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Mucosal Immune Responses in W/W^v and Sl/Sl^d Mutant Mice

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Abstract: In order to identify potential unanticipated side reactions and immune responses, the evaluation of candidate vaccines should include immunization of the murine model of the disease in question and mutant animals, as well as normal laboratory animals. We employed WBB6F₁- W/W^v and WBB6F₁- Sl/Sl^d mutant mice, which are genetically mast cell deficient and lack intestinal pacemaker activity due to a severe deficiency in interstitial cells of Cajal. Antigen-specific mucosal and systemic immune responses in the mutant and congenic normal mice were induced by intranasal or intragastric immunization with ovalbumin (OVA) plus cholera toxin as an adjuvant. It was found that the levels of the OVA-specific humoral immune response in the mucosal and systemic tissues of the mutant mice immunized intranasally were roughly equivalent to those of the congenic normal mice. In contrast, the specific humoral immune response in the intragastrically immunized mutant mice was greater than that observed in the congenic normal mice. Unexpectedly, the titers of OVA-specific IgA antibodies and total IgA antibodies in the fecal extracts of both intranasally and intragastrically immunized mutant mice were significantly lower than in those of the congenic normal mice. Although the detailed mechanisms leading to these differences remain unclear, the unexpected immune responses observed in the gastrointestinal tracts of the mice in this study may be related to an abnormality of gastrointestinal motility. Our data therefore suggest that studies using mutant mice and physiological assessments should be carried out during mucosal vaccine development.

Key words: gastrointestinal motility, mast cell deficiency, mucosal vaccine, mutant mice

Introduction

Due to the potential advantages of needle-free delivery and enhanced mucosal immune responses against pathogens, mucosal vaccination has received much attention in recent years [48]. The protective immune responses at mucosal surfaces can be enhanced by coadministration of effective mucosal adjuvants. For instance, enterotoxins such as cholera toxin (CT) and heat-labile toxin (LT) are powerful mucosal adjuvants that enhance both mu-

cosal and systemic immune responses to coadministered protein antigens (Ags) in mice [6, 11, 58]. The mucosal adjuvanticity of CT has also been demonstrated in non-human primates following intranasal immunization [23, 62]. However, a commercial nasal vaccine that included LT as an adjuvant has been withdrawn from the market due to safety concerns, including the possibility of side effects such as rhinorrhea, headache, and most seriously, facial palsy [16–18, 37].

In vaccine development, laboratory animals are used

(Received 28 October 2011 / Accepted 4 January 2012)

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to evaluate the immune responses elicited in response to candidate vaccines, as well as their safety. It is important to estimate accurately the effectiveness of any candidate vaccine in humans using results obtained from experiments performed on animals. However, the response to a vaccine may be different depending on the particular strain of laboratory animal used. Similarly, race and kindred can have an impact on immune responses to vaccination in humans [15]. As the immune response to a vaccine may also vary among patients with a disease and healthy subjects, the development of human vaccines may require studies to assess the vaccine-induced immune responses of the disease model and mutant animals, as well as normal animals, in order to fully understand the response to a vaccine.

The mutant *W/W^v* or *Sl/Sl^d* mice [28, 29, 31] used in this study carry mutations at the *W* (synonymous for dominant white spotting. Current gene symbol and gene name are *Kit* and *kit* oncogene.) and *Sl* (synonym for steel. Current symbol and gene name are *Kitl* and *kit* ligand.) loci, respectively [40, 63], and have widely been used as animal models. In *W/W^v* mice, an aberrant form of the c-Kit (CD117) protein is expressed [41], while *Sl/Sl^d* mice express a soluble truncated form of stem cell factor (SCF), a ligand of the CD117 protein [8, 12]. As these mutant mice are mast cell deficient, they also provide a unique opportunity to characterize the contribution of mast cells to many different disease processes and other biological responses [14, 39]. Mast cells are required for the enhanced recruitment of lymphocytes and Langerhans cells to draining lymph nodes, which is required for the induction of acquired immune responses [27, 34, 35]. Innate immunity is an important system that enhances vaccine-specific immune responses [1]. Herein, we employed both *W/W^v* and *Sl/Sl^d* mice in our investigation of immune responses to mucosal immunization.

Materials and Methods

Mice

Five-week-old female WBB6F₁-+/+, WBB6F₁-*W/W^v* and WBB6F₁-*Sl/Sl^d* mice were purchased from Japan SLC, Inc. (Yokohama, Japan). The mice were acclimated to the experimental animal facility for more than 1 week before being used in experiments. They were maintained in the facility under pathogen-free conditions. All experimental procedures were carried out fol-

lowing the guidelines established by The Committee of Animal Experiments, Iwate Medical University.

Immunization

For intranasal immunization, the mice were lightly anesthetized with ketamine before being immunized with a 5- μ l aliquot (2.5 μ l/nostril) of PBS containing 100 μ g of ovalbumin (OVA; Sigma-Aldrich, St. Louis, MO, USA) and 0.5 μ g of CT (List Biological Laboratories, Campbell, CA, USA), with a 5-min rest between each inoculation. For intragastric immunization, mice were deprived of food for 12 h before immunization, their stomach acidity was neutralized, and they were immunized with a 200- μ l aliquot of PBS containing 1,000 μ g OVA and 10 μ g CT. All groups of mice were immunized thrice at weekly intervals in every experiment. The dosage of CT and the immunization schedule were largely the same as those used in previous studies that examined the use of CT as a mucosal adjuvant in mice [20, 25, 57, 58, 61].

Sample collection and preparation of single-cell suspensions

Mucosal secretions (fecal extracts, nasal washes, and saliva) and blood samples were collected using methods described elsewhere [36, 51, 56, 61] and stored at -80°C until use. Single-cell suspensions were obtained from the nasal lamina propria (n-LP), submandibular lymph nodes (SMLNs), small intestinal lamina propria (i-LP), mesenteric lymph nodes (MLNs), and spleen, as previously described [36].

Detection of OVA-specific antibody (Ab) production and enumeration of OVA-specific Ab-forming cells

The titers of OVA-specific Abs in the plasma and mucosal secretions were determined using an endpoint ELISA. The endpoint titers were expressed as the last dilution that gave an optical density at 450 nm (OD₄₅₀) of ≥ 0.1 OD units above the OD₄₅₀ of the negative controls [56, 61]. The numbers of OVA-specific IgA and IgG Ab-forming cells (AFCs) in the n-LP, SMLNs, i-LP, MLNs, and spleens of the experimental mice were determined by enzyme-linked immunosorbent spot (ELISPOT) assay. OVA-specific AFCs were quantitated with the aid of a stereomicroscope [56, 61].

Quantitation of total IgA Abs in the fecal extracts

Fecal pellets (100 mg) were suspended in 1 ml of PBS

containing 0.1% sodium azide and were extracted by vortexing for 15 min. The samples were centrifuged at 10,000 g for 5 min, and the supernatants were collected as fecal extracts. The total amount of IgA Abs present in the fecal extracts was determined by performing a sandwich ELISA. Briefly, 96-well plates (Nunc A/S; Roskilde, Denmark) were coated with goat anti-mouse Ig Abs (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) prior to blocking with 1% bovine serum albumin and the addition of serial dilutions of fecal extract samples. Mouse IgA Abs (clone; J558, Southern Biotechnology Associates, Inc.) were used as a standard. After incubation, horseradish peroxidase-conjugated goat anti-mouse IgA Abs (Southern Biotechnology Associates, Inc.) were added to the wells. The resulting colorimetric reaction was developed with TMB+ substrate-chromogen (DAKO, Carpinteria, CA, USA).

Immunofluorescent assay of secretory component (SC)

The small intestines of naïve mice were resected and divided into approximately 0.5-cm-long pieces before being washed with PBS to remove fecal contents. The tissues were embedded in OCT compound and snap frozen with dry ice/acetone. Five-micrometer frozen sections of small intestine were air-dried and fixed in 4% paraformaldehyde in PBS for 10 min at 4°C, before being washed with PBS. After blocking, the slides were incubated with rabbit anti-SC polyclonal Abs (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4°C overnight. After washing with PBS, the slides were further incubated at room temperature for 30 min with a fluorescein isothiocyanate-labeled Fab fragment of goat anti-rabbit IgG Abs (Beckman Coulter, Inc., Miami, FL, USA). Digital images of the resulting fluorescence were obtained using a fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Statistical analysis

Normally distributed variables were compared by the two-tailed Student's *t*-test, and the results were expressed as means $\pm$ SD. Non-normally distributed variables were compared using the two-tailed Mann-Whitney U test, and the results were expressed as medians $\pm$ interquartile range (IQR). A *P* value of <0.05 was considered significant.

Results

Different enhanced Ab titers between congenic normal and mutant mice immunized intranasally or intragastrically

In order to assess the immune responses elicited by mucosal immunization, the levels of OVA-specific Abs in the mucosal secretions and plasma of congenic normal (+/+) mice and mutant (*W/W^v* and *Sl/Sl^d*) mice were determined using an endpoint ELISA. The samples were collected 1 week following the final immunization. When the mucosal secretions of the intranasally immunized mice were assessed, the titers of OVA-specific IgA Abs in the fecal extracts of congenic normal mice were significantly higher than in the mutant mice. Further, there was a significant difference in the titers of OVA-specific IgA Abs in the fecal extracts between *W/W^v* and *Sl/Sl^d* mice. In contrast, the titers of OVA-specific IgA Abs in the other mucosal secretions (nasal washes and saliva) were roughly equivalent. Moreover, no differences in the titers of OVA-specific IgG Abs in the plasma of any group were detected (Fig. 1).

When the fecal extracts were examined, the enhanced Ab responses of the mice immunized intragastrically resembled those of the mice immunized intranasally, with the titers of OVA-specific IgA Abs in the fecal extracts of congenic normal mice being significantly higher than in the mutant mice. Conversely, the titers of OVA-specific IgA Abs in the saliva and IgG Abs in the plasma of normal mice immunized intragastrically were lower than those of mutant mice (Fig. 2).

Assessment of OVA-specific AFCs in the mucosal and systemic tissues

In order to confirm OVA-specific Ab responses at the cellular level, we compared the number of OVA-specific AFCs in the mucosal and systemic lymphoid tissues 1 week after the final immunization. We assessed i-LP and n-LP as mucosal effector tissues and the spleen as a systemic tissue, as well as SMLNs from the intranasally immunized mice or MLNs from the intragastrically immunized mice as mucosal inductive sites [13]. Though the titers of OVA-specific IgA Abs in the fecal extracts of congenic normal mice immunized intranasally were significantly higher than in the mutant mice, the numbers of OVA-specific AFCs in the assessed tissues were roughly equivalent between each group (Fig. 3).

In contrast, the numbers of OVA-specific AFCs in the

Fig. 1. Titers of OVA-specific antibodies (Abs) in the mucosal secretions and plasma of intranasally immunized mice. Mice were sacrificed 1 week after the final immunization, and the titers of OVA-specific IgA Abs in the mucosal secretions (fecal extracts, nasal washes, and saliva) and IgG Abs in the plasma were determined by ELISA. Each dot represents an individual mouse. The horizontal and vertical bars indicate the mean $\pm$ SD or median $\pm$ IQR, respectively, of the 12 mice in each experimental group. Data are representative of 3 separate experiments. Significant differences between the $+/+$ group and W/W^v or SI/SI^d groups and between the W/W^v group and SI/SI^d group are indicated with an asterisk (**: $P < 0.005$).

Fig. 2. Titers of OVA-specific antibodies (Abs) in the mucosal secretions and plasma of intragastrically immunized mice. Mice were sacrificed 1 week after the final immunization, and the titers of OVA-specific IgA Abs in the mucosal secretions (fecal extracts, nasal washes, and saliva) and IgG Abs in the plasma were determined by ELISA. Each dot represents an individual mouse. The horizontal and vertical bars indicate the mean $\pm$ SD or median $\pm$ IQR, respectively, of the 12 mice in each experimental group. Data are representative of 3 separate experiments. Significant differences between the $+/+$ group and W/W^v or SI/SI^d groups are indicated with an asterisk (* and **: $P < 0.05$ and $P < 0.005$, respectively).

n-LP, MLNs, and spleens of intragastrically immunized mutant mice were significantly higher than in congenic normal mice. The enhanced numbers of OVA-specific IgA and IgG AFCs in the MLNs, which are the secondary lymphoid tissues for mucosal immunity in response to intragastric immunization, were particularly remarkable. Although no significant differences were observed between the numbers of OVA-specific AFCs in the i-LP of congenic normal and mutant mice, the number of AFCs in the mutant mice tended to be higher than in the congenic normal mice. The mean numbers of OVA-specific IgA AFCs in the i-LP of $+/+$, W/W^v and SI/SI^d

mice were 1,025, 1,406 ($P = 0.051$), and 1,394 ($P = 0.078$) cells/ 10^6 lymphocytes, respectively (Fig. 4).

Concentrations of total IgA Abs in the fecal extracts

The titers of OVA-specific IgA Abs in the fecal extracts of congenic normal mice were significantly higher than in mutant mice, regardless of the immunization route. We therefore also determined the concentrations of total IgA Abs in the fecal extracts of naïve and immunized mice, and it was found that the concentrations of total IgA Abs in the fecal extracts of naïve congenic normal mice were significantly higher than in the mutant

Fig. 3. Numbers of AFCs in the mucosal and systemic tissues of intranasally immunized mice. Mice were sacrificed 1 week after the final immunization, and the levels of OVA-specific IgA and IgG AFCs in the i-LP, n-LP, SMLNs, and spleen were determined by ELISPOT assay. The data shown represent the mean numbers of AFCs per 10⁶ cells $\pm$ SD of 12 mice over 3 separate experiments.

Fig. 4. Numbers of AFCs in the mucosal and systemic tissues of intragastrically immunized mice. Mice were sacrificed 1 week after the final immunization, and the levels of OVA-specific IgA and IgG AFCs in the i-LP, n-LP, SMLNs, and spleen were determined by ELISPOT assay. The data shown represent the mean numbers of AFCs per 10⁶ cells $\pm$ SD of 12 mice over 3 separate experiments. Significant differences between the +/+ group and W/W^u or S/S^H groups are indicated with an asterisk (* and **: $P < 0.05$ and $P < 0.005$, respectively).

mice. Similar results were also obtained after either intranasal or intragastric immunization (Fig. 5).

SC expression detected by immunofluorescent assay

Polymeric Ig receptors (pIgRs) are expressed on glan-

dular epithelial cells of the gastrointestinal (GI) tract and mediate the transport of IgA Abs into external body fluids. During transcellular transport of polymeric IgA Abs, pIgRs undergo proteolytic cleavage to generate a fragment called SC [24, 49, 53]. We therefore confirmed

Fig. 5. Total IgA Ab levels in the fecal extracts of +/+, *W/W^v*, and *SI/SI^d* mice. The levels of total IgA Abs were determined by sandwich ELISA. The data shown are the mean concentrations $\pm$ SD. Twelve mice were examined in each experimental group, and data are representative of 3 separate experiments. Significant differences between the +/+ group and *W/W^v* or *SI/SI^d* groups are indicated with an asterisk (**; $P < 0.005$).

the expression levels of SC in the tissue samples by immunofluorescent assay. The levels of SC expression detected in the small intestines of each group of mice were found to be roughly equivalent (data not shown).

Discussion

Studies that have compared immune responses between mast cell-deficient *W/W^v* mice and their mast cell-sufficient littermates have demonstrated that the Ag-specific IgG and IgM Ab responses of *W/W^v* mice after intravenous injection of sheep erythrocytes, polyvinylpyrrolidone, or bacterial lipopolysaccharide are similar to those of congenic normal mice [19]. Moreover, the contact sensitivity and delayed-type hypersensitivity of both groups of mice were also found to be similar [19, 26]. These studies concluded that there was no evidence of immune deficiency in *W/W^v* mice following intravenous immunization. Here, we demonstrated that intranasal immunization with OVA plus CT as a mucosal adjuvant could induce the presence of OVA-specific AFCs in the mucosal and systemic tissues of both *W/W^v* and *SI/SI^d* mice and that there were no differences in the number of AFCs detected in congenic normal and mutant mice. Specific immune responses following intranasal immunization may not be significantly dependent upon mast cells in the nasopharynx. These results suggest that mutations at the *W* and *SI* loci do not affect maturation of cells producing Ag-specific IgA and IgG Ab.

In contrast, when intragastric immunization was investigated in the present study, it was found that the

numbers of OVA-specific AFCs and titers of OVA-specific Abs in the saliva and plasma of the mutant mice were higher than those of the congenic normal mice. Interaction of CD117 and SCF plays an important role in lymphocyte development [10], and along with IL-7, it is involved in the organogenesis of MLNs [5]. SCF is also essential in the development of Peyer's patches (PPs) [5]. While the populations of T cells and B cells in the PPs of congenic normal and *W/W^v* mice are similar, the percentages of Ag-presenting cells (APCs) in the PPs of *W/W^v* mice are significantly lower than in normal mice [42]. Immunological differences in the development of intestinal lymphoid tissues and differences in the percentages of APCs in PPs between mutant and congenic normal mice may not fully explain our results, and the reasons for the differences in AFC and Ab numbers between the congenic normal and mutant mice in our study remain unclear.

One potential contributing factor is that the GI absorption of orally administered drugs is determined not only by the permeability of GI mucosa but also by the transit rate of drug delivery in the GI tract [45]. One factor that can impact this is the number of interstitial cells of Cajal (ICCs) within the GI tract. ICCs are found within the longitudinal and circular muscle layers, myenteric plexus, deep muscular plexus, and submucosal plexus of the intestinal wall [21], where they initiate slow waves in the smooth muscle cells of the GI tract through the generation of pacemaker activity [52]. As *W/W^v* and *SI/SI^d* mice are severely deficient in a subpopulation of ICCs [33, 55] and lack the network of ICCs associated with Auerbach's nerve plexus and intestinal pacemaker activity [22], this may be a source of discrepancy.

Moreover, spontaneous development of perforating stomach ulcer is found more frequently in mutant *W/W^v* and *SI/SI^d* mice than in congenic normal mice [30, 47]. Bile reflux is a cause of spontaneous gastric ulcers in mutant mice [2, 60]. Further, deoxycholic acid, which is included in bile, reduces mucin levels in differentiated enterocyte-like cells *in vitro* [46]. The mucin layer of the surface gastric epithelium was reduced in *W/W^v* mice [2]. The layer of mucus covering the epithelial surface of the gastric mucosa constitutes the first line of mucosal defense against insults by a variety of antigens and pathogens [7]. Bile reflux and reduced mucus gel due to the disturbance of gastric movement in mutant mice might be related different responses to intragastric immunization between congenic normal and mutant mice.

The enhanced humoral immunity in the assessed tissues, saliva, and plasma of intragastrically immunized mutant mice may be a result of an increased transit time through the GI tract and a reduced mucin layer in the mutant mice, further suggesting that physiological differences in gastric movement surpassed immunological differences between the immune cells such as APCs in PPs of the mutant and congenic normal mice. It has therefore been suggested that the transit rate, as well as the uptake of Ags in mucosal induction sites, should be considered when assessing oral vaccines.

Another interesting finding of this study was that the titers of OVA-specific secretory IgA Abs detected in the fecal extracts of the mutant mice were significantly lower than in both intranasally and intragastrically immunized congenic normal mice. Moreover, the levels of total IgA Abs secreted from the intestinal tracts and gallbladders of the mutant mice were also significantly lower than in congenic normal mice. *W/W^v* and *SI/Sl^d* mice failed to yield an intestinal secretory response after luminal CT challenge [32]. That *W/W^v* mice have aberrant secretory responses to Ag-induced intestinal anaphylaxis may potentially be explained by the mast cell-deficient status of these mice, as abnormalities in fluid secretion in the GI tracts of mast cell-deficient mice have been reported [43]. Mast cell interactions with enteric nerves also appear to be necessary for fluid secretion associated with intestinal anaphylaxis [3, 9, 43, 50]. Further, *SI/Sl^d* mice appeared to differ from *W/W^v* mice in their intestinal secretory response to CT [32]. In our study, it was also observed that the titers of OVA-specific IgA Abs in the fecal extracts of intranasally immunized *SI/Sl^d* mice were significantly higher than in *W/W^v* mice. However, the basis for the different IgA Ab secretions in *SI/Sl^d* versus *W/W^v* mice is unclear. Although we assessed the expression of SC in an attempt to elucidate the mechanisms responsible for the aberrant secretory IgA Ab responses in the mutant mice, SC was found to be expressed in the intestines of both congenic normal and mutant mice. These mechanisms, therefore, remain to be identified in further studies.

Our study has conclusively shown that mutant *W/W^v* and *SI/Sl^d* mice, which exhibit a defect in the ICC network, have different responses to mucosal immunization than congenic normal mice. Many GI motor disorders are related to changes in the number, structure, and/or density of ICCs. Indeed, the loss of ICCs or disruption of ICC networks has been reported in a wide range of

GI diseases in humans, including achalasia, chronic intestinal pseudoobstruction, Hirschsprung disease, inflammatory bowel diseases, and others [44, 54]. ICCs may also play an important role in the pathophysiology of slow-transit constipation [4, 54]. Moreover, it has been reported that there is disturbed GI motility and decreased levels of ICCs in the GI tracts of the diabetic C57BL/KsJ-*db/db* mouse model of type 2 diabetes mellitus [59]. A deficiency of ICCs may also be related to the pathogenesis of diabetic gastroenteropathy in humans [38]. Our findings could have important implications in GI disorders and diseases, as there may be inadequate immunity induced in patients vaccinated via mucosal routes, depending on the background of the patient.

Although we employed only genetically mast cell-deficient mice in our study, unexpected and novel findings were obtained. Fundamental studies of mucosal vaccines and adjuvants generally only include immunological analyses of normal animals; however, our study suggests that experiments examining only normal animals may be insufficient when considering the application of mucosal vaccines in humans. Furthermore, the contributions of intestinal peristaltic motility in mucosal immunity must be made clear in future studies. Our data, therefore, suggests that more effective and safer vaccines could be developed by studying both physiological factors and immunological factors in mice with different genetic backgrounds.

Acknowledgments

The authors thank Ms. Sumiko Yaegashi, Mr. Yuji Shibata, and Ms. Kumi Furusawa, School of Medicine, Iwate Medical University, for technical assistance and Ms. Ayako Sato, Ms. Rika Hatakeyama, and Mr. Tomoki Takahashi, Center for In Vivo Science, Iwate Medical University, for animal care. This work was supported by a Grant-in-Aid to the Strategic Medical Science Research Center from the Ministry of Education, Culture, Sports, Science and Technology of Japan (2010–2014). The authors declare that they have no conflicts of interest.

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