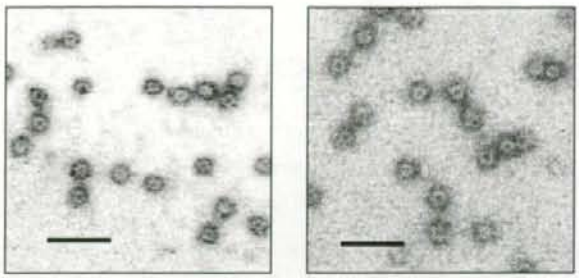

A. -52C B. w/o Tag

Fig. 4. Electron micrograph of VLP-52C. VLP-52C (A) and VLP without tag (B) were observed under electron microscopy after negative staining at a magnification of ×60,000. Inserted bar indicates 100 nm.

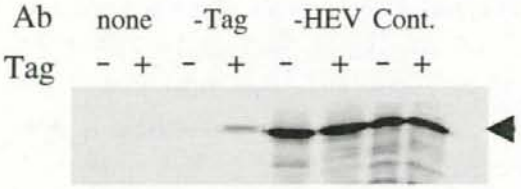

Fig. 5. Surface exposure of the tag epitope on VLP-52C. Surface exposure of the tag epitope on intact VLP-52C was examined by immunoprecipitation with the anti-tag antibody. Antibodies used are indicated at the top of panel. None, negative control without antibody; α-Tag, anti-tag antibody; α-HEV, anti-HEV antibody; cont., purified VLP-52C and VLP without tag were run as controls. The second row indicates either VLP with (+) or without the tag (−).

VLP without the tag (Fig. 4). Using two methods, we confirmed that the inserted epitope tag was exposed on the surface. The intact VLP-52C was immunoprecipitated with the anti-tag antibody, while the anti-HEV antibody immunoprecipitated both VLP-52C and the VLP without the tag (Fig. 5). Furthermore, the anti-tag antibody specifically reacted with the intact VLP-52C in an ELISA (data not shown). The results of immunoprecipitation and ELISA using intact chimeric VLP suggest that the tag epitope is exposed on the surface of the HEV-VLP.

Immune Responses to Chimeric HEV-VLPs through Oral Administration

Since many pathogenic viruses and bacteria establish their initial infections through the mucosal surface, vaccine strategies that can stimulate mucosal immunity have been widely studied (reviewed in Ogra *et al.*).[19] However, there are several difficulties in oral immunization with non-replicating molecules, such as low pH in the stomach, presence of proteolytic enzymes in the digestive tract, and presence of physical as well as biochemical barriers associated with the mucosal surface itself.[19] We previously reported that the HEV-VLP preserved original HEV construction and entered the epithelial cells of the small intestine by oral administration.[20] From these findings, mice were immunized with 50 μg of purified VLP-52C by the oral route four times at two-week intervals, with the mice having the ability to induce mucosal and systemic epitope-specific antibody responses. Specific IgG antibodies to the tag as well as to HEV were detected in intestinal fluids as early as two wpi (Fig. 6A). IgG levels in intestinal fluids continued to increase until the termination of the experiments. Specific IgA to both the tag and HEV also appeared in intestinal fluids from two wpi, paralleling the IgG levels (Fig. 6B). The IgA levels also continued to increase until the termination of experiments. As expected, the control mice immunized with VLP without the tag developed IgG and IgA only to HEV. In sera, levels of a specific IgG antibody to both the tag and HEV showed slightly higher OD values than those in non-immunized controls, but they never reached significant levels, as occurred in the intestinal fluids (Fig. 6C). The levels of specific IgA in sera were also low, although the OD values were also higher than the non-immunized controls (Fig. 6D). The control mice immunized with VLP without the tag showed similarly low OD values to HEV. The specific antibodies in the intestinal fluids were analyzed for their isotypes at 10 wpi. At this point, average OD values and SD for IgG and IgA in the intestinal fluids were 1.02±0.22 and 0.64±0.038, and 0.96±0.086 and 0.66±0.040, respectively, to HEV and the tag in three mice immunized with the chimeric VLP. In the control mice immunized with VLP without the tag, the average OD values and SD for IgG and IgA were 0.88±0.047 and 0.64±0.027, and 0.11±0.024 and 0.084±0.013, respectively, to HEV and the tag. All

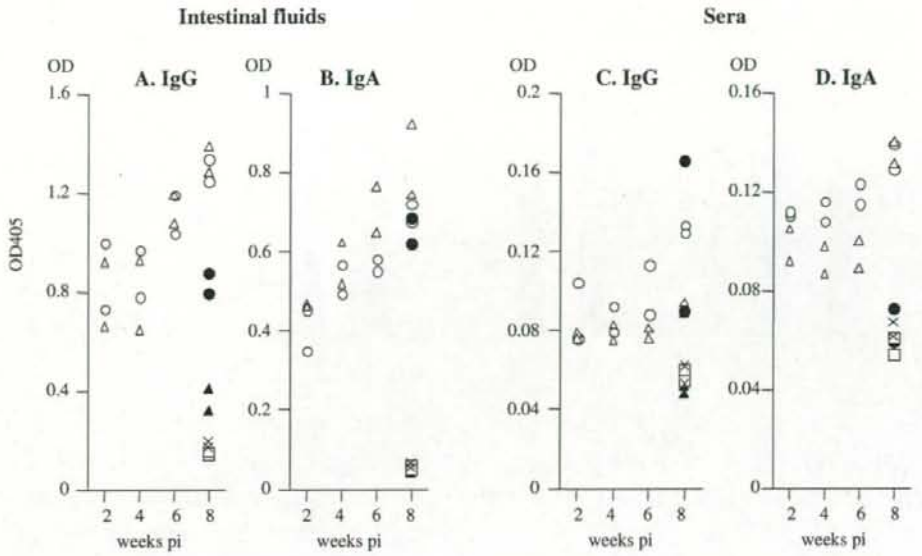

Fig. 6. IgG (A and C) and IgA (B and D) levels in intestinal fluids (A and B) and sera (C and D) of orally immunized mice. Circles and triangles indicate HEV-specific and the tag epitope-specific antibody levels, respectively, in individual mice. Two immunized mice were sacrificed at each time point (two, four, six and eight wpi). Specific antibody levels to HEV and the tag epitope of control mice immunized with VLP without the tag (closed circles and closed triangles, respectively) and background levels to HEV and the tag epitope of non-immunized mice (squares and crosses, respectively) are also shown. Antibody levels are indicated as OD405 in ELISA when sera and intestinal fluids were diluted at 1:100 and 1:2, respectively.

subclasses of IgGs to HEV — except IgG3, IgM, and IgA — were evident in all mice (Fig. 7B). Both to the tag and HEV, all mice failed to develop IgG3 above the detectable level (Figs. 7A and 7B). In the control mice immunized with VLPs without the tag, HEV-specific antibody reactions similar to the those with the chimeric VLPs were shown (Fig. 7B), while no detectable level of any isotype antibody specific to the tag was observed (Fig. 7A), as expected.

Induction of foreign epitope-specific antibody (Ab) responses by chimeric VLP administration is not easy compared with inducing cellular immune responses such as a cytotoxic T lymphocyte (CTL) response.[8,10,11,21,22] Moreover, our results showed Ab responses by oral

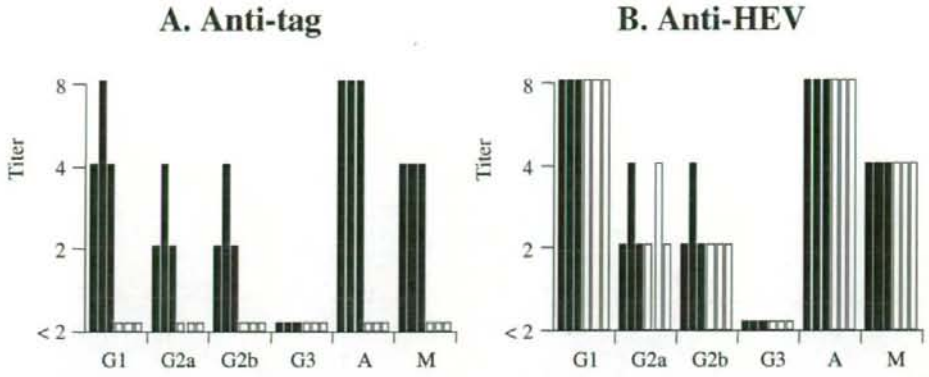

Fig. 7. Isotypes of antibodies specific to the tag epitope (A) and HEV (B) in intestinal fluids in orally immunized mice sacrificed at 10 wpi. Levels of IgA (A), IgM (M), and IgG subclasses (G1, G2a, G2b and G3) were examined by ELISA using isotype-specific secondary antibodies and are shown as end-point titers. Solid and open bars indicate antibody levels of each mouse immunized with the chimeric VLP and VLP without the tag epitope insertion, respectively.

administration to overcome the difficulties of a severe environment through the digestive tract. It is plausible that HEV-VLPs, which are derived from an orally transmissible virus, were incorporated into HEV-permissive epithelial cells in the small intestine because they retained structures and properties similar to those of HEV particles, producing an infection similar to that induced naturally.[17] It has been shown that the VLP structure should provide resistance to severe environments in the digestive tracts and enable specific binding to the mucosal surface if an appropriate VLP is chosen.[23] The delivery of a vaccine antigen (Ag) for induction of mucosal immune responses is usually achieved through the upper nasopharynx-associated lymphoid tissue (NALT), upper airway, salivary glands and tonsils.[24,25] Despite its obvious convenience, oral administration is rarely successful since it is quite difficult to protect vaccine Ag from the environment in the digestive tract.

The results of immunoprecipitation and ELISA using intact chimeric VLPs suggest that the tag epitope is exposed on the surface of the HEV-VLP. The successful induction of antibodies to the tag

also supports the hypothesis that the tag is exposed on the surface, since an internally localized B cell epitope in chimeric parvovirus-VLP failed to induce a specific antibody response.[11] Furthermore, this hypothesis is consistent with the results of three-dimensional analysis of the Norwalk virus-VLP particle, in which the C-terminal is exposed to the VLP surface.[26] Considering that B cell epitopes are generally hydrophilic and most likely exposed to the VLP surface, B cell epitope regions may not be directly involved in the protein-protein interactions to form a VLP. Our unsuccessful insertions into internal sites suggest that the integrity of internal regions must be maintained for proper protein folding and VLP formation. To find potential internal insertion sites, a precise three-dimensional structural map of the HEV-VLP may be necessary.

Oral vaccination has obvious advantages for a field trial in a large-scale public health vaccination program.[27] From a practical standpoint, oral administration is less stressful for vaccine recipients and does not require professional skill for administration. Moreover, delivery of vaccines via the intestinal tract is considered to be inherently safer than systemic injection. Encouraging results of phase I trials using Norwalk virus VLPs have recently been reported.[28] We also confirmed that chimeric HEV-VLPs carrying foreign CTL and B cell epitope at C-termini can elicit mucosal and systemic cellular immune responses as well as humoral immune responses by oral administration (submitted). It has become apparent that mucosal immune responses on different mucosal surfaces were achieved simultaneously, despite the initial stimulation of a single mucosal site.[29,30] Therefore, it is probable that oral administration of chimeric HEV-VLPs stimulates immune responses simultaneously on distant mucosal surfaces as well. This phenomenon significantly extends the potential use of chimeric HEV-VLPs as an oral vaccine vehicle.

A chimeric HEV-VLP has several advantages as an oral vaccine vector. First, large amounts can be easily obtained from standard cultivation protocols compared with amounts of other VLPs obtained. Second, the outcome of delivery of vaccine Ag in humans can be predicted using conventional laboratory animals, since HEV naturally infects various animals as well as humans through the same infectious

route and target cells.[6,31] Third, HEV-VLPs are stable at room temperature. Fourth, anti-HEV immune responses had no effect on boosting administration in the present study. Thus, HEV-VLPs are an attractive vaccine vector in developing countries because these VLPs can be preserved without the requirement of any particular equipment. These findings suggest that chimeric VLPs derived from orally transmissible viruses can be used as vaccine vectors to mucosal tissue by oral administration for the purpose of vaccination.

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Congenital Cystic Adenomatoid-like Malformation in a Cynomolgus Monkey (*Macaca fascicularis*)

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Abstract. Congenital cystic adenomatoid malformation (CCAM) is a developmental lung abnormality characterized by abnormal proliferation of mesenchymal elements and failure of bronchiolar structures to mature, ultimately resulting in the compression of normal pulmonary tissue and mediastinal shift with rapid expansion of cysts. Although various clinical and pathologic studies of CCAM in humans exist, CCAM has yet to be reported in animals, even in nonhuman primates. In the present study, histopathologic analyses of a neonatal cynomolgus monkey that died 17 days after birth revealed that normal lung architecture was replaced by disorganized overgrowths of cysts lined with simple cuboidal epithelium. The epithelium projected a few ciliates into the air spaces and produced mucus. To our knowledge, this is the first case study describing CCAM or a CCAM-like lesion in nonhuman primates.

Key words: Congenital cystic adenomatoid malformation; cynomolgus monkey; histopathological analyses; lung.

Congenital cystic adenomatoid malformation (CCAM) is a rare fetal developmental abnormality of the lung. First described by Ch'in and Tang in 1949,[2] it is characterized by abnormal development of terminal respiratory structures, resulting in an adenomatoid proliferation of bronchiolar elements and cyst formation.[11,18,19] CCAM is observed mainly during the neonatal period and infancy and can be diagnosed prenatally by ultrasonography after 16 weeks of gestation.[9,10,13,14] CCAM lesions are believed to be a consequence of abnormal embryogenesis during the first 6 to 7 weeks of pregnacy.[18]

Stoker et al.[18,19] classified CCAM into three types based on clinical and histologic criteria. In the neonatal period, the expansion of cysts and the compression of surrounding lung parenchyma cause progressive respiratory distress.[18,19] Moreover, emphysema-related lung masses also cause dyspnea, eventually leading to death.[18,19] Fetuses with CCAM tend to be born prematurely or are stillborn.[12] Due to the severity and impact of this disorder, numerous clinical and pathologic studies in humans have focused on determining the pathologic mechanisms underlying CCAM and the clinical management of CCAM.[1,4,8,15–17] Although CCAM is well characterized in humans, CCAM or CCAM-like pathogenesis has not been reported in animals. To our knowledge, we report here the first case of CCAM in a nonhuman primate, as demonstrated through histopathologic analyses of a neonatal cynomolgus monkey.

A male cynomolgus monkey (*Macaca fascicularis*) born in our colony died naturally 17 days after birth. The animal was housed in an individual cage with its mother and maintained according to the National Institute of Biomedical Innovation rules and guidelines for experimental animal welfare. The mother monkey had the experience to raise another 6 baby monkeys. In this case, the mother monkey embraced her baby as usual, and the lactation also was observed. After birth, breeding staff members observed the baby monkey to check its health status; however, they could not find any abnormalities until 3 days before it died. Necropsy was performed. Tissues were fixed in 10% neutral-buffered formalin, processed conventionally, embedded in paraffin, cut into 3-μm–thick sections, and stained with hematoxylin and eosin. Lung tissues were also stained with periodic acid–Schiff (PAS), Alcian blue (AB), Masson's trichrome, and elastica van Gieson stains.

For immunohistochemical analyses, sections were first deparaffinized by pretreatment with 0.3% H_2O_2 solution. Next, sections underwent antigen retrieval with citric acid buffer and heating in an autoclave at 121°C for 10 minutes. Finally, sections were incubated free floating in primary antibody solution overnight at 4°C. The primary antibodies were anti-cytokeratin antibody (AE1/AE3 clone; 1:50; Dako, Glostrup, Denmark), anti-vimentin antibody (1:100; Dako), and anti–von Willebrand factor VIII antibody (1:400; Dako). Following brief washes with buffer, the sections were sequentially incubated with Polymer immunocomplex (Dako) for 30 minutes. Immunoreactive elements were visualized by treating the sections with 3-3′ diaminobenzidine tetroxide (Dojin Kagaku, Kumamoto, Japan). The sections then were counterstained with hematoxylin.

Fig. 1. Lung, 17-day-old monkey. The right lower lobe contains a 3.5 cm × 2 cm × 1 cm, thin-walled, multicystic structure filled with air. The remaining lobes were pinkish-red and showed signs of atelectasis. Bar = 5 mm.

Macroscopically, lesions were confined to the right lower pulmonary lobe. In the lesion area, we found a thin-walled multicystic structure (3.5 cm × 2 cm × 1 cm) filled with air (Fig. 1). The other pulmonary lobes were pinkish-red and showed signs of atelectasis (Fig. 1). In heart, both atria dilated with clot. Microscopically, the affected lung displayed diffuse, abnormal architecture composed of numerous large, partially collapsed air spaces. These lesions looked like adenomatoids, and air spaces were lined with simple cuboidal epithelium surrounded by connective tissue and immature cartilage (Fig. 2). Within the air spaces, we found a few ciliates (Fig. 3), and PAS stained a little mucus on a part of epithelium (Fig. 4). AB weakly stained premature cartilage. Masson trichrome stained fibrous interstices, but smooth muscles were not observed. Elastica van Gieson stained elastic fibers around the air spaces. Other lung tissues were congested and were compressed by the surrounding mass. The border between the cystic lesion and normal tissue was not clear. In liver and kidney, congestion was observed.

Immunohistochemical analyses revealed that the epithelium of the lesion was immunopositive for cytokeratin but negative for vimentin and von Willebrand factor VIII. However, the fibrous interstices and premature cartilage of the lesion were immunopositive for vimentin.

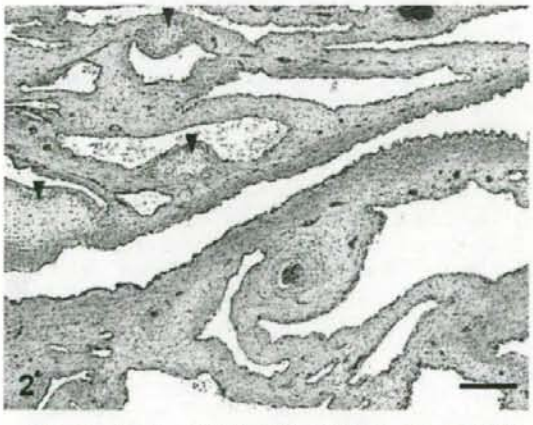

Fig. 2. Lung, 17-day-old monkey. Photomicrograph of affected lung showing diffusely abnormal architecture characterized by numerous large air spaces lined with epithelium surrounded by connective tissue and immature cartilage (arrowheads). HE. Bar = 250 μm.

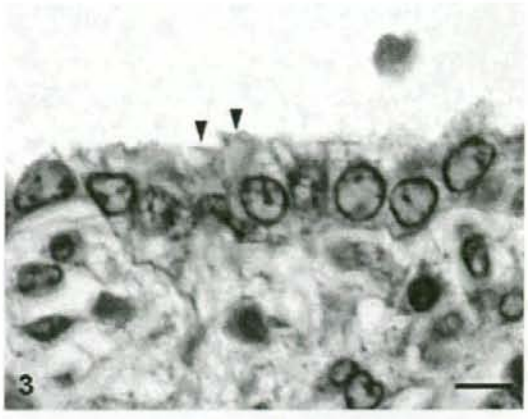

Fig. 3. Lung, 17-day-old monkey. Air spaces are lined with simple cuboidal epithelium. A few ciliated cells (arrowheads) were observed within some air spaces. HE. Bar = 6 μm.

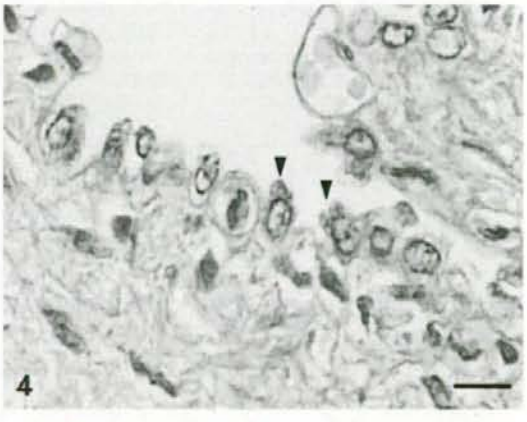

Fig. 4. Lung, 17-day-old monkey. Periodic acid–Shiff demonstrated mucus (arrowheads) on a part of the epithelium lining air spaces. Bar = 8 μm.

The congenital pulmonary abnormalities can be divided into some classifications.[5,6]

1. Bronchopulmonary sequestration:[5,6] bronchopulmonary sequestration is a pulmonary malformation in which a portion of lung parenchyma has no communication with the tracheobronchial tree and receives its blood supply via a systemic artery. The abnormality is observed as intralobar or extralobar. The first type (intralobar sequestration [ILS]) is contiguous with normal lung parenchyma and within the same visceral pleural envelope. The abnormal tissue is usually well demarcated from surrounding lung parenchyma and consists of one or more cystic spaces filled with mucus or pus. Microscopically, they resemble dilated bronchi, with respiratory epithelium and occasional mural cartilage plates. The latter type (extralobar sequestration [ELS]) is enclosed within its own pleural membrane, usually close to a normal lung. In addition to these types, the bronchopulmonary foregut malformation (BPFM)[7] is characterized by the pulmonary sequestrations that communicate with the upper gastrointestinal tract.

2. Bronchogenic cysts:[5,6] Bronchogenic cysts are lesions of congenital origin derived from the primitive foregut and are usually solitary, thin-walled, unilocular, and roughly spherical. They are filled with either mucus or serous fluid, and they do not communicate with the trancheobronchial tree. Microscopically, the cyst wall is lined by respiratory epithelium and contains cartilage, smooth muscle and, sometimes, seromucinous bronchial-type glands.

3. Congenital lobar emphysema (CLE):[5,6] CLE is characterized by severe overinflation of a pulmonary lobe. The pathogenesis is perhaps due to hypoplasia of bronchial cartilage. Microscopically, there is massive distention of alveolar spaces, but not tissue destruction.

4. Congenital cystic adenomatoid malformation (CCAM):[3,5,6,18,19] CCAM consists of an intralobar mass of disorganized pulmonary tissue that can exist with or without gross cyst formation. The anomaly is considered by some to represent a hamartoma, whereas others feel that it results from localized arrested development of the fetal bronchial tree. The cysts can usually be shown to communicate with normal airways; vascularization is by way of the pulmonary circulation. The histologic appearances give rise to the name, as there are numerous spaces of varying size, some of which resemble glandular acini, being lined cuboidal or columnar epithelium. According to the Stocker et al.[18,19] on the basis of 38 cases, CCAM is classified into three categories. Type I consists of some large cysts (>2 cm in diameter). The lesions are lined by ciliated cuboidal or columnar epithelium with elastic tissue or smooth muscle. Mucus-producing cells or cartilage may be present. Relatively normal alveoli may be seen between or adjacent to these cysts. Type II consists of small cysts (<2 cm in diameter) lacking mucus-producing cells and cartilage. Type III consists of bulky, firm, solid mass with small cysts (<0.5 cm in diameter). The lesions are lined with nonciliated cuboidal epithelium.

In this case, we found the abnormal architecture composed of numerous large air spaces. These cysts looked like adenomatoids, and they were lined with simple cuboidal epithelium surrounded by elastic tissue and premature cartilage. These pathologic features are different from that of ILS, ELS, BPFM, bronchogenic cysts, or CLE, because cysts were not unilocular, they did not include mucus or serous fluid, and they had neither abnormal artery nor communication with the upper gastrointestinal tract. Furthermore, the lesions were not massive distentions of alveolar spaces. Taken together, this monkey case is considered CCAM.

We applied the Stocker et al.[18,19] classification scheme to our monkey case, since no reports on CCAM in animals currently exist. In this monkey case, we observed large cysts greater than 2 cm in diameter (Fig. 1) lined with sparsely ciliated epithelium (Fig. 3) that produced a little mucus in a part (Fig. 4). These cysts also contained immature cartilage (Fig. 2). Thus, this monkey case exhibited sufficient pathologic features to meet the criteria for type I CCAM. We considered that pulmonary lobes were compressed by cysts and alveolars were collapsed, so that this monkey would suffer from respiratory distress and die. Moreover, the heart or great vessel may be compressed by pulmonary cysts, so that we consider liver and kidney congestion as a result of circulatory failure.

To our knowledge, this is the first case study describing CCAM or CCAM-like lesions in nonhuman primates. We have no information about the genetic background of the monkey in this case. Nonetheless, this case is quite noteworthy, because it provides an example of CCAM-like pathology in animal tissue. Should we encounter other CCAM-resembling or suspicious cases, we will carry out not only histopathologic analyses but also genetic analyses to determine the genetic background of these monkeys. Accumulating data from such analyses in nonhuman primates will contribute greatly to understanding CCAM pathology.

Acknowledgements

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Maternal Behavior of Laboratory-born, Individually Reared Long-tailed Macaques (*Macaca fascicularis*)

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To investigate maternal behavior in laboratory-born, individually reared monkeys, we carried out a statistical analysis of 896 long-tailed macaques (*Macaca fascicularis*) based on breeding records of the Tsukuba Primate Research Center (National Institute of Biomedical Innovation, Ibaraki, Japan). Data were obtained from 3266 cases of normal delivery between 1982 and 2004. In each case, maternal behavior was classified as either adequate or inadequate. We examined the effects of parity and the sex of the infant on maternal behavior. We also investigated the similarity of maternal behavior between mothers and their daughters and the effect of quality of maternal care received in infancy on maternal behavior as an adult. The results showed that only the mother's number of deliveries had a significant effect on maternal behavior. The greatest improvement of maternal behavior was observed at second delivery, and the incidence of improvement kept being above 0 thereafter. Our results suggest that, as reported previously, parity is an important factor in the adequacy of maternal behavior in individually reared monkeys.

Abbreviations: IR, improvement ratio; IA, individual adequacy; SPF, specific pathogen-free; TPRC, Tsukuba Primate Research Center

The Tsukuba Primate Research Center (TPRC; National Institute of Biomedical Innovation, Ibaraki, Japan) was established in 1978. The original purpose of monkey breeding in the TPRC was to supply the laboratory-bred monkeys for national vaccine safety testing performed in the National Institute of Infectious Diseases. All monkeys in the TPRC breeding colony were free from measles virus by 1982, and the breeding colony became free *Shigella*, *Salmonella*, *Mycobacterium tuberculosis*, *Mycobacterium bovis*, simian varicella virus, and herpes B virus by 2002.

The TPRC now has expanded its function to provide monkeys to medical researchers in a broad range of fields, such as infection and hyperimmunization restraint. These research areas often need monkeys that are specific-pathogen free (SPF) at its highest level. To effectively maintain and supply SPF monkeys and monkey fetuses that are appropriate for each experiment, the TPRC uses individual rearing. Monkeys are separated from their mothers 5 to 6 mo after birth and receive pair-rearing with an age-matched cagemate until 2 y of age. Thereafter, they are kept in individual cages except during mating periods. Even though TPRC monkeys always have visual, auditory, and olfactory contact with their conspecifics living in the same room, their social experience is quite limited compared with that of wild or group-reared captive monkeys.

Many previous studies indicated that monkeys with limited or no social experience show abnormal social behavior. The most extreme cases are reported in a particular series of studies.[1,7,8,19,20] For the duration of their infancy, monkeys were housed without their mothers and with little or no opportunity to interact with other monkeys. As a result, they had difficulty forming normal social relationships in many interactive situations, such as playing,[1,8,20] mating,[7] and mothering.[1,7,20] The abnormal maternal behavior of these monkeys evoked interest among researchers. Some of the females that had poor social experiences as infants abused or neglected their own infants, in some cases so severely that the infants died.[20]

Many of the cited studies were observations of monkeys reared under more severe circumstances than those in a normal breeding colony. Even in normal breeding colonies, most researchers observe only a portion of the colony animals.[12,14-17,21] No large-scale investigation has examined the effects of individual rearing on the maternal behavior of the monkeys. Here we report the statistical analysis of data accumulated over a period of 20 y on the maternal behavior of more than 3000 cases involving approximately 900 monkeys that were individually reared for most of their lives.

Materials and Methods

Rearing and breeding conditions of the TPRC. The rearing and breeding conditions of the TPRC have changed somewhat over time, depending on prevailing regulations and practices. However, what has never changed is the individual rearing of monkeys and the length of time during which monkeys are reared with their mothers or age-mates.

The rearing and breeding conditions were fixed as follows from 2005. Monkeys in the TPRC breeding colony are reared in individual cages (0.5 m wide × 0.8 m high × 0.9 m deep; stainless steel mesh). The breeding rooms are rectangular, and the individual cages are installed on the long sides of the room. Each room contains at least 90 cages. Most (90% to 95%) cages are occupied continuously. Therefore, monkeys can always make visual, auditory and olfactory contact with their room-mates. Ambient temperature in the rooms is kept about 25 °C, and humidity is set at 50% to 60%. The air is replaced 12 times hourly.

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Lighting is on for 12 h, from 0700 to 1900. Monkeys are provided with apples in the morning, and monkey chow is given to them twice in the afternoon. Water is available ad libitum.

Monkeys in the breeding colony are inspected daily by experienced animal technicians. When any abnormality is found, a veterinarian examines the monkey promptly and applies the appropriate treatment. Moreover, the monkeys are medically examined under anesthesia at least once every 2 y. The medical examination consists of body weight measurement, tuberculin test, blood sample, stool test, examination of the fundus, and a medicated bath.

Monkeys are separated from their mothers 5 to 6 mo after birth and receive peer-rearing with an age-mate until 2 y of age. After that, they are reared individually except during the mating period. The mating period begins 11 d after menstrual bleeding is observed, which occurs 1 d before the estimated ovulation. The monkey is anesthetized and moved to the cage next to the mating partner at least 1 d before the start of the mating period. The mating period is begun by removing a partition between the cages. After 3 d the partition is replaced.

Pregnancy diagnosis is conducted by the ultrasonography under anesthesia 5 wk after the end of the mating period. If the female is not pregnant, she begins the next mating period around the presumed ovulation day. During pregnancy, females are reared individually. After delivery, the dam and her infant are reared together for about 6 mo, after which they are separated. The offspring begin peer-rearing with an age-mate, and dams are reared individually again.

These rearing and breeding conditions are approved by the Institutional Animal Care and Use Committee of National Institute of Biomedical Innovation, Japan.

Laboratory procedures. Subjects were 896 laboratory-born long-tailed macaques (*Macaca fascicularis*) in the breeding colony of the TPRC. Data were obtained from breeding records collected between March 1982 and March 2004. Individual monkeys gave birth from 1 to 12 times (mean ± SD, 3.7 ± 2.3). The total number of normal births was 3266.

Animal technicians determined the adequacy of maternal behavior on the basis of daily inspection from the infant's birth to separation of mother and infant, about 6 mo.

Breeding records reported whether a dam showed inadequate maternal behavior, regardless of duration or frequency. Moreover, even if a mother that was once judged to be 'inadequate' never again showed inadequate behavior to the same offspring, the record was never changed. In short, only the cases in which inadequate maternal behavior was *never* observed by the animal technicians were recorded as adequate.

Inadequate maternal behavior was defined as rejection of the infant, holding an infant incorrectly, refusal to nurse, or violence against the infant. The dam's avoidance of, or escape from, physical contact with her infant was considered rejection of the infant. Holding an infant incorrectly means that the dam held her infant on her ventral side upside down. Refusal to nurse means that the dam held her infant correctly but prevented the infant's access to her nipples. Violence against the infant was physically hitting or stepping on her offspring.

When a dam showed inadequate maternal behavior, she and her infant were separated, and food and medical treatment were given to the infant as needed. If the infant regained his or her health, he or she was returned to the mother. In most cases, the infant was returned to the dam only once. The animal technician observed the pair for about 10 min, and if the dam took care of her infant normally, the infant was allowed to remain. If inadequate maternal behavior was observed at that time or at the inspection thereafter, the infant was separated from the mother again and was never returned to her.

If the infant was in poor physical condition for more than 2 to 3 d, or if the dam showed inadequate mothering after the infant's return, the infant was reared by artificial nursing or by foster mothers.

Consistency in adequacy of maternal behavior. Multiparous monkeys were classified into 4 groups based on changes in their adequacy of maternal behavior: good, improved, poor, and inconsistent. Monkeys that showed adequate care for all their offspring were classified as having good maternal behavior. The monkeys belonging to the improved group showed inadequate maternal behavior at the first delivery or between the first and a certain delivery number, but they never had inadequate behavior thereafter. Note that our definition of improvement is based on the change in maternal behavior between deliveries, not on a change with the same infant. Monkeys that showed inadequate maternal behavior with all their infants were classified as having poor maternal behavior. The remaining monkeys were classified as having inconsistent maternal behavior.

Primiparous monkeys were classified into 2 groups (good and poor), based on their maternal behavior.

Effects of parity on maternal behavior. To determine whether adequacy of maternal behavior improves with increasing parity, we compared the proportion of 'adequate care' cases at each number of deliveries. As shown in Figure 1, adequacy of maternal behavior appears to improve after giving birth at least twice. However, this analysis may not reflect the natural tendency for improvement of maternal behavior, as monkeys showing inadequate maternal behavior may have been preferentially been excluded from the breeding colony as a means of colony management. Therefore, in a second analysis, we evaluated dams that gave inadequate maternal care to their first offspring based on the adequacy of mothering toward the second offspring. The monkeys that did not improve their maternal behavior at the second delivery were classified again, this time according to their maternal adequacy toward the third offspring. Data from the inconsistent group were excluded from this analysis. We then calculated the improvement ratio (IR) for each delivery number as $N_s / (N_s + N_i)$, where N_s is the number of monkeys that improved their maternal behavior and N_i is the number of monkeys that continued inadequate maternal behavior. If all monkeys that showed inadequate mothering toward the previous offspring improved, the value of IR was 1. Conversely, if none improved, the value of IR was 0.

Relationship of the maternal behavior of the mother to that of the daughter. A total of 340 pairs of mothers and daughters among the monkeys were included in the study. Because the subjects in the present study consisted of the monkeys belonging to various generations, some subjects were represented both as mother and as daughter. When a monkey had several daughters who experienced delivery, the dam was paired with each daughter. In other words, the number of times that a monkey was represented as mother was the same as the number of her daughters who had experience of delivery. Two analyses were conducted using the data from these pairs..

First, to investigate similarity of maternal behavior between mother and daughter, we calculated the ratio of the number of observations of adequate maternal behavior to the total number of deliveries for each monkey. We defined this value as individual adequacy (IA). We then examined the correlation of IA between daughter and mother by Spearman rank correlation coefficient.

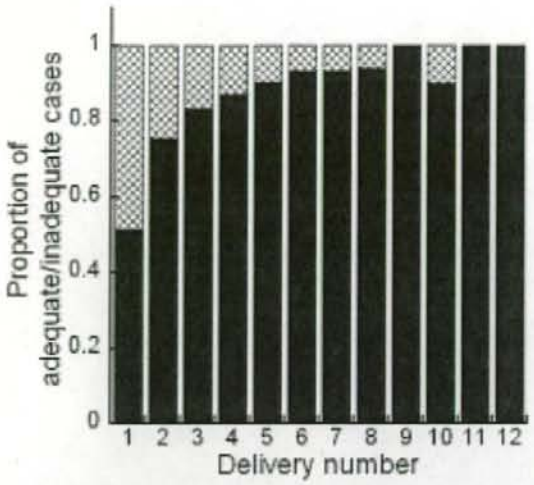

Figure 1. Apparent improvement of maternal behavior with childbirth. We classified each case by the number of deliveries and calculated the ratio of adequate to inadequate maternal behavior. Because there was a possibility that monkeys that showed inadequate behavior were excluded preferentially from the breeding colony, we conducted another analysis (see Figures 2 and 3).

Second, to investigate whether the quality of maternal care received in infancy influenced the maternal behavior of the daughter, we divided the daughters into 2 groups according to the quality of maternal care they received (adequate and inadequate). We then compared the IA value and the consistency in adequacy of maternal behavior in the 2 groups of daughters by *t* test.

Effects of sex of the infant on maternal behavior. We divided all cases by the sex of the infant. We then calculated the incidence of adequate and inadequate maternal behavior in each group and compared them by χ^2 test.

All statistical analyses were performed using StatView statistical software (version 5.0; SAS Institute, Inc., Cary, NC).

Results

Consistency in adequacy of maternal behavior. Of 3266 normal deliveries, 2459 (75.3%) were associated with adequate maternal behavior, and the remaining 807 (24.7%) were categorized as having inadequate maternal care. The severity and continuity of inadequate maternal behavior differed. As soon as inadequate behavior was observed by an animal technician, the infant was separated from the mother for treatment as needed. In 390 of the 807 (48.3%) cases with inadequate maternal behavior, the physical condition of the infant was good, and the dam showed signs of accepting her infant; therefore, the infant was returned to his or her biological mother and was reared by her until their separation. However, if the mother continued displaying inadequate behavior or if the physical condition of the infant was impaired, the infant was reared by artificial nursing (266 of the 807 cases, 33.0%) or by foster mothers (151 cases, 18.7%).

The results of classifying monkeys by the consistency in adequacy of maternal behavior are shown in Table 1. Of primiparous monkeys (n = 187), 42.2% were categorized as having good maternal behavior, and 57.8% were categorized as having poor maternal behavior. Of multiparous monkeys (n = 709), 52.0% were categorized as good; most of the remaining monkeys had maternal behavior that improved with subsequent births (28.8%) or that remained poor regardless of parity (15.0%). Only 4.2% of the multiparous monkeys were classified as having inconsistent maternal behavior regardless of parity.

Table 1. Distribution of monkeys, arranged by the consistency of adequacy of maternal behavior

	No. of subjects				
Parity	Good	Improved	Poor	Inconsistent	Total
1	79	na	108	na	187
2	77	30	44	2	153
3	56	58	29	6	149
4	67	34	17	5	123
5	56	24	11	3	94
6	43	22	0	7	72
7	32	15	2	5	54
8	20	11	3	1	35
9	12	7	0	0	19
10	5	2	0	1	8
11	0	1	0	0	1
12	1	0	0	0	1
Primiparous	79	na	108	na	187
Multiparous	369	204	106	30	709
Total	448	204	214	30	896

na, not applicable

In the improved group, the number (mean ± SD) of times that dams continued to show inadequate behavior from the first delivery was 1.5 ± 0.9, with 4.5 ± 2.1 deliveries. The total number of normal deliveries which dams in this group experienced was 909. In 614 of these cases, their maternal behaviors were determined as adequate. In the other 295 cases, the maternal behaviors were determined as inadequate. In the inconsistent group, the mean number of deliveries was 5.1 ± 1.9, and the total number of normal deliveries was 152. In 84 of these cases, maternal behavior of the dams was determined as adequate. In the other 68 cases, the maternal behavior was determined as inadequate.

Effects of parity on maternal behavior. As shown in Figures 2 and 3, the incidence of adequate maternal behavior at the first delivery was 51.7%. The highest IR was observed at the second delivery. After that, the value of IR was low but nonvanishing (Figure 3). However, only a few dams required more than deliveries to develop adequate maternal behavior (Figure 2).

Relationship of the maternal behavior of a mother to that of her daughter. The correlation between the IA of mothers and daughters was not statistically significant (Figure 4; Spearman's rank correlation coefficient, n = 340 pairs, $P > 0.1$). Furthermore, the IA value of the daughters that received adequate maternal care in infancy was not significantly different from that of the daughters that received inadequate care in infancy (*t* test, $t(338) = -0.1$, $P > 0.10$; IA of daughters that received adequate care, 0.5 ± 0.4; IA of daughters that received inadequate care, 0.5 ± 0.4). Moreover, consistency in adequacy of maternal behavior did not differ between the 2 groups of daughters (Figure 5).

Effect of sex of the infant on maternal behavior. In total, 1709 infants were male and 1557 were female. For 75.0% (1282) of the male infants and 75.6% (1177) of the female, mothers showed adequate maternal behavior. The quality of maternal care the infants received did not differ according to their sex (χ^2 test, $P > 0.1$).

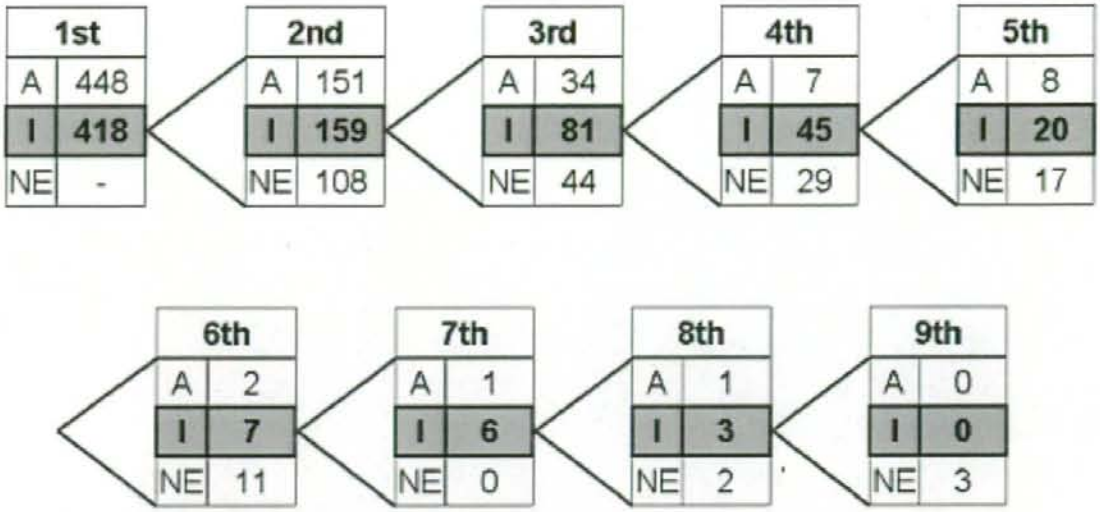

Figure 2. Schema for calculation of improvement ratio (IR) for each delivery number. The ordinal number at the top of each box is the number of deliveries. Inside each box is the number of subjects in each category: A, monkeys that showed *adequate* maternal behavior at delivery; I, monkeys that showed *inadequate* maternal behavior at delivery; NE, monkeys that *did not experience* that number of deliveries. The total number of monkeys at each delivery number is equal to the number of monkeys categorized as I at the previous delivery number. Because the number of monkeys belonging to I became 0 at the ninth delivery, we stopped the analysis at that step.

Discussion

One of the most important questions we asked is whether monkeys in our breeding colony show inadequate maternal behavior at a higher rate than do group-reared or free-ranging monkeys. The incidence of inadequate maternal behavior in the TPRC was 24.7% (807 of 3266 animals). However, our assessment of inadequacy is quite strict: a dam defined as having inadequate maternal performance as soon as she displays a single suboptimal rearing behavior. Moreover, this classification is never revised, even if the dam improves her maternal behavior by the time of protocol-defined separation from her infant. In approximately half (390 of 807) of the animals defined as having inadequate maternal behavior, the dam showed the suboptimal behavior only transiently and then reared her infant successfully without any intervention until the separation. Therefore, we consider the actual percentage of inadequate maternal behavior to be 12.8% (417 of 3266).

In 1 study, the incidence of inadequate maternal behavior in group-reared monkeys was 1.9% to 12.2%.[13] However, the authors stated that the actual incidence might be higher because they counted only severe cases of neglect and abuse. We are aware that not all infant deaths are the result of inadequate maternal care, and for comparison, infant mortality in a free-ranging troop was 6.7% to 16.2%.[9,10,18] Compared with these data, the incidence of inadequate maternal behavior in the TPRC is not unreasonably high.

However, our results do not suggest that the social experience of dams has no effect on their maternal behavior. In our study, the incidence of adequate maternal behavior by individually reared monkeys at the first delivery was 51.7%. In a 1981 study,[21] among 6 long-tailed macaques that received peer-rearing with 6 to 7 age-mates until sexual maturation, all but 1 showed normal maternal care to the first offspring. In a 1989 study of 10 long-tailed macaques reared in different social conditions,[11] 1 group consisted of monkeys that were born and reared in family groups until the first delivery. Another group consisted of monkeys that were born in family groups but were reared with 6 to 7 age-mates. All monkeys in both groups showed adequate maternal behavior. In other studies,[1,20] most of the monkeys that had limited social experience could not rear their first infants, but these authors observed only 4 to 8 monkeys. Of 50 rhesus monkeys that were reared without mothers, 34 (68.0%) abused or neglected their first infants.[19]

The monkeys that showed increased incidence of adequate maternal behavior[11,21] than that in the current study received peer-rearing for a longer time and with more age-mates than did those in the TPRC. In contrast, those that showed poorer rearing skills[1,19,20] were reared in a poorer social environment than those in the TPRC. These results suggest that the duration and complexity of social rearing affect maternal behavior at the first delivery.

As already mentioned, about half the monkeys in our study showed inadequate mothering at their first delivery, but about half of those monkeys improved their maternal behavior by their second delivery. Moreover, few monkeys showed inconsistent maternal behavior overall (Table 1). Overall, about 75% of the monkeys we studied had developed adequate maternal behavior by the time of their second delivery. These results suggest that the maternal behavior can be improved through the delivery experience. Regardless of whether the monkeys are captive or wild, many investigators have suggested that previous deliveries help the dam learn how to treat her infant and contribute to the overall improvement of infant survival rates.[6,9,10,13-15] Even monkeys that received no maternal care in infancy were able to improve their maternal behavior with repetition of delivery.[7,19]

We identified no factors other than parity that affected maternal behavior. Previous studies in group-living monkeys suggested that infant abuse, not neglect, tended to be observed only in particular matrilines.[12,13] We cannot know all the details regarding inadequate maternal behavior at the TPRC in the past, because the breeding records were designed to record

Figure 3. Improvement ratio (IR) for each delivery number. The open circle indicates the incidence of adequate maternal behavior at the first delivery for all subjects (n = 896). The closed circles indicate the IR. We calculated the IR for each delivery number by the formula $N_a / (N_a + N_i)$, where N_a is the number of monkeys that improved their maternal behavior, and N_i is the number of monkeys that continued inadequate maternal behavior. The actual values of N_a and N_i for each delivery number are shown in Figure 2.

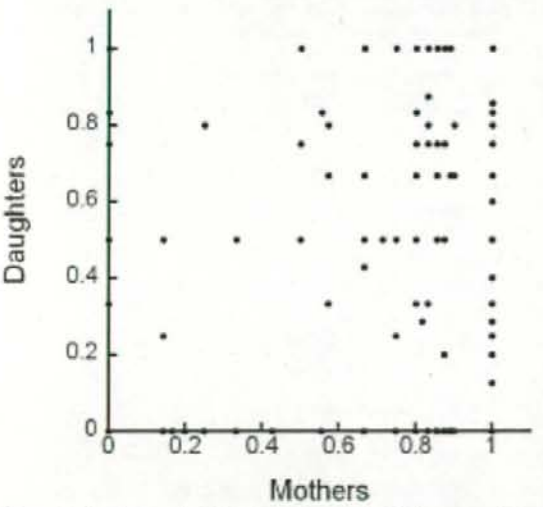

Figure 4. Correlation of individual adequacy (IA) between mothers and daughters (n = 340 pairs). Because some dots overlapped, the number of dots in the figure is less than the number of the pairs. There was no significant relationship between the IA of mothers and that of daughters.

maternal behavior only as adequate or inadequate. Perhaps we found no correlation in the adequacy of maternal behavior between mothers and daughters because of a limited choice of categories. We would need to examine the medical history for each infant until mother–infant separation to determine why maternal behavior was judged inadequate (for example, if the dam physically abused her infant). Moreover, although infant

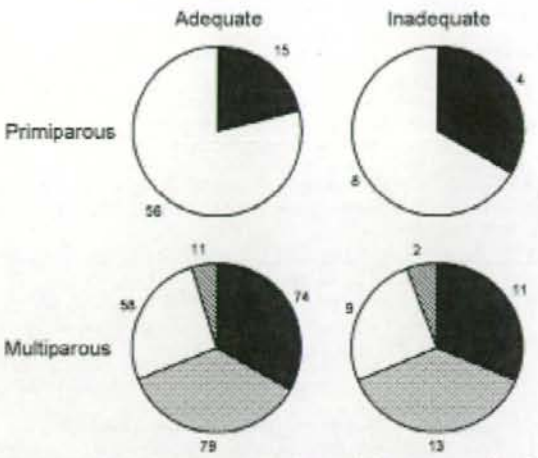

Figure 5. Relationship of maternal behavior of the mothers to that of their daughters. We divided the daughters into 4 groups according to the quality of maternal care they received (adequate or inadequate) and parity (primiparous or multiparous). Numbers show the number of subjects in each group. Black, good; cross-hatched, improved; white, poor; striped, inconsistent.

behavior between the sexes is quite different,[3,16] we did not find any effects of the infant's sex on maternal behavior. Supporting our finding, another study reported that sex of the infant was not a risk factor for abuse or neglect.[15]

Few previous reports about maternal behavior in long-tailed macaques are available; to our knowledge, there are only 2 observational experiments that compared maternal behavior in different rearing conditions.[11,17] Although another article[21] contains statistical data on maternal behavior in captive and peer-reared long-tailed macaques, only 6 subjects were assessed at their first delivery only. Most of the other studies we cite involve other *Macaca* species, and interspecies differences might not be negligible. Considering that long-tailed macaques are used in experiments as widely as other species, more information about their maternal behavior is needed.

Although the incidence of inadequate maternal behavior in the TPRC is not unreasonably high, we are apprehensive about the future. The maternal behavior of peer-reared or isolation-reared monkeys may differ from that of mother-reared monkeys,[4] and differences in maternal behavior may exist between laboratory-born and wild-born monkeys in the TPRC.[17] The differences might increase in each subsequent generation, as could the incidence of inadequate maternal behavior. Indeed, in the current study, the proportion of daughters in the group with good maternal behavior was lower than that overall and the ratio of the daughters in the group with poor maternal behavior was higher than that overall (Table 1 and Figure 5). Directly comparing the adequacy of maternal behavior between generations is difficult because the mean number of deliveries of each generation appears to differ. However, we need to determine whether the incidence of inadequate maternal behavior is likely to increase in future generations. Moreover, the SPF level required differs by experiment, and whether the level of social experience for each subgroup of monkeys can be changed depending on the purpose of the proposed research will be important to determine. Perhaps, for example, extension of the pair-rearing period[2] will decrease incidence of abnormal behavior in our monkeys. Further, modification of the cages may

make it possible to avoid disturbing the physical contact among the monkeys living in the adjoining cages.[5] For TPRC—and other facilities—to continue supplying high-quality monkeys, such possibilities need to be considered.

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ORIGINAL PAPER

Administration of Ag85B showed therapeutic effects to Th2-type cytokine-mediated acute phase atopic dermatitis by inducing regulatory T cells

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Abstract Increase in the number of patients with atopic dermatitis (AD) has been recently reported. T helper (Th) cells that infiltrate AD skin lesions are Th2-type dominant; reduced exposure to environmental Th1-cytokine-inducing microbes is believed to contribute to the increased number of AD patients. Regulatory type immune responses have been also associated with the occurrence of AD. It has been reported that antigen 85B (Ag85B) purified from mycobacteria is a potent inducer of Th1-type immune response in mice as well as in humans. In this study, we have examined the effect of plasmid DNA encoding Ag85B derived from *Mycobacterium kansasii* on AD skin lesions induced by oxazolone (OX) application. Th2-cytokine mediated mouse AD model with immediate type response followed by a late phase reaction was developed by repeated applications of low-dose OX to sensitized mice. Mice were immunized with plasmid DNA encoding cDNA of Ag85B before OX sensitization or during repeated elicitation phase. Both therapies were associated with significant suppression of immediate type response, clinical appearance, dermal cell infiltration, reduced IL-4 production, and augmented IFN-γ mRNA expression compared to placebo-treated mice. Additionally, increased number of Foxp3$^+$ regulatory T cells were observed in the skin sections in Ag85B treated mice. The results of this study suggest that Ag85B DNA vaccine is a potential therapy for Th2 type dermatitis.

Keywords Atopic dermatitis · Antigen 85B · Regulatory T cell

Abbreviations

AD	Atopic dermatitis
Th	T helper
BCG	*Bacillus Calmette-Güerin*
Treg	Regulatory T cell
Ag85B	Antigen 85B
OX	Oxazolone

Introduction

It is known that acute phase skin lesion in atopic dermatitis (AD) is associated with enhanced secretion of T helper (Th) 2-type cytokines [8]. Increased incidence of atopic disorders has been reported in industrialized countries; according to the hygiene hypothesis, the increase in the incidence of patients may be explained by a better lifestyle and less exposure to environmental microbes [5, 7, 28]. Environmental microbes such as mycobacteria or certain virus may promote Th1-type immune response and thus reducing atopy-associated Th2-type reaction. For instance, the study

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carried out in Japanese *Bacillus Calmette-Güerin* (BCG)-vaccinated school children showed that responders to tuberculin had a lower prevalence of atopic disease compared to tuberculin non-responders [28]. BCG-treated mice showed suppression of experimental allergic responses [12]. More recently, it has been shown that microbial stimulation can induce regulatory T (Treg) cells with the ability to suppress both Th1-type and Th2-type inflammation [35]. In the experimental model of pulmonary inflammation, *Mycobacterium vaccae* reduces allergic pulmonary inflammation significantly by increasing the number of Treg cells that secretes IL-10 and TGF-β[37]. These observations indicate that shift from Th2 to Th1 type immune response by mycobacteria may be used for the prevention and treatment of atopic disorders.

The specific antigens eliciting Th1-type immune responses in mycobacteria have not been elucidated so far; a recent study suggested that one of the specific proteins for Th1 development is antigen 85B (Ag85B) [31]. Ag85B is a 30-kDa major protein secreted from all *Mycobacterium* species and that belongs to the Ag85 family[4]. The Ag85B can induce a strong Th1-type immune response in mice as well as in humans [31], and DNA vaccines encoding Ag85B have been reported to protect animals from tuberculosis infection by inducing Th1 response [34, 36]. We have previously reported enhancement of anti-tumor specific CTL response using Ag85B-transfected tumor cells, and by inducing Th1-type immune responses as a vaccine adjuvant [22, 30].

The purpose of the present study was to evaluate the therapeutic efficacy of Ag85B derived from *M. kansasii* in acute phase dermatitis. Repeated applications of hapten such as oxazolone (OX) on BALB/c mice causes delayed type hypersensitivity in the beginning that changes to an immediate-type response in the late phases with elevated IgE production, and deviation of Th cell responses. The skin lesions that appear in late phases are compatible with the clinical findings as well as cytokine profile observed in AD [19, 21]. In all Ag85B-treated AD mice, the immediate type reaction is effectively suppressed and IL-4 is significantly reduced. The results of this study provide evidence for the potential usefulness of Ag85B as a novel approach for the treatment of Th2 type-mediated dermatitis such as AD.

Materials and methods

Animals

Six-week-old BALB/c male mice were purchased from Japan SLC Co. (Shizuoka, Japan) and used at the age of 7 weeks. Animal care was done according to ethical guidelines, and approved by the Institutional Board Committee for Animal Care and Use of Mie University.

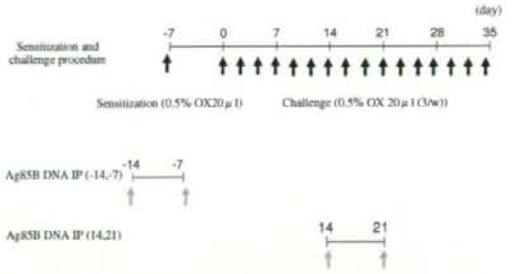

Fig. 1 Model of chronic contact hypersensitivity, and treatment with Ag85B DNA

Sensitization and challenge of animals

Oxazolone was purchased from Sigma (St Louis, MO, USA), and dissolved in acetone/olive oil (1:1). As shown in Fig. 1, mice were initially sensitized by pasting 20 μl of 0.5% OX solution to their left ear 7 days prior to the first challenge (day -7) and then 20 μl of 0.5% OX solution was repeatedly applied on the left ear three times per week from day 0. The ear swelling response was expressed as the difference between before and 30 min after application. The Ag85B expression vector pcDNA-Ag85B of *M. kansasii* open reading frame lacking a signal sequence has been constructed into KpnI–ApaI sites of pcDNA3.1 as described previously [22]. Plasmid DNAs were purified using the Plasmid Mega Kit (Qiagen, Chatsworth, CA, USA). The empty plasmid pcDNA3.1 was used as a control. Plasmid DNAs were diluted with sterilized physiological saline. Hundred micrograms per mouse of plasmid DNA was injected intraperitoneally on day -14, -7 to evaluate prophylactic effects, or on day 14 and 21 for the assessment of therapeutic effects.

Histological analysis

Skin specimens obtained 30 min after the final challenge were fixed in 10% buffered neutral formaldehyde and embedded in paraffin. Sections prepared of 7 μm thickness were stained with hematoxylin and eosin (H&E), or truidine blue.

Immunohistochemistry

The left ear was sacrificed on day 35, and was embedded in Tissue-Tek OCT compound (Miles, Elkhart, USA), frozen in liquid nitrogen, and cut with a cryostat into 7 μm-thick sections. The tissue preparations were then incubated with