NOTE *Virology*

Cytotoxic T-Lymphocyte Activity Specific for Hemagglutinin (H) Protein of Canine Distemper Virus in Dogs

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ABSTRACT. Cytotoxic T-lymphocyte (CTL) responses to hemagglutinin (H) protein of canine distemper virus (CDV) were evaluated in dogs using the replication-deficient adenovirus protein expression system. Skin fibroblasts were isolated from two dogs and were infected with recombinant adenovirus bearing the CDV-H gene (Ade-CDVH). CTL assay was performed using fibroblasts expressing CDV-H protein as target cells and peripheral blood lymphocytes (PBL) collected from the same dogs one week after immunization of CDV as effector cells. Specific cytotoxic activity was observed against autologous but not heterologous fibroblasts expressing CDV-H protein. These results indicate that the CTL epitope(s) were localized in the H protein.
KEY WORDS: canine distemper virus, cytotoxic T-lymphocyte, hemagglutinin.

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Canine distemper virus (CDV) belongs to the genus *Morbillivirus* family *Paramyxoviridae* and induces fatal diseases including encephalitis with demyelination, diarrhea and respiratory disorders in dogs. CDV infection had been well controlled by vaccination. However, epidemics of CDV infection have been appeared in the last decade even in vaccinated dogs [6, 7].

Cytotoxic T lymphocytes (CTL) play an important role in the immune system for the clearance and protection against morbilliviruses [3, 22]. CTL epitopes and responses to measles virus (MV) infection have been well studied in a mouse model. The major target antigen for CTL in mice was identified as the nucleocapsid (N) protein by several groups, which can induce cross reactive CTL between MV and CDV [1–3, 15, 19]. In addition, it was also reported that H protein of MV [1] and CDV [21] could be one of the CTL epitopes in mice. However, Jaye *et al*. reported that the fusion (F) and hemagglutinin (H) proteins were important targets for measles CTL responses in humans, a natural host of MV, receiving measles polypetides [11]. On the other hand, CTL responses in infection with another morbillivirus, rinderpest virus (RPV), were also analyzed in mice and cattle, the latter of which is a natural host of RPV. A CTL response against RPV N protein was detected and a CTL epitope within the N protein was identified in the mouse model [13]. However, the role of N protein in protection and induction of CTL responses against RPV infection in cattle was limited [16]. The CTL response against H protein in cattle is more effective and its effect lasts for at least two years [17, 20]. These discrepant results suggested that the CTL response in the mouse model does not always reflect that in the natural host, and analyses concerning the development of CTL activity using the virus and the natural host are required.

Restriction of the major histocompatibility complex (MHC) in CTL assay makes it difficult to develop an assay system in outbred animals. Recently, an easy method to produce recombinant adenoviruses based on a replication-deficient adenovirus vector was established, and this method has been shown to be useful for delivery of vectors for gene therapy and protein expression [12, 14]. The adenovirus vector possesses useful characteristics such as high level of protein expression, broad host cell range, applicability to non-replicating cells including neural cells, easy preparation of virus in high yield, insertion of large DNA fragments (approximately ~30 kb), and most importantly the viral vector is non-cytopathic because of its proliferation deficiency. Therefore, it can be used as a powerful expression vector to prepare target cells for CTL assay in outbred animals. The vector has been applied in analysis of CTL function in cattle against RPV infection by Ohishi *et al*., and they successfully detected RPV-specific activities [16]. The function of CTL against CDV infection has not yet been studied and thus the major target protein in dogs, the natural host animal, has not been determined. In the present study, we focused on the H protein, which is one of the membrane proteins of CDV and possesses antigenicity [9], for analysis of its inducibility of specific CTL responses to CDV in dogs using a recombinant adenovirus protein expression system.

The recombinant adenovirus expressing CDV-H protein was constructed and produced as described previously [14]. Briefly, a cDNA fragment encoding the full-length CDV-Yanaka H protein (1821 bp, nt 7079–8900) [10] was inserted into a cosmid vector, pAxCAwt (Takara, Kyoto, Japan). This cosmid vector was co-transfected with restriction enzyme-digested adenovirus DNA-terminal protein complex (TPC) into human embryonic kidney 293 cells.

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The recombinant adenovirus, designated as Ade-CDVH, was generated by overlapping recombination. The sequence of the H gene in the Ade-CDVH was identical to that of the parent virus, the CDV Yanaka strain (data not shown). The recombinant virus was expanded in 293 cells and yielded a maximum titer of 2×10^7 PFU/m*l*.

For preparation of target fibroblasts for CTL assay, serial dilution of recombinant virus were infected into fibroblasts established from skin tissues of two healthy beagle dogs (Nos. 1 and 2), and the H protein expression level and cell viability were examined to determine the optimal dose of Ade-CDVH. The two dogs used in this study were maintained in accordance with the Manual for Animal Experiments of the University of Tokyo. All procedures for animal experiments, including virus inoculations and surgical treatments, were also performed in accordance with this manual. At two days post-infection, the number of CDV-H-expressing fibroblasts was determined by fluorescence-activated cell sorter (FACS) analysis (Beckton-Dickinson, Franklin, NJ) using anti-CDV-H monoclonal antibody (d-7) [8]. The CDV-H-positive cell numbers increased in a viral dose-dependent manner with low multiplicity of infection (MOI) (Fig. 1a). The maximum numbers of CDV-H-positive cells were obtained (No. 1, 87%; No. 2, 69%) when the cells were infected with the virus at MOI of 0.1. The number of positive cells was decreased with MOI of more than 0.1. Cell viabilities of the Ade-CDVH-infected fibroblasts were also examined by measuring lactose dehydrogenase (LDH) activity in the culture supernatants (Promega, Madison, WI) (Fig. 1b). Uninfected cells showed viability of approximately 90% or greater after two days in culture. The fibroblasts inoculated with the virus at MOI of 0.01, 0.05 and 0.1 retained viability of more than 80%. However, the virus at MOI of 0.5 or 1.0 killed more than 40% of fibroblasts. From these findings, MOI of 0.1 was considered the optimal dose of Ade-CDVH to obtain the highest number of viable cells with the most efficient CDV-H expression. CDV-H-expressing fibroblasts generated under these conditions were subjected to the following CTL assay.

The same two dogs (Nos. 1 and 2) were then immunized with 1.5×10^4 50% tissue culture infective dose ($TCID_{50}$) of CDV Yanaka [10]. Booster immunization with same dose of the virus was performed two weeks after the first immunization. To confirm successful immunization, humoral immune response in the immunized dogs was confirmed by enzyme-linked immunosorbent assay (ELISA) as described previously [5]. Plasma samples from dogs prior to immunization showed no reactivity against CDV (Fig. 2). Two weeks after the first immunization, anti-CDV antibody responses were elicited in both dogs. Booster immunization at two weeks resulted in the enhancement of the humoral immune response at week 3. These results indicate the successful induction of humoral immunity in both dogs by the CDV Yanaka strain.

The induction of CDV-H-specific CTL in these two dogs was examined. CTL assay was carried out according to the procedure of Ohishi *et al.* with some modifications [16]. Briefly, to obtain an H-antigen mediated CTL response, peripheral blood lymphocytes (PBL) from the two immunized dogs were co-cultured for six days with autologous CDV-H expressing fibroblasts, which had been previously irradiated with 7,500 rad of gamma rays. Then, the stimu-

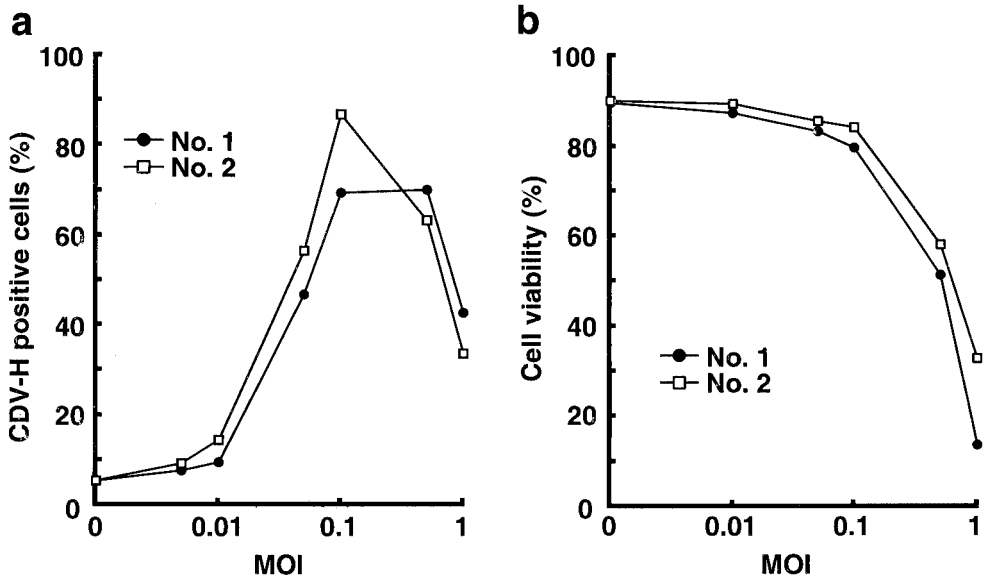

Fig. 1. CDV-H protein expression and viability of the Ade-CDVH-infected fibroblasts. (a) Fibroblasts were infected with Ade-CDVH at the indicated MOI and percentages of CDV-H-positive cells were determined by FACS using CDV-H-specific mAb d-7 two days post-infection. (b) The viabilities of these cells were analyzed by measuring the LDH activity in culture supernatants. Numbers (Nos. 1 and 2) indicate individual dogs.

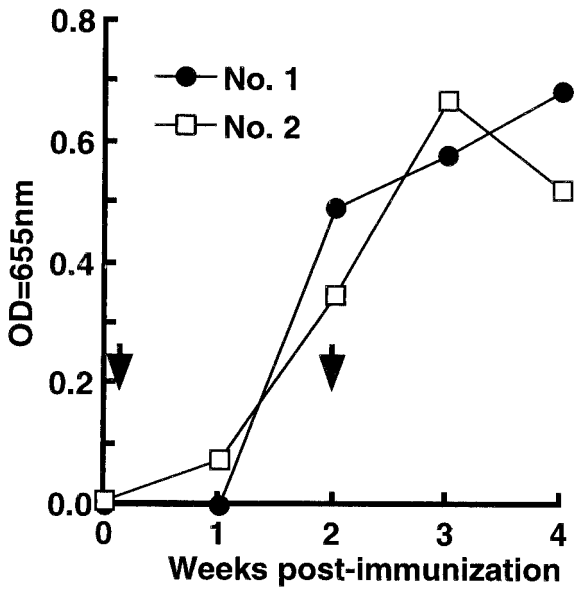

Fig. 2. Humoral immune responses in dogs immunized with the CDV Yanaka strain. Antibody titers in plasma samples collected at the indicated time points were analyzed by ELISA. Arrows show the week of immunization, and numbers (Nos. 1 and 2) indicate individual dog.

lated PBL were co-cultured with ^{51}Cr-labeled target cells, which were autologous fibroblasts infected with or without Ade-CDVH, and heterologous fibroblasts infected with Ade-CDVH. The effector/target (E:T) cell ratios ranged from 30:1 to 0.3:1. Four hours later, culture supernatants were harvested and ^{51}Cr radioactivity was measured. Percent specific lysis was calculated using the following formula: Percent specific lysis=(experimental release-spontaneous release)/(total release-spontaneous release) × 100. While the PBLs from both dogs prior to immunization did not show any specific CTL activity (data not shown), CDV-H-specific CTL responses were raised in both dogs by one week after the first immunization (Fig. 3). At an ET ratio of 30:1, the percentage of specific lysis of CDV-H-expressing autologous target cells were 25.4% and 18.9% from dogs No. 1 and No. 2, respectively. The same effector cells did not show any cytotoxicity towards uninfected mock control cells, wild-type adenovirus infected autologous cells or CDV-H-expressing heterologous cells, indicating that both dogs developed MHC restricted CTLs that recognized CDV-H protein.

One of the membrane proteins of morbilliviruses, H protein, mediates attachment of the virion to cellular receptor(s) and is expressed on the surface of the infected cells. H protein is a major inducer of the humoral immune response and neutralizing epitopes have been reported to be localized on this protein [18]. In the present study, we found that H protein is also a target antigen of specific CTL responses *in vivo*. Previously, Ohishi *et al*. reported that cattle immunized with recombinant vaccinia virus bearing the H gene of RPV were protected from virulent RPV challenge for up to

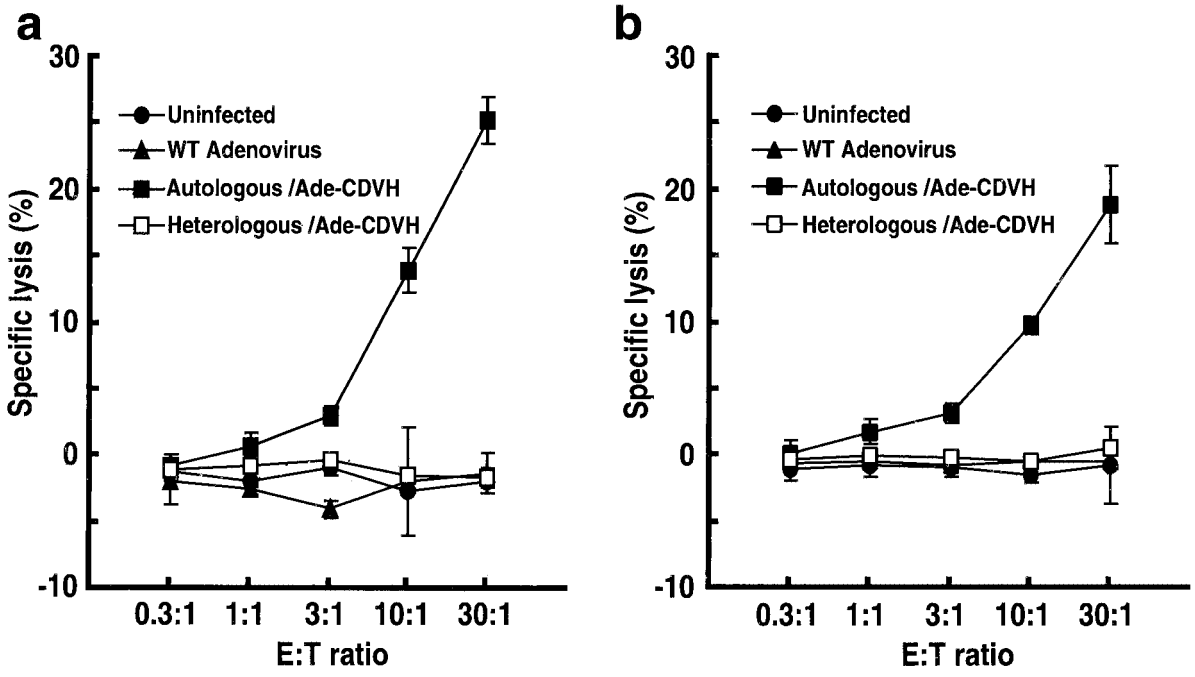

Fig. 3. Cytolytic activity of PBL from immunized dogs against CDV H protein expressing fibroblasts. Effector cells (a, No. 1; b, No. 2) were co-cultured with autologous (closed symbols) or heterologous (open symbols) target cells at E:T ratios ranging from 30:1 to 0.3:1. Results are average of three experiments and vertical bars indicate standard error of each data.

three years even though immunized cattle had an undetectable level of neutralizing antibody at the time of challenge [17]. These findings suggest that CTL responses against H protein play a major role in protective immunity against RPV infection. Both dogs used in this study developed CTL responses by one week post-immunization even though the serum antibody titer of both dogs was undetectable by ELISA at that time. Therefore, our findings also indicate that similar immunological events may occur in dogs immunized with live CDV.

By the end of the 1990s, CDV epidemics occurred even amongst vaccinated dogs. Molecular analyses of the viruses isolated from these dogs showed obvious differences from the vaccine strains such as those described in Onderstepoort and Convac [10]. These findings suggest the possibility that the protective efficacy of currently used vaccines against infection by prevalent CDV is insufficient. Recently, there has been a move towards developing new vaccine strategies against CDV infection using subunit vaccines or DNA vaccines [4]. Therefore, the evaluation of CTL responses would be indispensable to confirm the *in vivo* vaccine efficacy. In addition, it will be necessary to use antigens that can be mounted on all MHC haplotypes. Our strategy for CTL assay will be useful to evaluate the *in vivo* efficacy of new vaccines under development and to determine the function of CTLs in other canine infectious diseases.

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