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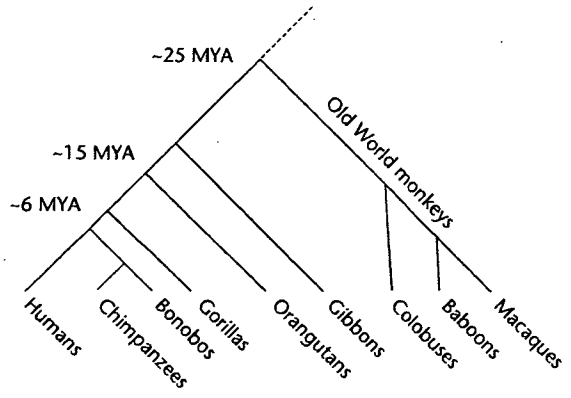
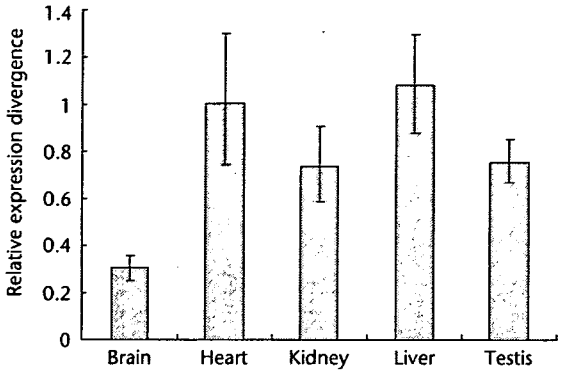
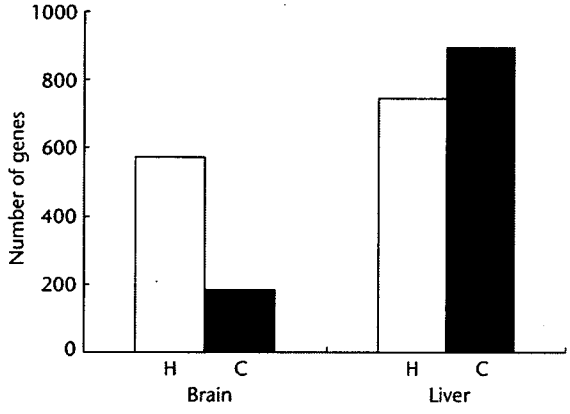
Author(s) and affiliation(s): Osada, Naoki / National Institute of Biomedical Innovation, Ibaraki, Osaka, Japan; Sumio, Sugano / University of Tokyo, Tokyo, Japan; Suzuki, Yutaka / University of Tokyo, Tokyo, Japan



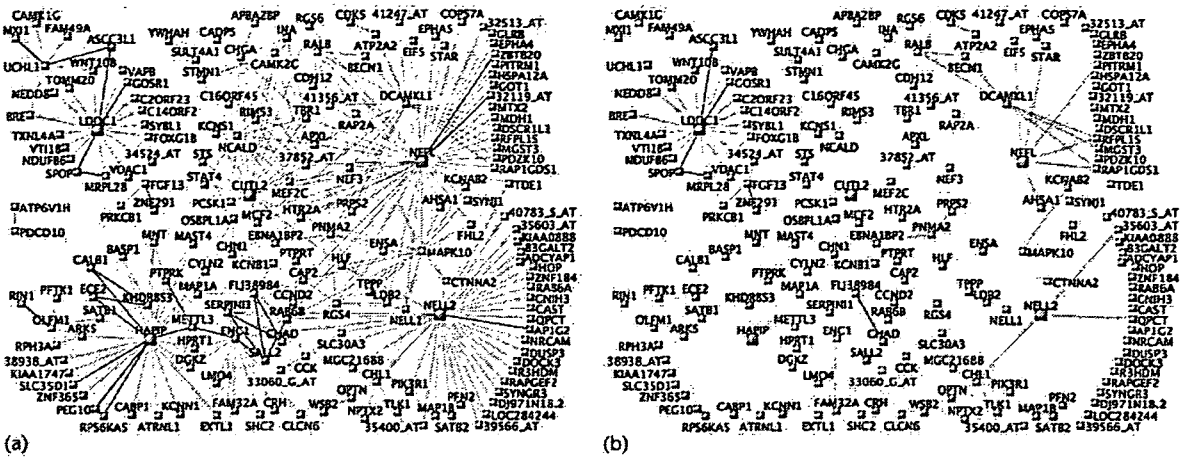
f0001 **Figure 1** Phylogenetic tree of the primates of the group *Catarrhini*. The closest relatives of humans are chimpanzees (*Pan troglodytes*) and bonobos (*Pan paniscus*); however, some parts of the human genome are more closely AU:8 related to the gorilla (*Gorilla gorilla*) genome.



f0002 **Figure 2** Divergence of gene expression between humans and chimpanzees in five tissues. Genes specifically expressed in the target tissues were analysed. The height of the bars represents the average amount of expression divergence over all possible pairwise comparisons. Error bars show 95% confidence intervals estimated by bootstrap resampling. Data are from AU: 9 Khaitovich *et al.* (2005).



f0003 **Figure 3** The number of genes expressed differentially between the lineages of the humans (open bars, H) and chimpanzees (solid bars, C) in the brains and livers ($P \leqslant 0.05$). Orangutans were used as the outgroup. The data was obtained by Enard *et al.* (2002) and reanalysed by Gu and Gu (2003).



f0004 **Figure 4** Human-specific hub genes identified by module visualization. (a) Three hundred pairs of genes with the greatest connectivity in the human cortex are represented by thin lines. Genes with expression levels that are negatively correlated are connected by thick lines. (b) Connections from (a) that are specific to the human cortex. Reproduced from Oldham *et al.* (2006) by permission of National Academy of Sciences, USA.

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Th1-type immune responses by Toll-like receptor 4 signaling are required for the development of myocarditis in mice with BCG-induced myocarditis

Kimiaki Nishikubo [a], Kyoko Imanaka-Yoshida [b], Shigenori Tamaki [a], Michiaki Hiroe [c], Toshimichi Yoshida [b], Yukihiko Adachi [d], Yasuhiro Yasutomi [e,f,*]

[a] *Department of Rheumatology, National Hospital Organization, Mie Chuo Medical Center, Mie 514-1101, Japan*
[b] *Department of Pathology, Mie University Graduate School of Medicine, Mie 514-8507, Japan*
[c] *Department of Nephrology and Cardiology, International Medical Center of Japan, Tokyo 162-8655, Japan*
[d] *Department of Gastroenterology and Hepatology, Mie University Graduate School of Medicine, Mie 514-8507, Japan*
[e] *Department of Bioregulation, Mie University Graduate School of Medicine, Mie 514-8507, Japan*
[f] *Laboratory of Immunoregulation and Vaccine Research, Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Ibaraki 305-0843, Japan*

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Abstract

The immunological aspects of autoimmune myocarditis are difficult to understand because of the existence of many infectious agents and animal models suggesting different mechanisms in autoimmune myocarditis. To overcome these difficulties, two strains of mice, C3H/HeN and C3H/HeJ, showing different immune responses to mycobacteria, were immunized with myosin mixed with BCG. The C3H/HeN mice with a wild-type Toll-like receptor 4 (TLR4) showed severe myocarditis, whereas the C3H/HeJ mice with nonfunctional mutated TLR4 did not. $CD4^+$ cells from both strains of mice exhibited appreciable proliferative responses following myosin stimulation; however, the cytokines from these cells differed between these two strains. The C3H/HeN mice showed T helper (Th)1-type cytokine responses, whereas the expressions of mRNA in C3H/HeJ mice were Th2-type cytokine. When both of these strains of immunized mice were inoculated with a plasmid encoding cDNA of interleukin (IL)-4 or agonistic IL-4, the development of myocarditis was inhibited in C3H/HeN mice. Moreover, C3H/HeJ mice, in which development of myocarditis was not induced by immunization of myosin mixed with BCG, showed myocarditis after injection of IL-4 antagonistic mutant DNA for the induction of Th1-type immune responses. The results suggested that the induction of autoimmune myocarditis by myosin is affected by Th1-type immune responses.

Keywords: Autoimmunity; Bacillus Calmette–Guérin; Myocarditis; Th1/Th2; TLR4

1. Introduction

Myocarditis is a potentially lethal disorder of various etiologies for which no treatment is currently satisfactory [1]. Although the etiology of dilated cardiomyopathy is unknown, more than 10% of cases are associated with a previous virus infection, such as Coxsackievirus B3 [2]. Since heart failure generally occurs long after infection with autoimmune responses, autoimmunity is thought to play an important role in myocarditis as well as contributing to the progression to cardiomyopathy and heart failure [3]. To explore the mechanisms

Abbreviations: DC, dendritic cell; DTH, delayed-type hypersensitivity; EAM, experimental autoimmune myocarditis; TLR, Toll-like receptor.

* Corresponding author. Laboratory of Immunoregulation and Vaccine Research, Tsukuba Primate Research Center, National Institute of Biomedical Innovation, 1-1 Hachimandai, Tsukuba, Ibaraki 305-0843, Japan. Tel.: +81 29 837 2121; fax: +81 29 837 0218.

E-mail addresses: yasutomi@doc.medic.mie-u.ac.jp, yasutomi@nibio.go.jp (Y. Yasutomi).

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of such immune system-mediated damage to the heart in this disease, various animal models have been established by infection of various pathogens and immunization of cardiac myosin (reviewed in [4]). Although animal models of experimental autoimmune myocarditis (EAM) have provided information on pathogenesis that is valuable for the prevention and treatment of myocarditis, an understanding of the pathogenesis of EAM in animal models is difficult to apply to human myocarditis. Animal models of EAM have been established in various species and strains of animals using various types of infectious pathogens and immunization of cardiac myosin, and the pathogenic mechanisms in these models have not shown identical immune responses [5,6]. To overcome these difficulties, animal models of EAM that are established for understanding immune response to myocytes should allow us to identify several factors that induce EAM such as pathogens and the genetic basis of animals.

T helper (Th) cells are thought to have crucial roles in both autoimmune diseases and immunological disorders. Th cells are identified by functions as Th1 or Th2 subsets secreting distinct cytokine patterns that demonstrate effector functions and cross inhibition [7]. Cytokines are important for controlling the response of Th cells to self antigens (Ags) and they play a critical role in shifting the immune response toward a Th1 or Th2 pattern. A Th1 response shifts the cytokine profile toward delayed-type hypersensitivity (DTH), macrophage activation, and proinflammatory T-cell response associated with interferon (IFN)-γ and interleukin (IL)-2 and -12, whereas a Th2 response is associated with B cell activation and humoral immunity and with IL-4, -5, -9, -13 and IgE production. As a result, understanding the Th cell responses to auto-Ags is important for the prevention and treatment of autoimmune diseases such as autoimmune myocarditis in human patients.

Mice with a C3H/He lineage were originally established in 1941, and two laboratories have maintained this strain as C3H/HeN and C3H/HeJ since 1947 and 1951, respectively. These two strains of mice showed different responses to some strains of bacteria, and the differences in the responses to some strains of bacteria have been thought to be caused by Toll-like receptor (TLR) 4 [8]. C3H/HeJ mice have an unfunctional TLR4 [9], and these mice are more susceptible to mycobacteria infection than are TLR4 wild-type mice [10–12]. TLR activation elicits adaptive immune responses with a bias towards Th1 T-cell response. It has also been reported that TLR4 wild-type C3H/HeN mice, but not mutated TLR4 C3H/HeJ mice, showed typical Th1-type immune responses to mycobacteria infection [10,12,13], although both strains of mice have the wild-type Bacillus Calmette–Guérin (BCG) resistant gene *(N-ramp)*.

To elucidate the immunological mechanisms by which cardiac myosin is recognized without various factors, we tried to establish an animal model of EAM by using the responses to mycobacteria in the present study. Two strains of mice, mycobacteria-susceptible C3H/HeJ mice and mycobacteria-resistant C3H/HeN mice, were immunized with porcine cardiac myosin mixed with BCG. Interestingly, mycobacteria-resistant C3H/HeN mice, but not mycobacteria-susceptible C3H/HeJ mice, developed myocarditis. We herein report the differences in immune responses to myosin in the development of an animal model of EAM in mice with a close genetic background.

2. Materials and methods

2.1. Mice

Six- to eight-week-old C3H/HeN (TLR4 wild type) and C3H/HeJ (TLR4 mutated) female mice were purchased from CLEA Japan (Osaka, Japan) and housed in the Laboratory Animal Center of Mie University School of Medicine.

2.2. Immunization of myosin

Each mouse was immunized with 100 μg of porcine cardiac myosin (Sigma) mixed with 1 mg of BCG Tokyo strain (Japan BCG Laboratory, Tokyo, Japan) in IFA into the footpad on day 0 and day 14. The BCG used for immunization was wellground and killed. This myosin and BCG mixture was completely emulsified with IFA. The injection site (footpad) and degree of emulsification are very important for the development EAM. The control mice were injected with the same amount of BCG alone in emulsified IFA. All mice were sacrificed on day 21 for pathological observations (Fig. 1a).

2.3. Administration of DNA

The plasmids encoding cDNA of antagonistic interleukin (IL)-4 double mutant (Q116D/Y119D) (IL-4DM) and agonistic IL-4 single mutant (Q116D) (IL-4SM) have been described previously [14]. The mice were intraperitoneally administered 100 μg of plasmid DNA encoding IL-4, IL-4SM or IL-4 DM on days −7, 0, 7 and 14 to regulate the Th balances. An empty plasmid (pcDNA 3.1) vector was used as a control (Fig. 1b).

2.4. Proliferative responses of spleen cells to porcine myosin

The responding spleen cells obtained from the immunized mice were depleted of $CD4^+$ or $CD8^+$ cells using a commercially available system of magnetic bead-coupled specific antibodies (Abs) to confirm the subset of effector cells. The purity of cells (CD4 or CD8 cells) was confirmed by FACS analysis. The proportion of targeted cells did not exceed 0.01%, and dead cells were removed after cell washing (viability >90%). The cells were resuspended in complete medium and cultured at a concentration of 2×10^5 cells per culture well in a total volume of 0.2 ml with 10 μg/ml of myosin. The same amount of OVA was used as a control Ag. Each culture was performed in triplicate in 96-well microculture plates and was then maintained in a humidified atmosphere of 5% CO_2 at 37 °C. The cultures were harvested using a cell harvester at 96 h after a 6-h pulse with 18.5 kBq/well of [^{3}H]thymidine. The results were calculated from the uptake of [^{3}H]thymidine and expressed as the mean uptake in cpm ± SD of triplicate cultures.

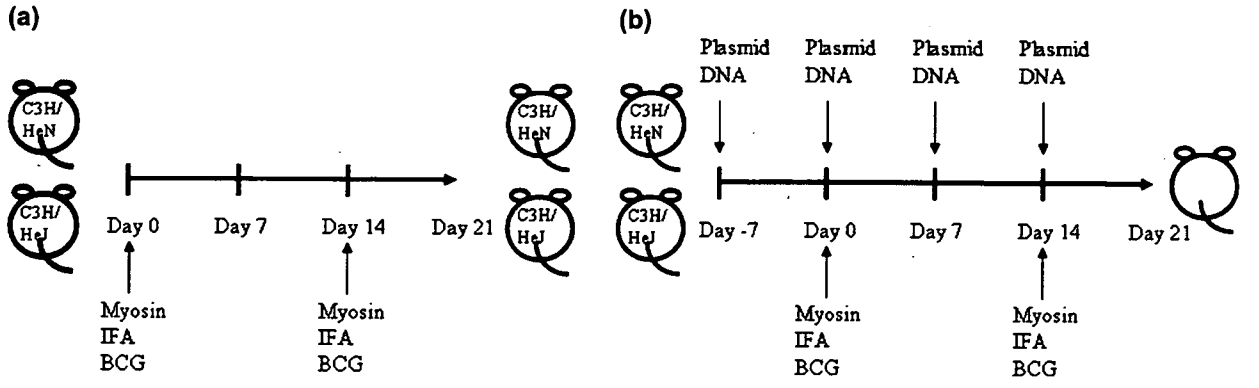

Fig. 1. Experimental design in this study. (a) Each mouse was immunized with 100 μg of porcine cardiac myosin mixed with 1 mg of BCG in IFA into the footpad on day 0 and day 14 (see Section 2). (b) Plasmid DNA of IL-4, IL-4SM, IL-4DM or control was intraperitoneally injected once on days −7, 0, 7 and 14.

2.5. Detection of cytokine mRNA from lymphocytes using RT–PCR

Total RNA was purified from the OVA (control)- or myosin-stimulated spleen cells using Isogen (Nippongene, Japan) following the manufacturer's instructions. For the RT reaction, a reverse transcription system (Promega, WI, USA) was used. PCR was performed in a total volume of 50 μl of 1× PCR buffer (Takara Shuzo, Japan) containing 0.5–1.0 μg of cDNA, 0.25 mM of each dNTP, 2 μM of each primer, and 2.5 U of *Taq* DNA polymerase (Takara Shuzo, Japan). The specific primer pairs used were as follows: IL-2, 5′-AAGAT GAACTTGGACCTCTGCGG-3′ (sense) and 5′-CCTTATG TGTTGTAAGCAGGAGG-3′ (antisense); IL-4, 5′-ATGGGT CTCAACCCCCAGCTAGT-3′ (sense) and 5′-GCTCTTTAG GCTTTCCAGGAAGTC-3′ (antisense); IL-12p40, 5′-TCC TGCACTGCTGAAGACATC-3′ (sense) and 5′-TCTCGCCA TTATAGATTCAGAGAC-3′ (antisense); IL-13, 5′-GACCCA GAGGATATTGCATG-3′ (sense) and 5′-CCAGCAAAGTCT GATGTGAG-3′ (antisense); and mouse HPRT, 5′-GATAC AGGCCAGACTTTGTTGG-3′ (sense) and 5′-GAGGGTA GGCTGGCCTATAGG-3′ (antisense). The samples were amplified for 30–35 cycles under the following conditions: annealing for 30 s at 56 °C, extension for 1 min at 73 °C, and denaturation for 30 s at 93 °C. The reaction products were analyzed on 2% agarose, Tris-buffered EDTA TBE gel.

2.6. Measurement of interferon-γ (IFN-γ)

Spleen cells from immunized mice (5×10^6) were cultured with 10 μg/ml of myosin in 24-well culture plates at a volume of 2 ml. After incubation at 37 °C in a humidified incubator (5% CO_2) for 96 h, culture supernatants were quantified by using a standard ELISA kit (BioSource International, CA, USA).

2.7. Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test and the Kruskal–Wallis test. The values are expressed as means ± SD. A 95% confidence limit was considered to be significant ($p < 0.05$).

3. Results

3.1. Development of EAM

Although rodent models of EAM have been established in various species and strains, it is not easy to understand the mechanisms underlying the development of EAM by recognition of autologous myosin Ags because of the effects of genetic backgrounds. To determine whether cardiac myosin immunization can induce EAM in strains of mice with the same genetic background except for TLR4, C3H/HeN and C3H/HeJ mice were immunized with cardiac myosin mixed with BCG. The histological findings were classified into severe (>50%), moderate (10–49%) and mild (<9%) depending on the ratio of affected area to total myocardium. The C3H/HeN mice developed mild (2/20), moderate (14/20) and severe (4/20) myocarditis, whereas only one C3H/HeJ mouse showed mild myocarditis (1/20) by histopathological observations on day 21 (Fig. 2). Two C3H/HeN mice died before sacrifice on day 18 (severe and moderate myocarditis). Moreover, EAM did not develop in either strain of mice immunized with myosin and IFA without BCG, and the control mice immunized with IFA emulsion containing BCG or myosin alone did not show any abnormalities (data not shown). These results demonstrated that immunization of an emulsion containing myosin and BCG induced the development of EAM in wild-type C3H/HeN mice but not in TLR4-mutated C3H/HeJ mice.

3.2. Myosin-specific spleen cell proliferative responses in myosin-immunized mice

We next confirmed the presence of effector cells that recognize the myosin induced by the immunization of myosin mixed with BCG in *in vivo* experiments. Spleen cells from both strains of mice immunized with myosin mixed with BCG were assessed for their proliferative responses after stimulation *in vitro* with myosin. Spleen cells from both C3H/HeN mice with myocarditis and C3H/HeJ mice without myocarditis exhibited proliferative responses after *in vitro* stimulation with myosin (Fig. 3). These proliferative responses were not observed in the case of stimulation with an irrelevant Ag

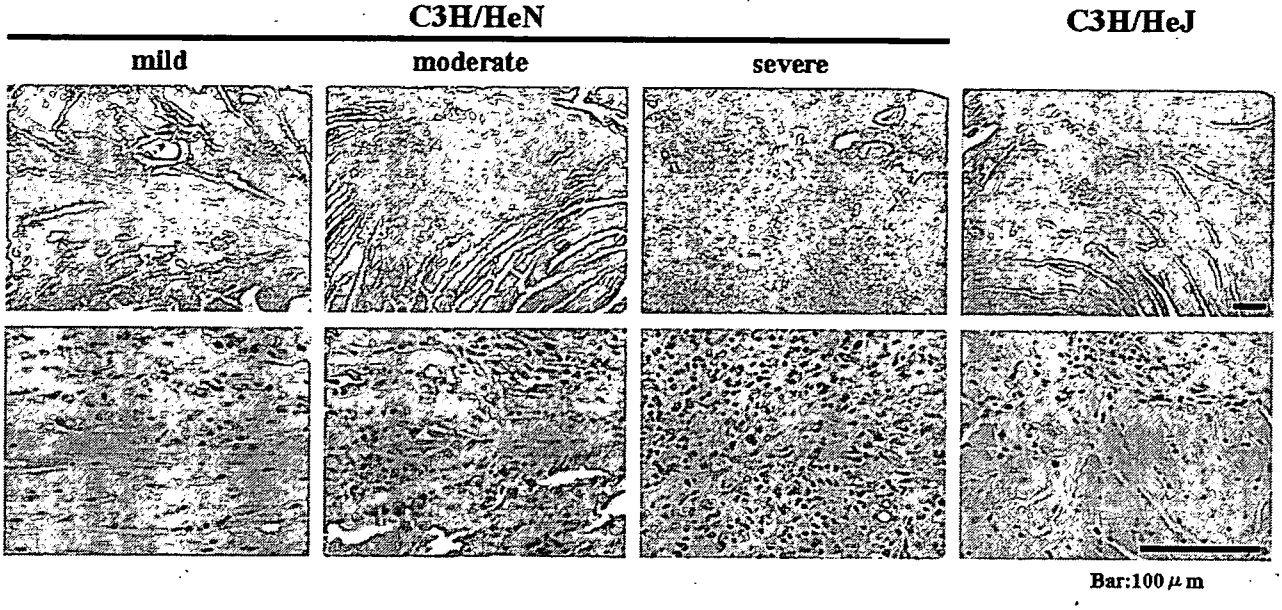

Fig. 2. Results of histopathological examination of hearts from mice that had been immunized with cardiac myosin mixed with BCG in IFA. The histological findings were classified into severe (>50%), moderate (10–49%) and mild (<9%) depending on the ratio of affected area to total myocardium. All tissue specimens were obtained 21 days after the first myosin immunization. The tissue specimens were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. These results are representative of five independent experiments. Bars represent 100 μm.

(OVA). A significant component of this proliferative response was attributed to the presence of $CD4^+$ cells, because $CD4^+$ cell-depleted spleen cells exhibited a substantially reduced myosin-specific proliferative response. Moreover, the C3H/HeN mice did not develop myocarditis when they received a monoclonal Ab to CD4 for depleting $CD4^+$ cells (data not shown). The observation that immunization of myosin mixed with BCG in both strains of mice elicited a $CD4^+$ proliferative T-lymphocyte response suggested that myosin mixed with BCG induces a myosin-specific Th cell response in not only mice with myocarditis but also in mice without myocarditis.

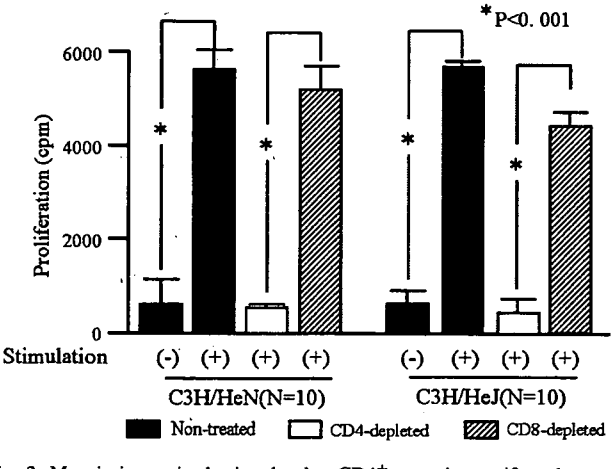

Fig. 3. Myosin-immunized mice develop $CD4^+$, myosin-specific spleen cell proliferative responses. Responding spleen cells were depleted of $CD4^+$ or $CD8^+$ cells using a commercially available system of magnetic bead-coupled specific Abs and co-cultured with myosin. Each value is the mean cpm ± SE of ten mice/group. $*p < 0.001$.

3.3. Myosin-specific cytokine responses of spleen cells from immunized mice

To elucidate the immunological qualities of myosin-specific $CD4^+$ T cells, myosin-specific cytokine responses were analyzed in experimental mice. The responses of myosin-specific cytokines in spleen cells obtained from the experimental mice on day 21 were examined by two different methods. The production of IFN-γ from spleen cells after stimulation *in vitro* with myosin or OVA (control) was assessed by ELISA. Spleen cells from C3H/HeN mice with myocarditis immunized with myosin mixed with BCG produced a significantly larger amount of IFN-γ in the supernatant of the culture than did spleen cells from C3H/HeJ mice without myocarditis after stimulation *in vitro* with myosin (Fig. 4a). We next assessed the mRNA expression levels of Th1-type cytokines (IL-2 and -12) and Th2-type cytokines (IL-4 and -13) in spleen cells after *in vitro* stimulation with myosin or OVA (control). Spleen cells from C3H/HeN mice with myocarditis showed strong IL-2 and -12 expression and weak IL-4 and -13 expression of mRNA, whereas completely opposite results were obtained for spleen cells from C3H/HeJ mice without myocarditis. Spleen cells from C3H/HeJ mice without myocarditis showed strong expression of mRNA of Th2-type cytokines (IL-4 and -13) and weak expression of Th1-type cytokines (IL-2 and -12). Moreover, spleen cells from C3H/HeN mice treated with myosin and IFA without BCG showed Th2-type immune responses (data not shown). Upstream of the release of some Th1-type cytokines are the TLRs, and C3H/HeN mice with wild-type TLR4 developed EAM while also showing a Th1-type immune response to myosin. Although induction of another T-cell linage, Treg, was also assessed by the expression

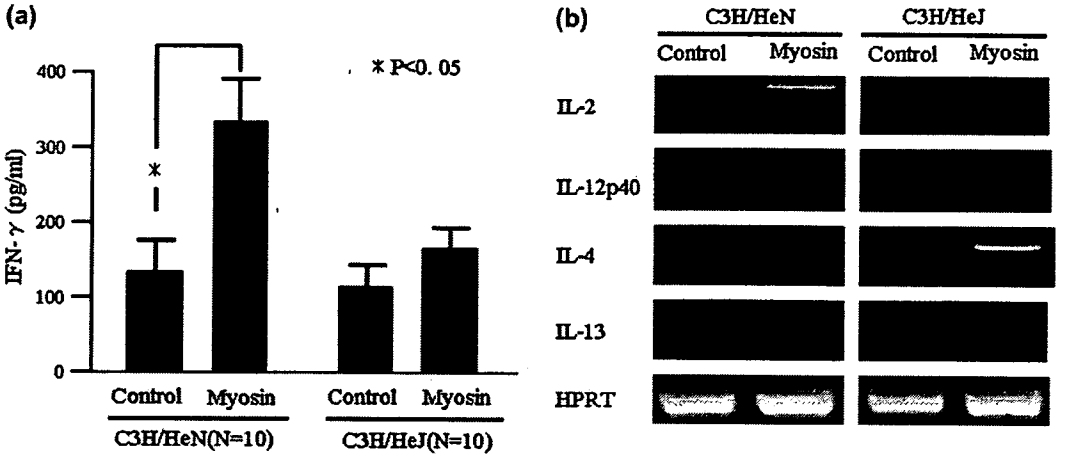

Fig. 4. Cytokine production in culture supernatant and expression of mRNAs of cytokines from spleen cells. (a) The amount of IFN-γ in the culture supernatant was measured by ELISA 21 days after the first myosin immunization. Each value shown is the mean and SD of ten mice per group. (b) Spleen cells were stimulated *in vitro* with myosin for 1 day in culture. Spleen cells stimulated with OVA were used as controls. The reaction products were analyzed on 2% agarose, Tris-buffered EDTA TBE gels. The profiles are representative of three independent experiments.

of mRNA of IL-10 and transforming growth factor-β, these cytokines were not different in C3H/HeN mice with myocarditis and C3H/HeJ mice without myocarditis (data not shown). These results indicated that the $CD4^+$ cells of C3H/HeN mice (TLR4 wild type) and C3H/HeJ mice (TLR4 unfunctional mutated) were polarized toward different Th responses after immunization by an emulsion of myosin mixed with BCG followed by proliferation.

3.4. Effects of IL-4, IL-4SM or IL-4DM DNA administration on the development of myocarditis in mice immunized with myosin mixed with BCG

To examine the effects of regulating Th responses using IL-4, IL-4SM (agonistic IL-4 single mutant) and IL-4DM (antagonistic IL-4 double mutant) DNA on the development of myocarditis in both strains of mice immunized with myosin mixed with BCG, the mice were intraperitoneally administered 100 μg of DNA vaccine or control plasmid on days −7, 0, 7 and 14 (Fig. 1b). The IL-4 mutant Q116D/Y119D, which forms unproductive complexes with the IL-4Rα-chain, acts as an antagonist by inhibiting the formation of heterodimers with other receptors [15]. These IL-4-binding inhibitors act not only by inhibiting IL-4 binding to its receptor but also by preventing IL-13 from eliciting its activity, since the IL-4Rα-chain also forms a functional signaling component of the IL-13R heterodimer [16,17]. We previously reported that such plasmid administration can regulate the systemic Th immune responses in autoimmune and allergic diseases by only a single injection [14]. C3H/HeN mice, in which the development of myocarditis was induced by immunization of myosin mixed with BCG, did not develop myocarditis when they were injected with the IL-4 and IL-4SM (agonistic IL-4 mutant) DNA vaccines to inhibit the Th1-type immune response ($n = 10$ respectively) (Fig. 5). On the other hand, C3H/HeJ mice, in which the development of myocarditis was not induced by the same immunization, were not affected by the injection of IL-4 and IL-4SM DNA vaccines ($n = 10$ respectively). However, surprisingly, C3H/HeJ mice developed myocarditis (mild in 3/10 and moderate in 7/10) when they were injected with antagonistic IL4 mutant, IL-4DM, DNA vaccine for inhibition of Th2-type immune responses by prevention of IL-4 signaling (Fig. 5). The injection of control plasmid did not influence the development of myocarditis in either strain of mice ($n = 10$ respectively) (Fig. 5). Mice did not develop myocarditis without BCG in any plasmid DNA injected (data not shown). These results were derived by four injections of DNA, although the experimental model of allergic inflammation was inhibited by only a single injection of DNA. We previously reported that administration of IL-4DM DNA did not change the Ag-specific Th responses in cytokine production by *in vitro* stimulation of Ag without the presence of IL-4DM protein [14]. In fact, the results for myosin-specific cytokine production from spleen cells of both strains of mice administered IL-4, IL-4SM or IL-4DM DNA vaccines were the same as the results shown in Fig. 4 (without injection of DNA vaccines) after *in vitro* stimulation of myosin (data not shown). The existence of a small amount of IL-4 or IL-4 mutant for a long time *in vivo* might have played a role in the development of EAM. Since pharmacokinetic half-lives of IL-4 and IL-4 mutant proteins are very short *in vivo* ($t_{1/2} = 0.83$ h), a high concentration of these molecules in plasma must be maintained for a long period in order for effects on various phenotypes to be obtained. These effects usually disappeared immediately after discontinuing administration of these proteins. The antagonistic IL-4 is more effective than neutralizing Ab to IL-4, and commercially available Abs to IL-4 do not have sufficient effects to inhibit the activity of IL-4. These results indicated that the regulation of functions of IL-4 played an important role in the development of myocarditis induced by the immunization of myosin mixed with BCG in the strains of C3H/He mice. These results also indicated the possibility

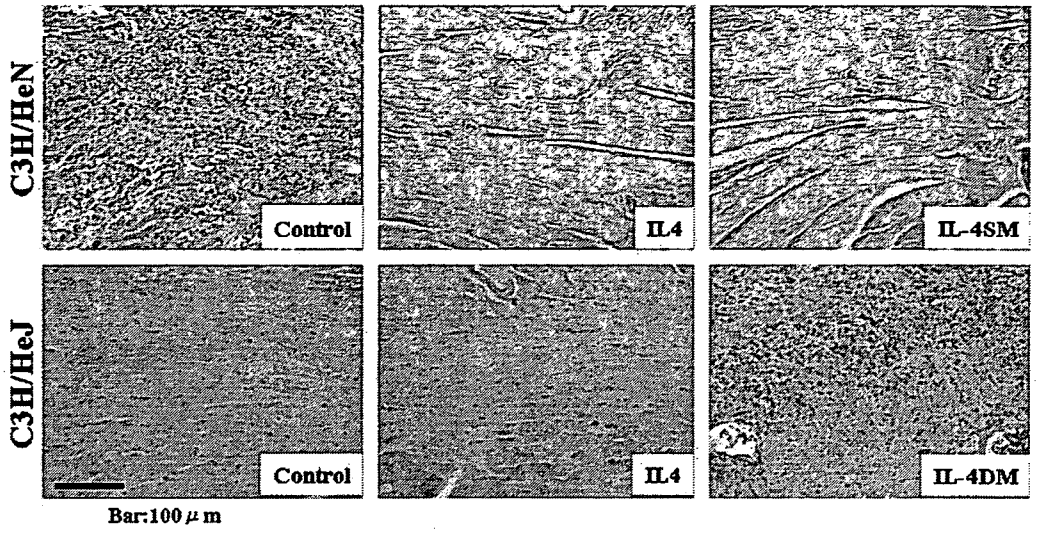

Fig. 5. Results of histopathological examination of hearts from myosin-immunized mice that had been administered IL-4, IL-4SM, IL-4DM or control. All tissue specimens were obtained 21 days after the first myosin immunization. The tissues specimens were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Mice that had been immunized with myosin mixed with BCG in IFA were each injected with 100 μg of IL-4, IL-4SM, IL-4DM or control plasmid DNA four times on days −7, 0, 7 and 14. These results are representative of three independent experiments. Bars represent 100 μm.

that myocarditis is associated with Th1-type $CD4^+$T-cells in these strains of mice.

4. Discussion

$CD4^+$ T cells have been reported to be required for the induction of EAM in mice. The development of EAM in A/J mice was prevented by the depletion of $CD4^+$ T cells, and disease severity was reduced by depleting $CD8^+$T cells (reviewed in [18,19]). A widely held belief is that when the cytokine profile of autoreactive T cells shifts toward an inflammatory Th1 type, the result is pathogenicity and autoimmune diseases (reviewed in [20,21]). Autoimmune myocarditis in the Lewis rat model was promoted by Th1-type immune responses in the same manner as that seen in our experiments [22]. On the other hand, it is difficult to understand the development of myocarditis in the EAM mouse model based on the Th1/Th2 paradigm. Although Th2-type immune responses played a critical role in the development of myocarditis in the mouse model of EAM using A/J and BALB/C strains [23,24], Th1-type immune responses were also suggested to participate in the development of EAM. Moreover, it has been reported that another novel population of T cells, regulatory T cell (Treg), are also important for controlling development of EAM as well as other disease [25–30]. These reports suggested that loss of immune tolerance regulated by Treg cells are one of the mechanisms of development of EAM. It has been reported that EAM did not develop in mice deficient in the Th1-type cytokine IL-12 or its receptor after administration of myosin or myosin Ag peptide, and that the CC-chemokine secreted by Th1-type T cells mediates EAM [31–33]. These differences in immune responses in EAM models are thought to be due to the correlation between mouse strain and mycobacteria species. The EAM model of A/J was established by using a large amount of virulent mycobacteria (*Mycobacterium tuberculosis*), and a small amount of the same bacteria was used for BALB/c mice. We used avirulent *Mycobacteria bovis* BCG (vaccine strain) for establishment of EAM. The A/J mouse strain is susceptible to *Mycobacterium tuberculosis* and resistant to BCG. C3H/HeN mice are resistant to both strains of mycobacteria with the same responses as those in humans, and BALB/c mice show responses opposite to those of A/J mice (resistant to *Mycobacterium tuberculosis* and susceptible to BCG) [34–36]. These differences are dependent on various genetic factors such as *Nramp* gene.

Some studies have suggested a relationship between TLR4 and myocarditis. Infection with a Coxsackievirus, which is a well-known agent of myocarditis, was found to upregulate TLR4 on mast cells and macrophages immediately following infection. TLR4 signaling also increases the occurrence of acute myocarditis and production of proinflammatory cytokines in the heart [37]. Moreover, the critical requirement of TLR4 signaling in dendritic cells (DCs) for myocarditis induction was genetically proven by the fact that myosin Ag peptide (MYHC-α)-pulsed and TLR4/CD40-activated DCs isolated from TLR4-deficient mice did not induce myocarditis in wild-type recipients when DCs isolated by the same procedure from TLR4-wild type transfer elicited myocarditis in a wild-type recipient [38]. In our system, two important points regarding the establishment of EAM were observed. Porcine myosin must be mixed well in IFA, and EAM was only observed by immunization of this emulsion into the footpad. These observations suggest that myosin Ag is incorporated in the same APCs such as macrophages or DCs together with BCG for a long period of time as oil particles, and then myosin-specific immune responses are induced by the influence of the characteristic immune responses of a large quantity of BCG associated with TLR4.

Relationships between TLRs and mycobacteria have been reported. TLR2, TLR4 and TLR1/TLR6 heterodimers with

TLR2 have been implicated in the recognition of mycobacterial Ags [39]. The emerging concept of TLRs as key molecules for shaping the quality of immune responses against microbes is further supported by results of experiments showing that mice lacking MyD88 are incapable of developing Ag-specific Th1 responses after immunization with OVA mixed with CFA (containing dead mycobacteria as an active component) [40]. These results are thought to be due to a mechanism involving both TLR2 and TLR4, since CFA contains a complex mixture of mycobacterial components, some of which are recognized by different members of the Toll family, TLR2 and TLR4. The EAM established in our system utilized these TLRs by immunization with extremely large amounts of BCG and cardiac myosin as a mixed emulsion, thus suggesting the importance of TLR4 for the induction of Th1-type immune responses related to EAM. The C3H/HeJ mouse, which has unfunctional TLR4, showed Th2-type immune responses to myosin after immunization of cardiac myosin mixed with BCG (Fig. 4). Mycobacteria induce Th1-type immune responses through TLR2 and TLR4 stimulation; however, our results showed Th2-type immune responses through TLR2 stimulation without TLR4 by BCG as an adjuvant. Similar results have also been reported by other investigators. Th2-type cytokines were induced from DCs by mycobacteria dependent on TLR2-mediated recognition but not TLR4-mediated recognition [41]. In our experiment, TLR4 mutant C3H/HeJ and wild-type C3H/HeN mice were used for analysis of Th responses in EAM. Unfunctional TLR4 mutations in humans have also been reported (reviewed in [42]). Studies using TLR4 knockout mice are needed to clarify this. Since the commonly used mouse background to generate knockout mice is associated with an increased susceptibility to mycobacteria, extensive backcrossing of such mice is required [43].

Many animal models of myocarditis are available to investigate the optimal therapy for myocarditis. However, the establishment of new animal models of myocarditis is still necessary to better understand myocarditis, because the understanding of myocarditis in humans is still insufficient. In the present study, we utilized two strains of C3H/He mice, which showed different susceptibilities to BCG, for the establishment of EAM involving Th1-type immune responses. The results of this study provide evidence of the potential utility of studying immunological mechanisms in order to both treat and prevent myocarditis.

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Chapter 20

Chimeric Recombinant Hepatitis E Virus-like Particles Presenting Foreign Epitopes as a Novel Vector of Vaccine by Oral Administration

Yasuhiro Yasutomi[*,†]

Virus-like particles (VLPs) are useful for studies on virion formation, host immune responses to viruses, and vaccines in no practical cell culture systems to allow the growth of virus. Among the various non-replicating molecules, VLP, an empty particle with a structure similar to that of an authentic virus particle, offers the possibility of a new approach for these studies. Hepatitis E virus (HEV) is an unclassified calicivirus-like, positive-strand RNA virus that causes human acute hepatitis by fecal-oral transmission. HEV first infects epithelial cells of the small intestine and then reaches the liver through the portal vein. It has been reported that intact open reading frame 2 (ORF2) of HEV is expressed as a membrane glycoprotein when artificially expressed in mammalian cells *in vitro*, probably because the N-terminal amino acid sequence serves as a signal peptide.[1,2] On the other hand, the intact ORF2 expression in insect cells resulted in various sizes of proteins with cleavages on both N- and C-termini, of which 53 kD polypeptides secreted in the culture supernatant.[3–5] It has also been reported that only after cleavage of C-terminal, which results in molecular weight reduction to

*Laboratory of Immunoregulation and Vaccine Research, Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Ibaraki 305-0843, Japan.
†Department of Immunoregulation, Mie University Graduate School of Medicine, Mie 514-8507, Japan. E-mail: yasutomi@nibio.go.jp; yasutomi@doc.medic.mie-u.ac.jp.

54 kD, does ORF2 participate in the VLP formation.[6,7] In this chapter, investigation of HEV-VLP could be carried a molecule for foreign antigenic epitopes and to stimulate mucosal immunity without the need for adjuvant.

Chimerization of VLP

Chimerization of VLP is a unique and useful method for studying morphology, assembly and host recognition of a virus. However, there are not many reports about chimeric VLP in the fields of virology and immunology. Moreover, the induction of immune responses through natural infectious route against not only VLPs but also carrying epitopes is limited.[8–12] Some chimeric plant virus particles carrying foreign epitopes have been reported.[13] These chimeric virus particles are replication-competent and elicit immune responses through mucosal immunization. These particles were derived by foreign epitope insertion in a cDNA of a virus. Chimeric VLPs obtained by the same method have also been reported.[8–11] Successful chimerization is dependent on selection of an appropriate insertion site in VLPs. Another system for chimerization of VLP is co-infection of a couple of baculoviruses in the same cells, which allows VLPs to be obtained as chimeric VLPs.[12] This method is an easy way to obtain the chimeric VLP, although the stability of molecular constructs and the characteristics of morphology to original virus are not promised. Chimeric VLPs obtained by foreign-molecule insertion are suitable for studying morphogenesis of viruses and host recognition to both VLPs and inserted molecules.

HEV-VLP

Hepatitis E is an acute viral hepatitis caused by infection with HEV that was first recognized in India 1955. The HEV has been isolated from various animals, suggesting that hepatitis E is a zoonosis.[14,15] Although an *in vitro* culture system to amplify HEV has not been developed, over-expression of a part of ORF2 in a baculovirus expression system allows this protein to assemble into a VLP.[6] Formation of this VLP occurs only when N-terminal of ORF2 — where potential

signal sequence is encoded — was deleted from the expression construct.[6,16] It has also been reported that additional endogenous cleavage of 52 amino acids at the C-terminal is necessary for the assembly of a VLP.[6,7] HEV-VLP appears as an empty particle of a slightly smaller size than that of a mature HEV particle.[6,7] An HEV-VLP has several advantages for studying virus formation or host recognition. In our experience, large amounts can be easily obtained from standard cultivation protocols compared with amounts of other VLPs obtained. The amount of purified HEV-VLPs collected from culture supernatant of 50 to 100 μg/ml is more than 100 times greater than that of other VLPs. It has recently been found that the VLPs elicit strong immune responses when administrated orally into mice as same to a natural infection route.[17] Moreover, it has been shown that oral inoculation of cynomolgus monkeys with HEV-VLP prevents the infection of native HEV by intravenous injection.[18] These findings indicated that HEV-VLPs conserved original HEV construction to enter the target cells. Conservation of the virus construct in VLPs is very attractive for vaccines inducing the same type of immune responses to virus infection.

Chimeric HEV-VLP Carrying Foreign Epitope

pVL5480/7126, a baculovirus transfer vector that includes a portion of the ORF2 from HEV (dORF2), was described previously.[6] To insert the tag sequence within dORF2, oligonucleotides that encode the tag amino acid sequence were synthesized as shown in Table 1, and described previously.[9] The restriction sites used for insertion sites 1 to 4 were *Hind*III, *Sac*II, *Bss*HII, and *Sac*II sites at nucleotide positions 5679, 6245, 6664, and 6773, respectively. For each site, oligonucleotide pairs of Htg5(0) and Htg3(GA), Htg5(+1) and Htg3(0), Htg5(0) and Htg3(GG), and Htg5(+1) and Htg3(0) were used, respectively. A C-terminal tag was added at a position 52 amino acids upstream from the translational terminal. This site was chosen because the last 52 amino acids at the C-terminal of ORF2 are cleaved off during the formation of VLPs. The nucleotide sequences around the inserted tag are schematically shown in Fig. 1. The plasmid containing the chimeric dORF2 was co-transfected

Table 1. Oligonucleotides Used in this Study

Oligonucleotide	Sequence (5′ to 3′)
HEVBacBg	CGCAGATCTATGGCGGTCGCTCCAGCCC
HEV52Pr	CTGCAGCTATGCTAGCGCAGAGTG
Htg5(0)	CAGCCTGAACTCGCTCCAGAGGA
Htg5(+1)	GCCAGCCTGAACTCGCTCCAGAGGA
Htg3(0)	ATCTTCCGGATCCTCTGGAGCGAG
Htg3(GA)	TCATCTTCCGGATCCTCTGGAGCGAG
Htg3(GG)	CCATCTTCCGGATCCTCTGGAGCGAG
Tag(-52)	CTGCAGCTAATCTTCGGGGTCCTCCGGGGCGAGCT CAGGCTGTGCTAGCGCAGAGTGG
BglTag	AGATCTATGGCGCAGCCTGAACTCGCTCCAGAGGA TCCAGAAGATGCGGTCGCTCCAGCCCATGAC

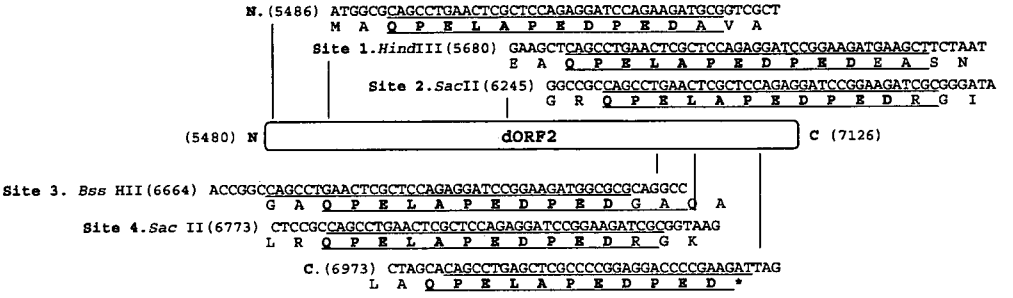

Fig. 1. Schematic diagram and sequences around the tag epitope insertion sites in dORF2. Upper rows show nucleotide sequences and lower rows show the corresponding amino acid sequences. Amino acid numbers relative to the full-length ORF2 are indicated next to the amino acid before the inserted amino acids. Nucleotide numbers referring to HEV genome are in parenthesis. Inserted sequences are underlined. The tag epitope amino acid sequence is in bold face.

with baculovirus DNA, and the recombinant baculovirus was generated as described previously.[6] The production and purification of HEV-VLP was performed as described previously.[6,7]

Utilizing any of the insertion sites, the chimeric dORF2 was expressed at almost equal levels in the cell lysates (Fig. 2A). The antigenicity of the tag epitope was maintained in all cases, as shown by

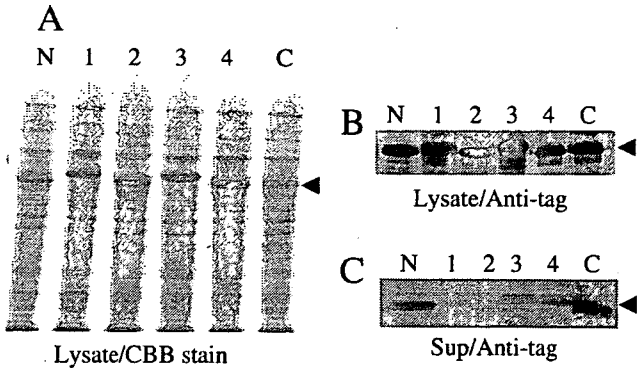

Fig. 2. Expression of chimeric dORF2. A. Expression in the cell lysates was examined by Coomasie brilliant blue staining. B. Antigenicity of the tag epitope in the cell lysates was confirmed by Western blotting with the anti-tag antibody. C. Presence of each chimeric dORF2 in cell supernatant (8 μl) was examined by Western blotting with the anti-tag antibody. The insertion site for each chimera is indicated at the top of the panel. N; N-terminal, 1 to 4; sites 1 to 4, respectively, C; C-terminal. The arrowhead on the right of each panel indicates the position of the chimeric dORF2.

Western blot analysis (Fig. 2B). Even at the C-terminal region, where the native HEV amino acid sequence is cleaved, the tag was not cleaved off from dORF2 (Fig. 2B, lane C). Among these chimeras, only the N- and C-terminal insertions resulted in release of a large amount of chimeric dORF2 into the culture supernatant (Figs. 2C and 2D), although small amounts were released when the insertions were made at either site 3 or 4. These results indicate that internal insertions somehow disturbed the release of dORF2 into the culture supernatant. The precise mechanisms involved in the HEV virion formation are not yet clear. The added tag at 52 amino acids upstream from the C-terminal region, where dORF2 is normally cleaved in insect cells, was not cleaved off in the infected cells during the generation of the chimeric VLPs. This is most likely due to alteration of the amino acid sequence recognized by the proteolytic enzyme involved in the C-terminal modification of HEV-VLP. The successful addition of extra amino acid sequences to the C-terminal of dORF2 suggests that the presence of extra amino acids at the C-terminal is not crucial