

Characterization of Canine Herpesvirus Glycoprotein D (Hemagglutinin)

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ABSTRACT. Glycoprotein D (gD) of canine herpesvirus (CHV) YP2 strain was expressed in COS-7 and insect (*Spodoptera frugiperda*; Sf9) cells. The gDs expressed in COS-7 and Sf9 cells reacted with a panel of monoclonal antibodies (MAbs) against CHV gD (hemagglutinin) and an MAbs 25C9 against feline herpesvirus type 1 (FHV-1) gD by indirect immunofluorescence assay, and possessed a molecular weight (MW) of approximately 51–55 and 41–46 kilodalton (kDa), respectively, when examined by immunoblot analysis. After treatment with tunicamycin, the MW of the gD expressed in Sf9 cells became approximately 37 kDa. By hemadsorption (HAD) tests using canine or feline red blood cells (RBC), COS-7 cells expressing CHV gD adsorbed only canine RBC, but not feline RBC, whereas control COS-7 cells expressing FHV-1 gD adsorbed feline RBC, but not canine RBC. By hemagglutination (HA) tests, lysates of Sf9 cells expressing CHV gD agglutinated canine RBC, but not feline RBC. These HA and HAD activities were inhibited by HA-inhibition MAbs against CHV gD. Control lysates of Sf9 cells expressing FHV-1 gD agglutinated only feline RBC. Serum from mice inoculated with lysates of Sf9 cells expressing CHV gD possessed a high titer of virus-neutralizing activities against CHV infection. These results indicated that CHV gD is structurally similar to FHV-1 gD, but is functionally different from FHV-1 gD. — **KEY WORDS:** canine herpesvirus, feline herpesvirus type 1, glycoprotein D, hemagglutinin.

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Canine herpesvirus (CHV) belongs to family *Herpesviridae*, subfamily *Alphaherpesvirinae* [26]. CHV causes a fatal hemorrhagic disease in neonatal puppies and an upper respiratory tract infection in adult dogs [3].

By using monoclonal antibodies (MAbs) against CHV YP11mu strain, three glycoproteins, gB, gC, and gD, were identified [33, 35, 36]. Almost all MAbs against gB and gC had complement-dependent or complement-enhanced virus-neutralizing (VN) activity. All of the MAbs against gD possessed complement-independent VN activity [35]. In addition, four of the five MAbs against CHV gD inhibited hemagglutination (HA) activity of CHV against canine red blood cells (RBC) [34].

Similarly, we reported that gD of feline herpesvirus type 1 (FHV-1) agglutinated feline RBC and cells expressing the gD adsorbed the RBC [17, 18]. Further, insect cells expressing FHV-1 gD on their cell surface were adhered to several cell lines originating from *Felidae* but not those from other animals [19]. Therefore, we speculated that the FHV-1 gD might restrict receptor(s) of cells from *Felidae*. One MAbs 25C9 against FHV-1 gD recognized CHV gD (hemagglutinin) by indirect immunofluorescence assay

(IFA) and immunoblot analysis, and inhibited HA activity of CHV [15]. CHV agglutinates only canine RBC [23] while FHV-1 agglutinates only feline RBC [6, 22]. The reason for these different HA activities has never been studied.

In herpes simplex virus (HSV), gD seems to have specific receptors on the surface of cells [9, 10]. In particular, Brunetti *et al.* reported that gD binds to mannose-6-phosphate receptors [2] and that this interaction is important for virus entry into cells and cell-to-cell transmission [1]. The gDs of alphaherpesviruses were important for virus penetration to cells [5, 8, 13, 24]. However, the role of gD in the virus penetration process remains to be further analyzed.

In this communication, we expressed the gD of CHV YP2 strain in COS-7 and insect cells. A molecular weight (MW) of CHV gD expressed in COS-7 cells was approximately 51–55 kilodaltons (kDa), and that in insect cells was 41–46 kDa. The expressed CHV gD adsorbed and agglutinated canine RBC, but not feline RBC. Furthermore, antibodies raised in mice immunized with recombinant CHV gD neutralized CHV infection *in vitro*.

MATERIALS AND METHODS

Viruses and cells: CHV YP2 strain [34] was propagated in Madin-Darby canine kidney (MDCK) cells which were grown in Dulbecco's modified Eagle's medium (DMEM) (SIGMA, MO, U.S.A.) supplemented with 5% heat-inactivated fetal calf serum (FCS) and 100 units of penicillin

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and 100 μ g of streptomycin per ml. COS-7 cells were cultured in DMEM supplemented with 10% FCS and antibiotics. Recombinant *Autographa californica* nuclear polyhedrosis viruses (rAcNPVs) were grown in *Spodoptera frugiperda* (Sf9) cells in TC100 medium (GIBCO, Grand Island, N. Y.) supplemented with 10% FCS, 0.3% tryptose phosphate broth (Difco, Detroit, Mich.), and antibiotics. As a control, AcfgD which expresses FHV-1 gD in Sf9 cells [18] was used.

Extraction of viral DNA: Monolayers of MDCK cells were infected with CHV at a multiplicity of infection of 1 plaque forming unit per cell. When the monolayers showed extensive cytopathic effects, the cells were washed once with phosphate-buffered saline (PBS), and lysed in 0.1 M Tris-HCl (pH 9.0) containing 1% sodium dodecyl sulfate (SDS), 0.1 M NaCl and 1 mM EDTA and then reacted overnight with 1 mg/ml of pronase E at 37°C. Viral DNA was extracted with phenol and precipitated with ethanol.

Polymerase chain reaction (PCR) amplification: Two primers, 5'-GGGAATTCATGATTAACTTCTATTTAT-3' (CGD-UP) and 5'-TTCTCGAGCTAAACATTGTGTGAATT-3' (CGD-DOWN), were prepared based on the sequence of CHV gD [14]. The former, CGD-UP, contains *Eco*RI site at the 5'-end and a translational initiation codon, ATG, and the latter, CGD-DOWN, does *Xho*I site at the 5'-end and a stop codon, TAG. Viral DNA was amplified by 30 cycles of denaturation (94°C, 1 min), annealing (60°C, 1 min), and polymerization (72°C, 2 min). The amplified fragment was purified by using Suprec-02 (TAKARA, Kyoto, Japan), digested with restriction enzymes, *Eco*RI and *Xho*I, subjected to electrophoresis on 1% agarose gel, and electrophoretically extracted from the gel. Then the fragment was cloned into *Eco*RI and *Xho*I sites of pBluescripts KS-, and designated as pBS-cgD (YP2).

Construction of expression plasmids: The pME18S expression vector contains the SR α promoter consisting of the simian virus 40 early promoter and the R segment and part of the U5 sequence of the long terminal repeat of human T-cell leukemia virus type 1 [32]. pME-cgD(YP2) was constructed by inserting an entire open reading frame (ORF) of gD(YP2) in pBS-cgD(YP2) at the *Eco*RI and *Xho*I sites of pME18S. As a control, pME-fgD which expresses FHV-1gD in COS-7 cells [17] was used.

Expression in COS-7 cells: COS-7 cells were transfected with the constructed plasmids according to the methods described previously [28] with minor modifications. Briefly, when COS-7 cells were grown in a 100 mm dish, 7.5 μ g of plasmid DNA prepared in 5 ml of DMEM/DEAE-dextran solution was added to the cells. After incubation for 3 hr at 37°C, the solution was removed. The cells were treated with 5 ml of 10% dimethyl sulfoxide for 1 min and returned to DMEM containing 10% FCS. After 72 hr post-transfection, the transfected cells were scraped off the plates and analyzed by indirect immunofluorescence assay (IFA), immunoblot analysis, and hemadsorption (HAD) test.

Construction of rAcNPV transfer vector: Plasmid pBS-cgD(YP2) was digested with restriction endonucleases,

*Eco*RI and *Xho*I. This digested fragment was electrophoretically isolated, blunted with Klenow fragment and cloned into the blunt-ended *Bam*HI site of pAcYM1 [20] for producing a transfer vector pAccgD (YP2). Restriction enzyme analysis of this transfer vector showed the entire sequence of the gD gene under the control of a baculovirus polyhedrin promoter.

Transfection and selection of recombinant viruses: Sf9 cells were co-transfected with linealized BaculoGoldTM baculovirus (AcNPV) DNA (PharMingen, San Diego, CA) and pAccgD(YP2) by use of Lipofectin reagent (GIBCO BRL, Gaithersburg, MD). After three cycles of plaque purification, the recombinant virus was isolated, and was designated as AccgD (YP2).

MAbs: MAbs used in this study were previously produced and characterized [7, 15, 34, 35].

IFA: For detection of CHV gD in IFA, transfected or infected cells were smeared on glass slides, air-dried and then fixed with acetone. The fixed cells were incubated for 30 min at 37°C with MAbs against CHV glycoproteins. After incubation, the slides were washed three times with PBS, and then anti-mouse immunoglobulins (G+M+A) goat antibody conjugated with fluorescein isothiocyanate (FITC) (Cappel, PA, U.S.A.) was applied. After incubation for 30 min at 37°C, the slides were washed again, mounted in buffered glycerol, and examined by fluorescence microscopy.

For membrane immunofluorescence, transfected or infected cells were suspended in ice-cold PBS containing 3% FCS and 0.1% sodium azide, and then reacted with MAbs for 30 min at 4°C. After washing three times by ice-cold PBS containing 3% FCS and 0.1% sodium azide, FITC-conjugated anti-mouse immunoglobulins were added and the cells were reincubated for 30 min at 4°C. After further washings for three times, the cells were resuspended in buffered glycerol and mounted for immunofluorescence microscopy.

Immunoblot analysis: SDS-polyacrylamide gel electrophoresis (PAGE) was carried out according to the discontinuous Laemmli buffer system [12]. All samples were dissolved in the buffer (62.5 mM Tris-HCl (pH 6.8), 20% glycerol, and 0.001% bromophenol blue), and then disrupted by heating for 2 min at 100°C. Polypeptides were separated on an SDS-polyacrylamide gel and electrophoretically transferred to polyvinylidene difluoride membrane (Immobilon, Millipore, MA, U.S.A.). The blotting papers were incubated for 30 min at 37°C with a mixture of four MAbs, 11F7, 09D1, 10C10, and 05B7, against CHV gD, or MAbs 10C10 and 25C9 against FHV-1 gD. Afterwards, they were washed three times, and incubated with anti-mouse immunoglobulins (G+M+A) peroxidase conjugate (Cappel, PA, U.S.A.) for 30 min at 37°C. The reaction was visualized by addition of a diaminobenzidine-hydrogen peroxidase substrate.

Tunicamycin (TM) treatment: Recombinant baculovirus-infected Sf9 cells were cultured in the media containing 10 μ g/ml TM from 1 to 48 hr post-infection. The infected

cells were then harvested and subjected to immunoblot analysis.

HAD test: At 72 hr post-transfection, COS-7 cells transfected with pME-cgD(YP2) or pME-fgD were washed three times with ice-cold PBS containing 3% FCS and 0.1% gelatin. The cells were incubated with 0.4% canine or feline RBC in PBS for 30 min at 4°C, and then were observed.

HA and HA-inhibition (HI) tests: Sf9 cells infected with recombinant viruses and MDCK cells infected with CHV YP2 strain were harvested after 48 hr post-infection. These cells were scraped off from the plates, washed three times with dilution buffer (PBS containing 0.1 (w/v)% bovine serum albumin and 0.002% gelatin) and then suspended in the dilution buffer. The suspensions were subjected to three cycles of freeze-and-thaw treatment, treated by ultrasonicator for 20 sec on ice and then centrifuged at 4,000 rpm for 10 min to remove the cell debris. The supernatants were serially diluted by a two-fold manner at 50 μ l per well of 96 well V-bottomed microplates in the dilution buffer. An equal volume of 0.3% canine or 0.5% feline RBC, washed and suspended in the dilution buffer, was added to each well. The HA titer was defined as the reciprocal of the highest dilution showing complete HA after incubation for 2 hr at 37°C.

To determine the HI activity of MABs, heat-inactivated MABs were treated with 12.5% kaoline and then adsorbed with 5.0% canine RBC to remove non-specific reactions. MABs were diluted at 25 μ l per well in the dilution buffer in 96 well V-bottomed microplates and mixed with an equal volume of a solution containing 4 HA units of hemagglutinin, prepared from Sf9 cells infected with AccgD (YP2). After plates were gently agitated and incubated at 37°C for 1 hr, 50 μ l of 0.3% canine RBC or 0.5% feline RBC suspension in the dilution buffer was added to each well. After 2 hr of further incubation at 37°C, the plate was read for HI activity and HI titer was expressed as the reciprocal of the highest serum dilution showing complete HI patterns.

Immunization of mice: Mice (BALB/c, 8 weeks old) were intraperitoneally immunized three times at 2 week intervals. The injections were done with lysates of AccgD (YP2)- or AcYM-infected Sf9 cells (1×10^6) in complete or incomplete Freund's adjuvant. The mice were bled 14 days after the final injection.

VN assay: The VN assays were performed in the presence or absence of complement as described earlier [24].

RESULTS

Expression of CHV gD in COS-7 cells: Expression of CHV gD (YP2) in COS-7 cells was examined by IFA using MABs against CHV glycoproteins [35]. An MAb 11F7 against CHV gD reacted with pME-cgD (YP2)-transfected cells regardless of fixation of the cells (Fig. 1 A, C). In addition, other three MABs including 10C10, which recognized distinct epitopes of CHVgD, reacted with pME-cgD (YP2)-transfected cells (data not shown). The

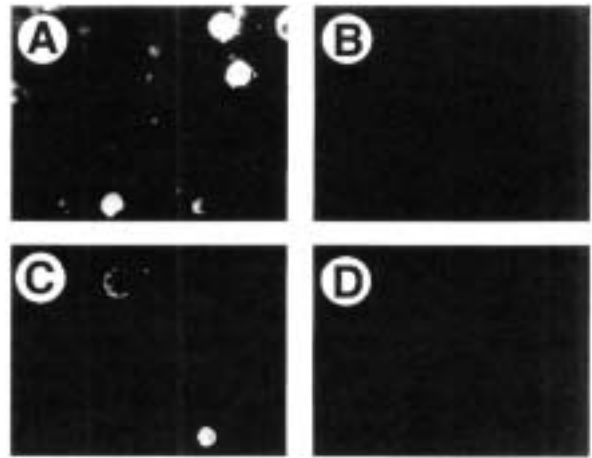


Fig. 1. IFA of COS-7 cells transfected with expression plasmids. Cells were incubated with an MAb 11F7, washed in PBS, and stained for fluorescence microscopy with fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulins. (A) and (C) pME-cgD (YP2)-transfected COS-7 cells. (B) and (D) pME-fgD-transfected COS-7 cells. Transfected cells were fixed by acetone (A and B) or unfixed (C and D).

specificity of the reaction was confirmed by the absence of fluorescence on pME-fgD-transfected COS-7 cells (Fig. 1 B, D). These results indicate that expressed proteins were recognized by MABs against CHV gD and located on the surface of transfected cells. In addition, pME-cgD (YP2)- and pME-fgD-transfected COS-7 cells were recognized by an MAb 25C9 against FHV-1 gD (data not shown). This observation consists with that of previous study using CHV-infected MDCK cells [15]. CHV gD must have at least an epitope similar to that of FHV-1 gD.

Expression of CHV gD (YP2) in COS-7 cells was further confirmed by immunoblot analysis using a mixture of four MABs against gD (Fig. 2A lane 1). The MW of authentic CHV-derived gD was approximately 51–55 kDa with a minor band of 48 kDa which seems to be a precursor form of the CHV gD. These bands were similar to those of CHV YP2-infected MDCK cells. In pME-fgD-transfected cells, no specific band was detected (Fig. 2A lane2).

Expression of CHV gD in insect cells: Expression of CHV gD(YP2) in Sf9 cells was examined by IFA using MABs against CHV glycoproteins. All of the MABs against CHV gD reacted with AccgD (YP2)-infected Sf9 cells. The specificity of the reaction was confirmed by the absence of fluorescence on AcfgD-infected Sf9 cells. In addition, an MAb 25C9 against FHV-1 gD also reacted with the AccgD (YP2)-infected Sf9 cells (data not shown).

Expression of CHV gD (YP2) was further confirmed by immunoblot analysis using an MAb 10C10 against CHV gD or an MAb 25C9 against FHV-1 gD (Fig. 2B and C). The MAb 10C10 detected a specific band of 41–46 kDa in AccgD (YP2)-infected Sf9 cells, but not in AcfgD-infected Sf9 cells (Fig. 2C, lanes 1 and 2). In addition, both CHV

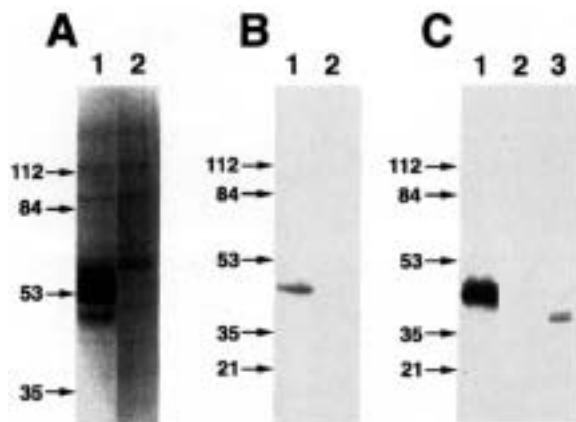


Fig. 2. Immunoblot analysis of gDs expressed in COS-7 or Sf9 cells. (A) COS-7 cells were transfected with the expression plasmid, pME-cgD (YP2) or pME-fgD. Transfected cells were lysed into gel sample buffer. Samples were run on SDS-PAGE, transferred to nylon membrane, and reacted with a mixture of MAb against CHV gD. Lane 1, pME-cgD (YP2)-transfected COS-7 cells; Lane 2, pME-fgD-transfected COS-7 cells. (B) Immunoblot analysis was carried out using an MAb 25C9. Lane 1, AccgD(YP2)-infected Sf9 cells; Lane 2, AcfgD-infected Sf9 cells. (C) Immunoblot analysis was carried out using an MAb 10C10. Lane 1, AccgD (YP2)-infected Sf9 cells; Lane 2, AcfgD-infected Sf9 cells; Lane 3, AccgD (YP2)-infected cells treated with 10 μ g/ml of TM. Molecular weight (in kilodaltons) are indicated at the left.

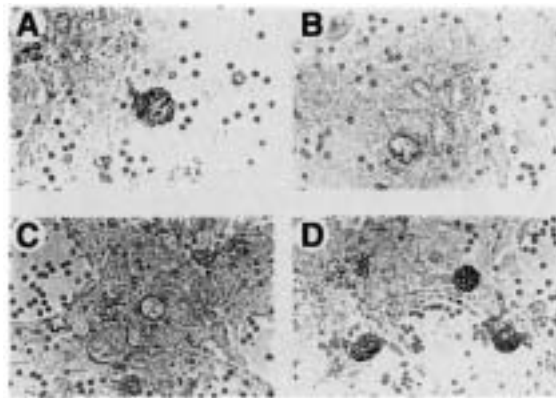


Fig. 3. HAD assays of COS-7 cells expressing gDs. COS-7 cells transfected with pME-cgD (YP2) (A, C), or pME-fgD (B, D) were mixed with 0.4% canine RBC (A and B) or feline RBC (C and D) and incubated for 30 min at 4°C.

gD (YP2) and FHV-1 gD expressed in Sf9 cells were detected by an MAb 25C9 against FHV-1 gD (Fig. 2B). When AccgD (YP2)-infected Sf9 cells were treated with 10 μ g/ml of TM, the MW of the TM-treated gD (YP2) was 37 kDa (Fig. 2C, lane 3). As CHV gD consists of 345 amino acid residues with a predicted MW of approximately 38 kDa [14], the estimated MW of the TM-treated gD seems to be reasonable.

HAD and HA tests: We reported that CHV has HA

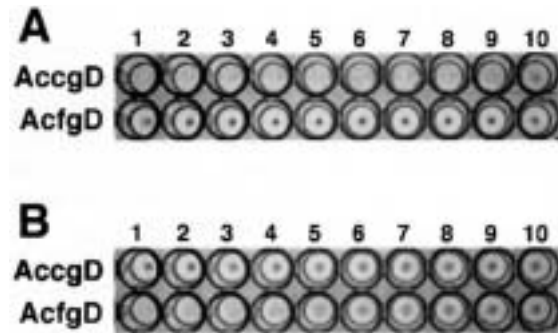


Fig. 4. HA test of the expressed gDs. HA activities of extracts from Sf9 cells infected with AccgD (YP2) or AcfgD by using canine RBC (A) or feline RBC (B). Numbers on the top of wells represent the inverse of \log_2 dilutions.

Table 1. Immunogenic properties of recombinant gD

Serum against	VN titer ^{a)}	
	w/o C'	w C'
AccgD (YP2)	80	2560
AcYM	<40	<40

a) Neutralization titer was expressed as the reciprocal of a serum dilution giving a 50% reduction in plaque number compared with the control.

w/o C'; without complement

w C'; with complement

activity, which is inhibited by four MAb against CHV gD, and that CHVgD might be a hemagglutinin [23, 34]. Recently, Xuan *et al.* [33] reported that CHVgD expressed by recombinant vaccinia virus agglutinated canine RBC. Therefore we speculated that CHV gD-expressing COS-7 cells might adsorb canine RBC. The transfected cells were used for HAD test using canine RBC or feline RBC. Figure 3 shows that pME-cgD (YP2)-transfected COS-7 cells adsorbed canine RBC, but not feline RBC, whereas pME-fgD-transfected COS-7 cells adsorbed feline RBC, but not canine RBC. These HAD reactions of CHV gD (YP2)-expressing COS-7 cells were inhibited by treatment of the cells with HI MAb against CHV gD (data not shown). These results indicate that CHV gD specifically adsorbed canine RBC.

Next we examined whether the expressed CHV gD could agglutinate canine RBC. Extracts of Sf9 cells infected with recombinant baculoviruses were used for HA test using canine RBC or feline RBC. Figure 4 showed that extracts of Sf9 cells infected with AccgD (YP2) agglutinated canine RBC, but not feline RBC, whereas extracts of Sf9 cells infected with AcfgD agglutinated feline RBC, but not canine RBC.

In addition, HI analysis was carried out using HI MAb 11F7 against CHV gD, and 25C9 against FHV-1 gD, and non-HI MAb 10C10 against CHV gD. Four HA units of extracts from AccgD (YP2)-infected Sf9 cells were used.

This HA activity was inhibited by HI MAbs 11F7 and 25C9, but not by non-HI MAb 10C10 (data not shown). These results indicate that CHV gD specifically agglutinated canine RBC.

Immunogenicity of the gD expressed in Sf9 cells against mice: Mice were inoculated three times with AccgD (YP2)- or AcYM-infected Sf9 cell lysates. As shown in Table 1, pooled serum from mice immunized with lysates from Sf9 cells infected with AccgD (YP2) possessed high titers of VN activity. No VN activity was detected in serum from mice immunized with AcYM-infected Sf9 cell lysates.

DISCUSSION

In this paper, CHV gD (YP2) was expressed in COS-7 and Sf9 cells and compared with FHV-1 gD by HAD, HA, and HI tests.

The CHV gD expressed by recombinant baculovirus in insect cells appeared to correspond in size to the partially glycosylated form of authentic gD, which has an apparent molecular mass of 47 kDa [34]. This suggests that recombinant gD undergoes glycosylation but no further processing to complex forms. A similar incomplete processing has been observed in HSV-1 gD [11], bovine herpesvirus-1 (BHV-1) gD (gIV) [38], equine herpesvirus type 1 (EHV-1) gD [16], and FHV-1 gD [18]. Recent studies on the role of carbohydrates of HSV-1 gD and BHV-1 gD have shown that antigenic properties are not altered by carbohydrate removal [29, 37], indicating that the CHV gD expressed in insect cells might be also useful as an immunogen to control CHV infection.

The COS-7 cells expressing CHV gD adsorbed canine RBC, but not feline RBC. The expressed CHV gD agglutinated canine RBC, but not feline RBC. We previously reported that FHV-1 gD could agglutinate feline RBC and that insect cells expressing FHV-1 gD adhered to cell lines originating from *Felidae*, but not from other animals [17–19]. We speculated that FHV-1 gD might restrict the narrow host range of FHV-1. In CHV, dog is the only known natural host for this agent. *In vitro*, the virus grows exclusively in primary canine kidney cell cultures and MDCK cells [3, 4, 31]. In addition, we reported that CHV agglutinated canine RBC, but not RBC of other animals [23]. In this study, CHV gD and FHV-1 gD adsorbed and agglutinated RBC from respective natural hosts (Figs. 3 and 4). However both CHV gD and FHV-1 gD were recognized by one complement-independent VN and HI MAb, 25C9 against FHV-1 gD (Fig. 2). These results might indicate that CHV gD is structurally similar to FHV-1 gD, but is functionally different from FHV-1 gD.

Infection of CHV and FHV-1 to susceptible cell lines, MDCK and CRFK cells, respectively, was inhibited by heparin (Maeda *et al.*, manuscript in preparation). At least one glycoprotein of FHV-1, gC, binds to heparin column (Maeda *et al.*, manuscript in preparation). Therefore, adsorption of these two viruses, CHV and FHV-1, to cells might be similar to those of other herpesviruses [30].

Nevertheless, these two viruses possess very narrow host range, in comparison with other herpesviruses, such as HSV and pseudorabies virus. Thus we speculated that these narrow host range might be restricted by post-adsorption events. It is unknown what restricts the difference in host range of two viruses. In previous study, FHV-1 gD recognized molecule(s) on the surface of feline cells, but not that of other animals [19]. In this study, CHV gD adsorbed canine RBC, but not feline RBC. These functions of gDs seem to correlate with host range of two viruses.

Limbach *et al.* [14] reported that nucleotide sequences of CHV gB, gC, and gD were the most homologous to that of FHV-1 among those of well-known alphaherpesviruses. By using polyvalent and monoclonal antibodies, CHV gB and gD were cross-reactive with FHV-1 gB and gD, respectively [15]. By Southern-blot analysis, it was revealed that genomic DNA of CHV is hybridized to that of FHV-1 [27]. Therefore, it is considered that CHV is very similar to FHV-1 regarding antigenic products or nucleotide sequence. On the other hand, EHV-1 or infectious laryngotracheitis virus (ILTV) also agglutinates RBC of natural host, horse or chicken, respectively [21, 25]. It is unknown which gene products of EHV-1 and ILTV agglutinate RBC of natural host. This interaction between viral proteins and RBC of natural host seems to be important for studies of host range of alphaherpesviruses.

In this study, the CHV gD adsorbed and agglutinated canine RBC, but not feline RBC. The difference in HAD and HA activities between CHV gD and FHV-1 gD might be useful for studies of receptors against these gDs. In addition, these data suggest that recombinant gDs produced in insect cells may be useful as an immunogen in CHV infection.

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