JOURNAL OF VIROLOGY, Jan. 1997, p. 42–49 Vol. 71, No. 1
0022-538X/97/$04.00+0

CD9, a Tetraspan Transmembrane Protein, Renders Cells Susceptible to Canine Distemper Virus

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Received 20 July 1996/Accepted 16 September 1996

Canine distemper virus (CDV), a lymphotropic and neurotropic negative-stranded RNA virus of the *Morbillivirus* genus, causes a life-threatening disease in several carnivores, including domestic dogs. To identify the cellular receptor(s) involved in the uptake of CDV by susceptible cells, we isolated a monoclonal antibody (MAb K41) which binds to the cell surface and inhibits the CDV infection of several cell lines from various species. Pretreatment of cells with MAb K41 reduces the number of infectious centers and the size of the syncytia. Using affinity chromatography with MAb K41, we purified from HeLa and Vero cell extracts a 26-kDa protein which contained the amino acid sequence TKDEPQRETLK of human CD9, a member of the tetraspan transmembrane or transmembrane 4 superfamily of cell surface proteins. Transfection of NIH 3T3 or MDBK cells with a CD9 expression plasmid rendered these cells permissive for viral infection and raised virus production by a factor of 10 to 100. The mechanism involved is still unclear, since we were unable to detect direct binding of CDV to CD9 by using immunoprecipitation and a virus overlay protein binding assay. These findings indicate that human CD9 and its homologs in other species are necessary factors for the uptake of CDV by target cells, the formation of syncytia, and the production of progeny virus.

Canine distemper virus (CDV) is a member of the genus of morbilliviruses, which includes measles virus (MV), rinderpest virus, peste des petits ruminants virus, porpoise morbillivirus, and phocine distemper virus (5, 15, 16, 33–35, 38, 39). CDV causes a highly contagious disease with many similarities to human measles in carnivores (canines, felids, ferrets, raccoons, and seals). Recently, it was identified as the etiologic agent in fatal epidemics in the Serengeti-Mara ecosystem in East Africa affecting silver-backed jackals, bat-eared foxes, wild dogs, and lions (1, 40). Sequence analyses of the P and H genes of CDV isolates revealed clustering of strains according to geographical distribution rather than to host species origin (16).

Canine distemper is characterized by fever, coryza, conjunctivitis, gastroenteritis, and pneumonitis. The mortality rates following CDV infection vary with the host species, ranging from 0% in domestic cats to approximately 50% in domestic dogs and 100% in ferrets. Encephalomyelitis is the most common cause of death (3, 48). After infecting epithelial cells of the respiratory tract, CDV spreads to bronchial lymph nodes and tonsils and then to the circulatory system as leukocyte-associated viremia. The virus reaches the central nervous system either by infection of endothelial cells of the blood-brain barrier or by transport within infected macrophages (2, 23, 49). In dogs, CDV infection results in a progressive demyelinating encephalomyelitis, presumably due to a bystander mechanism in which macrophages play an important role (51). Canine distemper is also associated with transient immunosuppression that may result in significant morbidity and mortality through opportunistic infections (10, 23).

The cellular receptor for MV, a closely related morbillivirus, was recently identified (11, 12, 25–27, 30, 31, 42, 43), but the receptor for CDV is still unknown. Because the host specificity and the tropism of CDV are different from those of MV, the cellular receptor for CDV is likely to be different from that of MV. Recently, it has been shown by complementation analysis, with the help of recombinant envelope proteins of CDV and MV, that the CDV H protein (CDV-H) is responsible for the selective tropism of CDV in cell culture (47). Human-mouse somatic cell hybrids were used to identify the human chromosome which encodes the CDV receptor on human cells. Two of twelve hybrids screened which were able to form syncytia following CDV infection contained human chromosome 19 (47).

To identify the molecule acting as a cellular receptor for CDV, we raised monoclonal antibodies (MAbs) against cell surface proteins of CDV-susceptible target cells. We obtained one MAb (K41) which was able to inhibit CDV infection and bound to a protein known as CD9. Further experiments using recombinant CD9 and commercially available antibodies supported the hypothesis that human CD9 and its homologs in other species are necessary factors for the infection of target cells with CDV.

MATERIALS AND METHODS

Propagation of cells and CDV. The cell lines HeLa (human cervix carcinoma; ATCC CCL 2), AV3 (human amnion cells; ATCC CCL 21), 293 (transformed primary human embryonal kidney cells; ATCC CRL 1573), Vero (African green monkey cells; ATCC CRL 6318), MDCK (dog kidney cells; ATCC CCL 34), BHK (baby hamster kidney cells; ATCC CRL 6281), NBL9 (raccoon uterus cells; ATCC CCL 74), CRFK (cat kidney cells; ATCC CCL 94), LLCPK (pig kidney cells; ATCC CL 101), RK13 (rabbit kidney cells; ATCC CCL 37), MDBK (bovine kidney cells; ATCC CCL 22), and NIH 3T3 (NIH Swiss mouse embryonic fibroblasts; ATCC CRL 1658) were cultured in minimal essential medium (MEM) medium containing 10% fetal calf serum (FCS) and additives as described previously (42).

The CDV strain used, Onderstepoort, (a gift from B. Rima, Queens University

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of Belfast, Belfast, United Kingdom), was propagated like MV as described previously (42). Briefly, Vero cells in MEM containing 5% FCS were infected with a multiplicity of infection (MOI) of 0.01 at 37°C and incubated at 37°C for 3 to 5 days, depending on the optimal titer of infectious CDV produced. CDV was harvested by one freeze-thaw cycle and centrifugation at 200 × *g* for 10 min to remove cell debris and stored at −70°C.

Infection inhibition assays. The infection inhibition assay to screen the activity of antibodies was done in microtiter plates. Vero cells were seeded into 96-well plates and grown to 80% confluency. Antibodies in dilutions were added to each test well and incubated at room temperature for 30 min. After incubation, CDV at an MOI of 0.1 was added and monolayers were incubated for 48 to 72 h at 37°C in the presence of MAbs. Inhibition of virus induced cytopathic effect was observed microscopically.

The plaque reduction assay was performed with Vero cell monolayers in six-well plastic dishes as described previously (12). Confluent cell monolayers (10^6 cells per well) were washed with phosphate-buffered saline (PBS), and 100 μl of protein G-purified antibodies was added in various concentrations. After incubation at room temperature for 45 min, the monolayers were washed once with PBS, and 100 μl of PBS containing 100 PFU of CDV was added to each test well. Further incubation at 37°C for 1 h was followed by washing with PBS prior to overlaying the wells with 5 ml of Eagle's MEM supplemented with 1% FCS and 1% Noble agar (Sigma). Plaques were visualized after 3 to 5 days of incubation at 37°C by staining with neutral red.

Antibodies. Mouse MAbs were raised against cell surface epitopes by inoculating BALB/c mice intraperitoneally with 1×10^6 to 2×10^6 Vero cells. Four weeks later, the mice were boosted with an additional intraperitoneal inoculation of Vero cells. Eight weeks after the initial immunization, mice were intravenously challenged, and spleens were harvested 3 to 4 days thereafter. BALB/c spleen cells were fused with the myeloma cell line Sp2/0-Ag14 and seeded into 96-well dishes for selection of hybridomas. Hybridoma cells were grown in RPMI 1640 medium as described previously (12). In a first round of screening, MAbs which recognized the cell surface of Vero cells were identified by flow cytometry and the corresponding hybridoma lines were selected. Supernatants of selected hybridomas were then screened for the capacity to inhibit the infection with CDV.

MAbs 1.347, 3.734, and 4.074 recognizing CDV H were a gift of C. Örvell, Laboratory of Clinical Virology, University Hospital, Huddinge, Sweden (32). The rabbit polyclonal anti-CDV serum was a gift of M. Appel, Veterinary Virus Research Institute, Cornell University, Ithaca, N.Y. The MAbs against CD9 were purchased from Dianova (ALB6), from The Binding Site (BU16), and from Serotec (MM2/57). MAb 13/42 recognizing CD46 and the anti-major histocompatibility complex (MHC) class I MAb W6/32 used as controls were produced in our laboratory. The MAbs were purified over protein G affinity columns.

Western blotting. Semidry Western blot analyses were performed as described previously (42). Blots were incubated with 1 μg of MAb against CD9 per ml in blocking buffer (5% dry milk powder–0.02% Tween in PBS) followed by peroxidase-conjugated anti-mouse immunoglobulin (Ig; 1:2,000; Dako). Bands were detected with an enhanced chemiluminescence (ECL) system (Amersham).

Flow cytometry. Flow cytometric analyses were performed as described previously (42). Briefly, 10^5 cells were incubated for 30 min on ice with 1 μg of MAb in 100 μl of fluorescence-activated cell sorting (FACS) buffer (PBS containing 0.4% bovine serum albumin and 0.02% sodium azide). Cells were washed twice in FACS buffer and incubated with 200 μl of a 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig (Dako) on ice for a further 30 min. After three washes with FACS buffer, flow cytometric analysis was performed on a FACScan (Becton Dickinson).

Immunoaffinity purification of proteins. The protein G-purified MAb K41 was coupled to CNBr-activated Sepharose 4B beads (Pharmacia) to form an immunoaffinity matrix as described in the manufacturer's protocol. The affinity column was equilibrated with 0.2% Nonidet P-40 (NP-40) in PBS. Extracts of HeLa or Vero cells in lysis buffer containing 1% NP-40, 10 mM Tris (pH 7.8), 0.15 M NaCl, 0.5 M KCl, 5 mM EDTA, and protease inhibitors (Boehringer Mannheim) were bound as batch to the column material for 24 h at 4°C. Bound proteins were eluted with 0.2 M glycine (pH 2.8)–0.15 M NaCl–0.2% NP-40.

Amino acid sequencing. For direct amino acid microsequencing, the affinity-purified proteins were concentrated with Centricon-10 tubes (10-kDa molecular mass exclusion; Amicon), size fractionated by electrophoresis on a sodium dodecyl sulfate (SDS)–14% polyacrylamide gel, and blotted onto Glassybond (Biometra, Göttingen, Germany) as described by Eckerskorn et al. (13). For internal sequencing, the protein was cleaved in the gel with protease K12 (Boehringer, Tutzing, Germany), and the peptides were eluted and separated by reversed-phase high-pressure liquid chromatography on Superspher 60 RP Select B (125 by 2 mm) (13). Amino acid sequence analyses were performed on a model 477A pulse liquid-phase sequencer equipped with a 120A phenylthiohydantoin analyzer (Applied Biosystems, Weiterstadt, Germany).

Immunoprecipitation. For immunoprecipitation, 2×10^6 cells were labeled with 50 μCi of [^{35}S]methionine and -cysteine (TranS-label; ICN) overnight, detached from the plastic dish with Ca^{2+}- and Mg^{2+}-free PBS containing 1 mM EDTA, suspended in 100 μl of PBS, lysed by addition of 1 volume of radioimmunoprecipitation assay-detergent buffer (150 mM NaCl, 10 mM Tris [pH 7.4], 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100) in the presence of protease inhibitors, vortexed, and centrifuged at 10,000 × *g* for 10 min. Five-microgram aliquots of antibodies were added to the supernatants for 1 h on ice followed by addition of protein G-Sepharose beads (Pharmacia) for 45 min. Sepharose beads were washed three times with ice-cold PBS, and the proteins were dissolved in gel loading buffer for SDS-polyacrylamide gel electrophoresis (PAGE).

Transfection of cells with a CD9 expression plasmid. MDBK and NIH 3T3 cells were transfected by the Lipofectin method (Gibco BRL) with a plasmid derived from pRC/CMV (Invitrogen) containing a 1.3-kb cDNA insert encoding the full-length human CD9 (24). Cells were seeded on microtiter plates and selected in MEM containing 1 mg of G418 (Gibco BRL) per ml. After selection of resistant cells, cells were further cultured in the presence of 500 μg of G418 per ml. The expression of CD9 was measured by flow cytometry using MAb K41.

Virus overlay protein binding assay (VOPBA). Binding of CDV to proteins blotted on nitrocellulose was analyzed as described previously (25). Briefly, total cell extracts from Vero cells (approximately 50 μg of protein/lane) containing 1% octylglucoside (Boehringer) or 1% Triton X-100 (Sigma) were separated by SDS-PAGE (14% gel) under reducing or nonreducing conditions, blotted on a polyvinylidene difluoride membrane (Immobilon P; Millipore), and blocked overnight with PBS containing 10% nonfat dry milk. Approximately 10 μg of sucrose gradient-purified CDV in 1 ml of PBS containing 0.1% Tween was incubated with the membrane for 1 h at 4°C. After washing, CDV proteins were detected with anti-CDV-H MAb 1.347, horseradish peroxidase-conjugated rabbit anti-mouse antibodies, and an ECL system (Amersham).

RESULTS

Screening for infection-inhibiting antibodies. To isolate an infection-inhibiting MAb, mice were immunized with CDV-permissive Vero cells. Using the infection inhibition assay in microtiter plates, we screened approximately 3,000 antibody-producing hybridomas before detecting clone K41, which produced a MAb K41 (IgG1κ) that recognized the cell surface of Vero cells and considerably reduced the cytopathic effect of CDV. After confirming the inhibitory effect of MAb K41 on the CDV infection using HeLa cells, we investigated the expression of the K41 epitope by flow cytometry on cells of various species, including canine cells. Human, monkey, dog, raccoon, cat, and rabbit cell lines were positive, whereas hamster, pig, bovine, and mouse cell lines were negative (Table 1). With one exception (BHK cells), the infectivity of the tissue culture cells tested correlated well with expression of the K41 epitope. Although BHK cells did not express the K41 epitope, they were readily infected with CDV, and the infection could not be blocked with MAb K41. Infection with CDV was inhibited by MAb K41 on all cell lines which expressed the K41 epitope (Table 1).

Identification of the protein recognized by MAb K41. Western blot experiments were performed with extracts from various cell lines to identify cellular proteins recognized by MAb K41. A diffuse band of a protein with the approximate molecular mass of 26 kDa was detected in extracts of cells analyzed under nonreducing conditions (Fig. 1A). Under reducing conditions, the antibody recognized a protein of similar mass, although the signal was much less intense than under nonreducing conditions (Fig. 6B).

To identify the protein recognized by MAb K41, the antibody was coupled to CNBr-activated Sepharose to facilitate affinity chromatography. A protein of 26 kDa was purified from HeLa and Vero cell extracts by using the K41 affinity column, size fractionated by SDS-PAGE (14% gel), digested in the gel with protease K12, and subjected to amino acid sequence analysis. The sequence of one internal peptide was determined to be TKDEPQRETLK. Comparison of the 11 amino acids with the sequences available in the EMBL database showed that the sequence was identical to that of peptide 134-144 of human CD9 (6, 24).

To confirm that MAb K41 recognizes the human CD9 and homologs of other species as described in Table 1, Western blot analyses were done with the eluate from the K41 affinity column and three commercial anti-CD9 MAbs, clones ALB6, BU16, and MM2/57. All three commercial antibodies recog-

TABLE 1. Expression of the epitope recognized by MAb K41, determination of the permissivity for CDV, and inhibition of the CDV infection with MAb K41 on tissue culture cells of various species

Cell line	Species	% Expression of the K41 epitope (MFI)[a]	% Infectivity with CDV (MFI)[b]	% Inhibition of CDV infection with MAb K41[e]
HeLa	Human	76 (156)	57 (333)	50
AV3	Human	92 (82)	88 (552)	49
293	Human	96 (232)	84 (1,087)	41
Vero	Monkey	98 (895)	87 (525)	61
MDCK	Dog	97 (244)	61 (243)	25
CRFK	Cat	87 (215)	26 (159)	37
NBL9	Raccoon	98 (281)	29 (48)	32
RK13	Rabbit	72 (118)	36 (30)	85
BHK	Hamster	0 (21)	57 (642)[c]	0
MDBK	Cattle	1 (30)	1 (8)[d]	ND
LLCPK	Pig	2 (28)	1 (6)[d]	ND
NIH 3T3	Mouse	0 (18)	0 (5)	ND

[a] Measured by flow cytometry and given as percentage of positive cells and mean fluorescence intensity (MFI).

[b] Cells were infected with CDV (strain Onderstepoort; MOI of 1) for 24 h, and expression of the viral hemagglutinin was measured by flow cytometry. To compare the relative infectivities of the cells, the percentage of CDV-H-positive cells and the mean fluorescence intensity (MFI) are given.

[c] BHK cells do not express the K41 epitope, but MAb MM2/57 interacts with these cells (Fig. 1).

[d] Approximately 1% of cells of cell lines MDBK and LLCPK can be infected with CDV.

[e] The reduction of CDV-positive cells was measured by flow cytometry. Cells preincubated with and without MAb K41 (100 μg/ml) for 1 h were infected with CDV (MOI of 0.1) for 24 h, and the percentage of CDV-positive cells was determined by using a polyclonal dog anti-CDV hyperimmune serum and FITC-conjugated second antibodies. ND, not done.

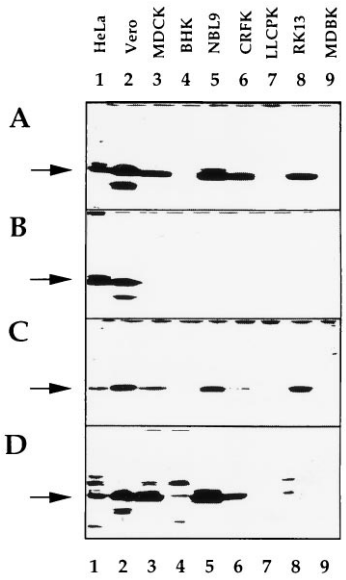

FIG. 1. Western blot of extracts of cell lines of various species. Crude extracts of the cell lines HeLa (human; lane 1), Vero (monkey; lane 2), MDCK (canine; lane 3), BHK (hamster; lane 4), NBL9 (raccoon; lane 5), CRFK (cat; lane 6), LLCPK (pig; lane 7), RK13 (rabbit; lane 8), and MDBK (bovine; lane 9) were run on an SDS–14% polyacrylamide gel and blotted on filters. The blots were developed with the anti-CD9 antibodies K41 (A), ALB6 (B), BU16 (C), and MM2/57 (D), peroxidase-conjugated secondary antibodies, and ECL (Amersham). The arrows mark the position of CD9 (26 kDa).

nized the eluted 26-kDa protein (not shown). In addition, MAb K41 recognizes recombinant CD9 expressed after transfection of CD9-negative cells with a CD9 expression vector (see below). In cell extracts from cell lines of different species, the commercial MAbs against CD9 recognized proteins of the same molecular weight as did MAb K41 but had different species specificities (Fig. 1). Whereas MAb K41 recognized its epitope in extracts of human, monkey, canine, raccoon, cat, and rabbit cells (Fig. 1A), MAb ALB6 did so in extracts of human and monkey cells (Fig. 1B, lanes 1 and 2), MAb BU16 did so in extracts of human, monkey, dog, raccoon, and rabbit cells (Fig. 1C lanes 1 to 3, 5, and 8), and MAb MM2/57 did so in extracts of human, monkey, dog, raccoon, and cat cells (Fig. 1D lanes 1 to 3, 5, and 6). In addition, MAb MM2/57 recognized a molecule similar in size to CD9 in extracts of the hamster cell line BHK (Fig. 1D, lane 4). This result suggests that these cells contain a hamster-specific CD9 which is not recognized by MAb K41 but confers susceptibility to the CDV infection (Table 1).

Inhibition of the CDV infection with anti-CD9 antibodies. As shown in Table 1, MAb K41 inhibited the infection of K41-positive cells with CDV. These results were determined by flow cytometry. To test by a second method the capacity of anti-CD9 antibodies to inhibit the uptake of virus and/or syncytium formation, a range of MOIs of CDV and constant concentrations of the MAbs were used in a plaque reduction assay. The experiment was done with MAb K41 (100 μg/ml), the commercial antibodies BU16 (50 μg/ml) and MM2/57 (50 μg/ml) against CD9, and anti-CD46 (MAb 13/42; 100 μg/ml) inhibiting MV infection of cells (42) and anti-MHC class I (MAb W6/32; 50 μg/ml) as controls. Two different striking effects were caused by the anti-CD9 antibodies: a reduction of the plaque size and a reduction of the number of plaques. The drastic reduction in both size and number of the plaques induced by CDV was visible in cultures preincubated with anti-CD9 MAbs after immunofluorescent staining of CDV antigens (Fig. 2). The plaque formation under the conditions used was inhibited by the MAbs against CD9 by up to approximately 70 to 80% after infection of cells with an MOI of 0.01 to 0.05 (Fig. 3).

Decreasing concentrations of anti-CD9 and control antibodies and a constant MOI of 0.01 of CDV were used in an infection inhibition test performed in microtiter plates. Fifty percent inhibition of syncytium formation was achieved with approximately 50 μg of K41, 25 μg of ALB6, 25 μg of BU16, and 12.5 μg of MM2/57 per ml in assays using HeLa cells and by 25 μg of K41, 50 μg of mALB6, 25 μg of BU16, and 25 μg of MM2/57 per ml in assays using Vero cells. Unrelated antibodies recognizing the cell surface as anti-MHC class I (MAb W6/32) or anti-CD46 (MAb 13/42) did not inhibit the syncytium formation after CDV infection.

When BHK cells were pretreated with antibody MM2/57 (100 μg/ml; in an experiment as described in Table 1 for MAb K41), the CDV infection (MOI = 0.1) could be inhibited by approximately 25%. Thus, the infection of all susceptible cells with CDV was inhibited with antibodies against CD9 and its homologs of other species.

Transfection of cells with a CD9 expression plasmid and CDV infection. The function of CD9 as a putative receptor for CDV was assessed by transfection with an expression plasmid containing the 1.3-kb full-length cDNA of CD9 under the control of the SRα promoter/enhancer (24). For these experiments, we selected mouse NIH 3T3 and bovine MDBK cells, which do not express the CD9 epitope recognized by the antibodies used. NIH 3T3 cells are not permissive for CDV, and MDBK cells are infectable at a very low level (Table 1). Transient expression of CD9 in MDBK cells led to a considerable

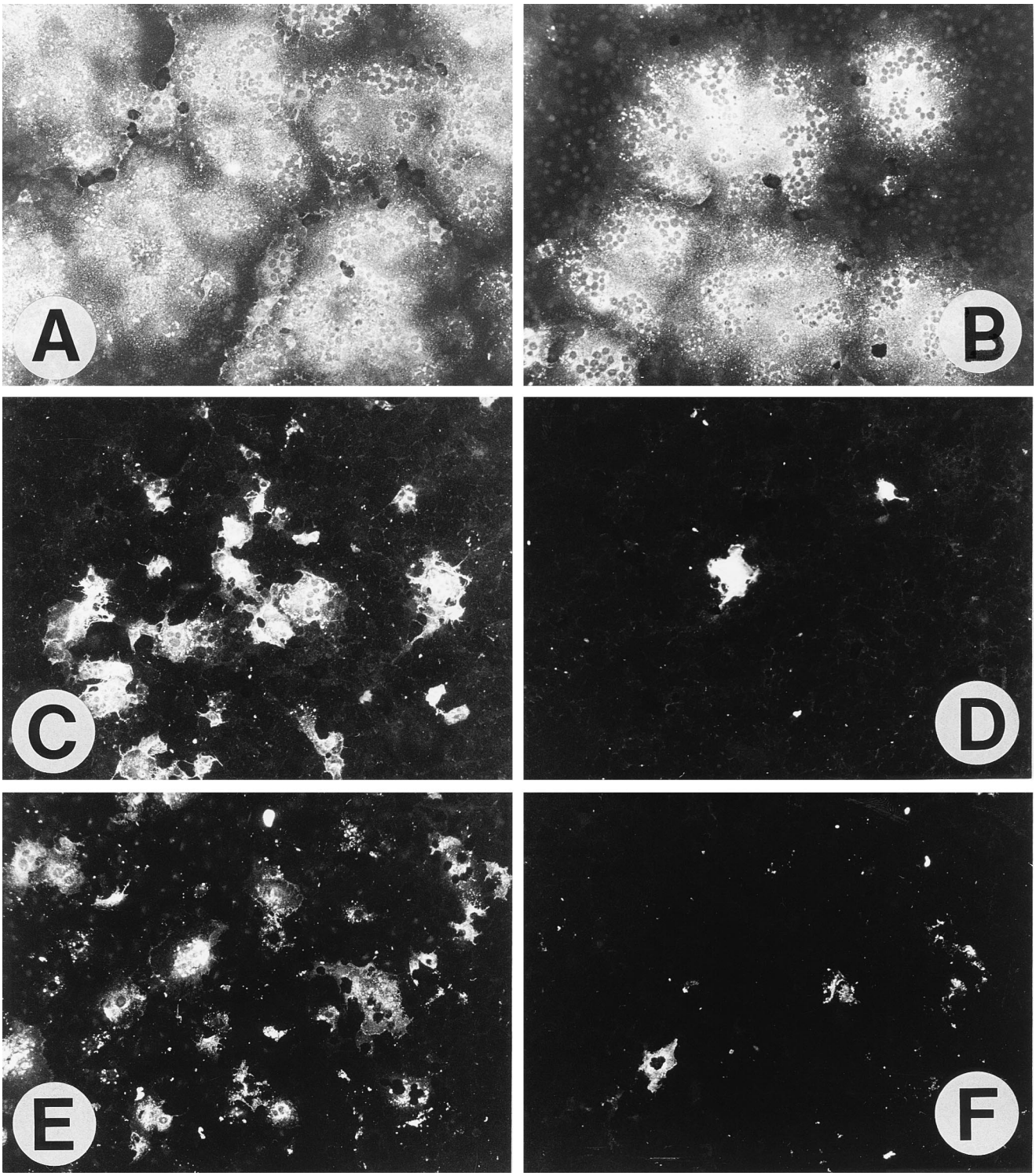

FIG. 2. Reduction of plaque size and the number of plaques by MAbs against CD9, determined by immunofluorescent staining of viral antigens in infected Vero cells with polyclonal anti-CDV serum and FITC-conjugated second antibodies. Vero cells were infected with CDV at MOIs of 1 (A, C, and E) and 0.05 (B, D, and F). Cells were pretreated with medium (A and B), anti-CD9 MAb K41 (C and D), and anti-CD9 MAb BU16 (E and F). Scale, 3 cm = 100 μm (magnification, ×300).

increase in both the number of infectious centers and the size of syncytia (Fig. 4). The propagation of CDV in transiently CD9-transfected MDBK cells was measured by titration of newly synthesized CDV after 3 days of infection. Transfection of cells with 1 and 5 μg of plasmid/well led to a dose-dependent increase in the CDV production (Fig. 5A).

To establish stably transfected cell lines, we transfected MDBK and NIH 3T3 cells with the CD9 expression plasmid

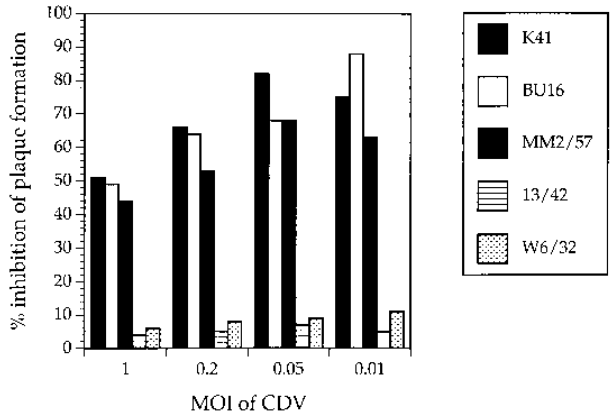

FIG. 3. Percentage inhibition of plaque formation by antibodies against CD9. Vero cells were pretreated with MAbs against CD9 (K41, BU16, and MM2/57) or against CD46 (13/42) and MHC class I (W6/32) as controls. Thereafter, cells were infected with different MOIs of CDV as indicated, incubated for 48 h, fixed, permeabilized, and stained with a polyclonal dog anti-CDV hyperimmune serum and FITC-conjugated second antibodies to count the plaques.

and selected with G418 for resistant cells. Using MAb K41 in flow cytometry, we selected lines expressing various defined levels of CD9 for further experiments. The number of CDV-infected cells and the number of giant cells were markedly increased in CD9-transfected cell lines in comparison to mock-transfected cells (not shown). The CDV production was measured in six stably transfected NIH 3T3 cell lines expressing various levels of CD9 (mean fluorescence intensities of between 255 and 114 [Fig. 5B]) and the original cell line (Fig. 5B, lane 7). In comparison to CD9-negative cells, the CDV production in CD9-expressing transfected NIH 3T3 cells was enhanced by a factor of 10 to 100, and the virus yield was roughly correlated with the expression level of CD9. Fully permissive cells, like Vero and HeLa cells, produce virus titers of approximately 10^6 PFU/ml under the same conditions. Thus, in these cells, yields are higher by a factor of at least 100 in comparison to the CD9-transfected, naturally nonpermissive cells.

Lack of evidence for a direct binding of CDV to CD9. The binding capacity of CDV to CD9 was first assessed by immunoprecipitation with antibodies against CDV and CD9 to detect possible coprecipitated proteins. For immunoprecipitation, HeLa cells were infected with CDV and after 24 h labeled metabolically with [^{35}S]methionine and -cysteine. Viral proteins were precipitated with a polyclonal serum against CDV and a MAb against CDV-H. CD9 was precipitated by both

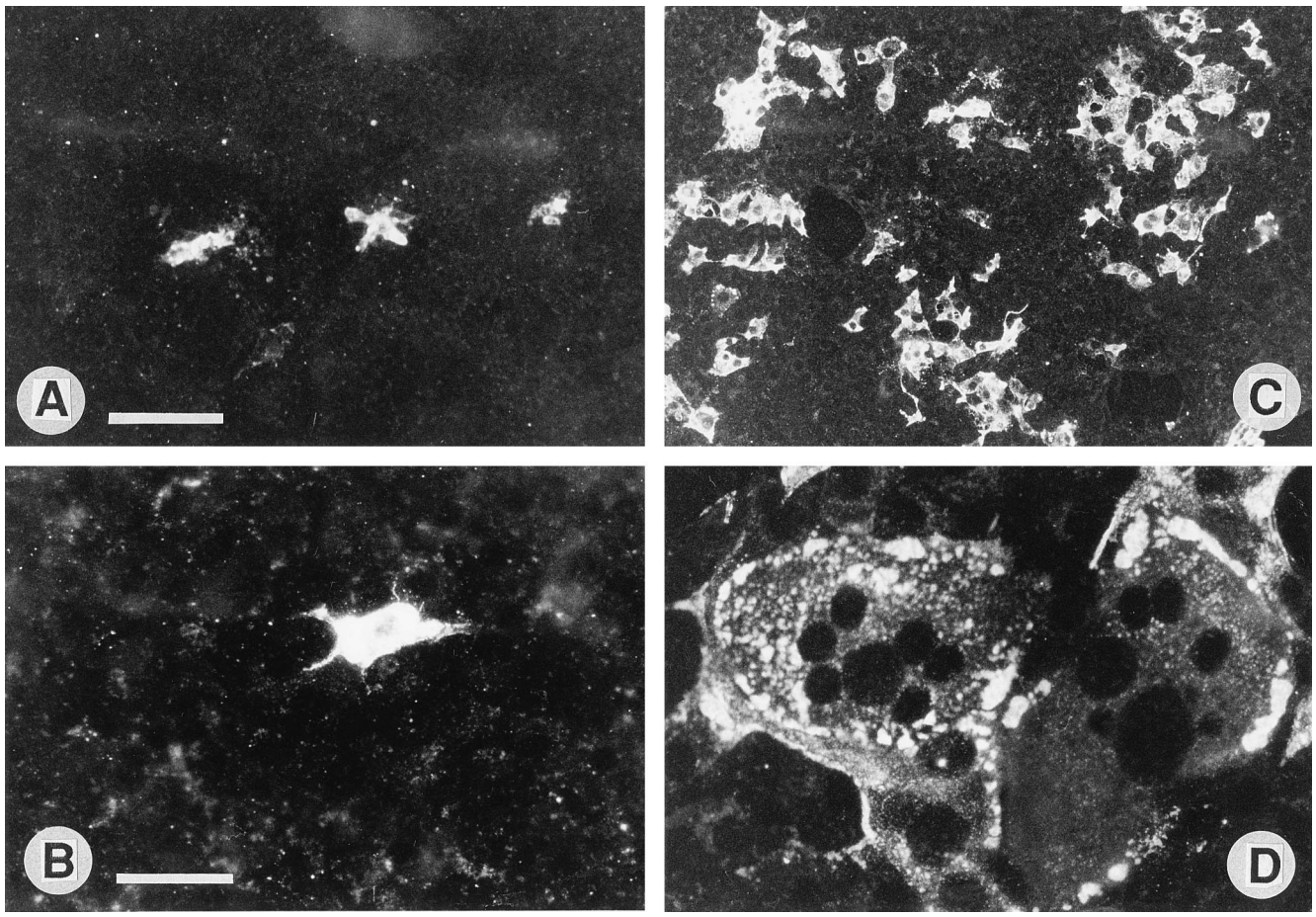

FIG. 4. Immunofluorescence analysis of CDV antigen expression in 48-h CDV-infected normal MDBK cells (A and B) and MDBK cells transiently transfected with the CD9 expression plasmid (C and D) (lower magnification in panels A and C [the bar in panel A represents 50 μm] and higher magnification in panels B and D [the bar in panel B represents 12.5 μm]). Single cells in the normal MDBK cell monolayer were found to have taken up CDV, whereas giant cell formation with many dark nuclei in CDV-infected syncytia was found for CD9-transfected cells.

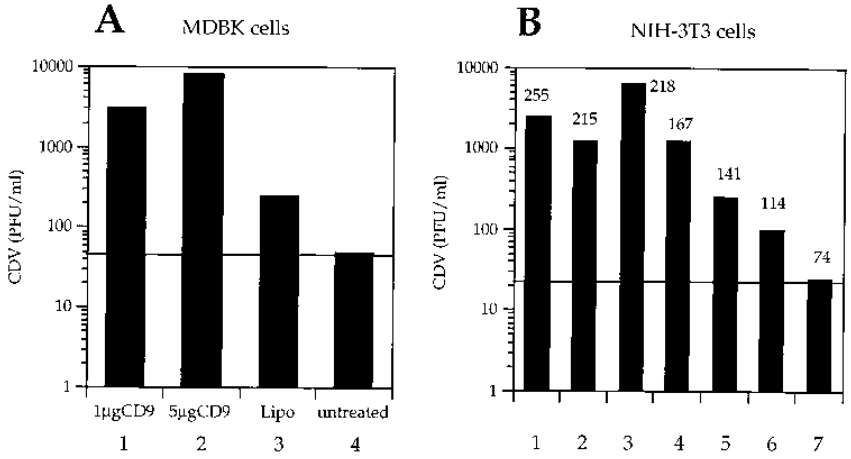

FIG. 5. Transfection of MDBK and NIH 3T3 cells with a CD9 expression plasmid leads to enhanced virus production. (A) MDBK cells (10^6 in each case) were transfected with 1 μg (lane 1) and 5 μg (lane 2) of CD9 plasmid, mock treated with Lipofectin (lane 3), or not treated (lane 4). After 48 h, the cells were infected with CDV (MOI = 0.1) for 4 days, and progeny virus (cell bound plus supernatant) was titrated on Vero cells. (B) NIH 3T3 cells were stably transfected with the CD9 plasmid and selected with G418. The relative expression of CD9 on transfected cells (lanes 1 to 6) and the parental line (lane 7) was measured by flow cytometry using MAb K41, and the mean fluorescence intensities of CD9 are given above the columns. NIH 3T3 cells were infected with CDV (MOI = 0.5) for 4 days, and progeny virus (cell bound plus supernatant) was titrated on Vero cells.

MAbs K41 and MM2/57. The specifically precipitated proteins recognized by the different antibodies were readily visible after short exposure. In addition to the 26-kDa band corresponding to CD9, anti-CD9 antibodies precipitated a major protein of approximately 130 kDa. This is likely to be the β1 chain of integrins, which is known to be noncovalently associated with CD9 (41). Additional coprecipitated proteins could not be distinguished clearly from background (data not shown).

To test by a second method whether CDV binds to CD9, a VOPBA was used. Although the protein extracts of Vero cells contained high amounts of CD9, as determined by Western blotting using MAb K41 (Fig. 6B), CDV did not bind to proteins in the molecular range of CD9. CDV did, however, recognize protein bands of approximately 55 to 65 kDa under nonreducing conditions and 65 to 70 kDa under reducing conditions (Fig. 6A). Interestingly, the strength of the interaction between CDV and this unknown protein was strongly dependent on the detergent used for preparation of the cell extracts. Best signals were detected with octylglucoside, whereas Triton X-100, sodium deoxycholate, 3-[3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), and NP-40 were less active.

DISCUSSION

In this report, we describe the isolation of a MAb which inhibits the infection of cells with CDV and recognizes the human transmembrane protein CD9 and homologs of several other species. CD9 is a widely distributed surface protein present on pre-B cells, eosinophilic, basophilic, activated T cells, and various nonhematopoietic cells and tissues, including the central nervous system (6). CD9 is present in all megakaryocytic leukemias, in 90% of nonacute T-lymphoblastoid leukemia cells, and in 50% of acute myeloid and chronic lymphoid leukemia cells (6, 22). It is not expressed by hematopoietic progenitor or resting mature T and B cells (6). The CD9 protein has a molecular mass of 22 to 28 kDa and belongs to the tetraspan or transmembrane 4 (TM4) superfamily of proteins, which contains CD9, CD37, CD53, CD63, CD81, and CD82 (18). TM4 proteins cross the cellular membrane four times and have both NH_2 and COOH termini inside the cell. They have two extracellular loops with the first loop bearing a possible glycosylation site (24). Among the known functions of CD9 is the activation of platelet aggregation, which can be induced by MAbs against CD9. Several molecules are involved in this process: CD9, the FcγRII receptor, and also the platelet integrins αIIbβ3 (GPIIbIIIa or CD41 [21, 46]). CD9 is also known to participate in adhesive and migratory events via integrins of the β1 family, especially the α4β1 and α5β1 integrins (VLA-4 and VLA-5). CD9 has been reported to be noncovalently associated with these receptors in various hematopoietic cell lines (41).

Immunoprecipitation experiments and the VOPBA assays did not support a direct association between CDV and CD9: the MAb against CDV-H did not precipitate a protein consistent with CD9, and similarly, CDV did not bind CD9 in the VOPBA. Instead, a protein of 65 to 70 kDa specifically bound CDV, suggesting that this protein and not CD9 is the attach-

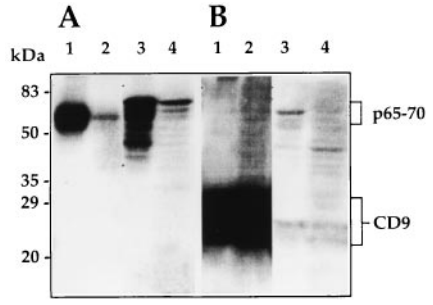

FIG. 6. (A) VOPBA with cell extracts blotted on filters, overlayed with CDV, and visualized with anti-CDV MAbs. (B) Western blot of the same cell extracts developed with anti-CD9 MAb K41. Vero cell extracts were prepared by using octylglucoside (lanes 1 and 3) or Triton X-100 (lanes 2 and 4). The SDS–14% polyacrylamide gels were run under nonreducing (lanes 1 and 2) and reducing (lanes 3 and 4) conditions. CDV (A) binds to a protein with an approximate molecular mass of 55 to 65 kDa under nonreducing conditions and 65 to 70 kDa under reducing conditions. Additional bands visible in lanes 3 and 4 are most likely degradation products of this protein. The anti-CD9 MAb K41 (B) detects the diffuse CD9 band at 26 kDa predominantly under nonreducing conditions (lanes 1 and 2) and weakly under reducing conditions (lanes 3 and 4). In addition, in lanes 3 and 4, other proteins cross-react with MAb K41.

ment receptor for CDV. Although we could not detect direct binding of CDV to CD9, antibodies against CD9 inhibited the infection with CDV and transfection of CD9-negative cells with an expression vector for CD9 rendered these cells susceptible to CDV infection. These data indicate that CD9 has an important function for the infection of cells with CDV and might act as a cofactor. The inhibition by anti-CD9 antibodies reducing the number of infectious centers and the plaque size could be due to steric hindrance of the virus attachment or fusion. Therefore, these data may indicate that CD9 is a component of a receptor complex for CDV, but it is also possible that CD9 acts via intracellular signalling, enhancing the expression or activity of a receptor molecule. Alternatively, CD9 could with lower affinity than necessary for the VOPBA directly interact with a viral protein. Further experiments are required to solve these questions.

Because of the tight association of integrins with CD9, members of this class of surface adhesion proteins are candidates for being the directly CDV-H binding molecules. In addition to integrins, an unknown 45-kDa molecule and the 20-kDa diphtheria toxin receptor have been reported to be tightly associated with CD9 (8, 20, 29, 41). This property of CD9 to associate could apply to other unknown proteins as well. The association of CD9 with other proteins may vary from cell to cell, and several of these molecules may exist in multiprotein complexes as found for other TM4 family members, such as CD37, CD53, CD81, and CD82 (7, 18). All of these proteins have characteristics clearly different from those of the 65- to 70-kDa protein found with the VOPBA. Therefore, the identification of this protein will require its purification and sequence determination.

Stern et al. recently demonstrated with human-mouse somatic cell hybrids that a putative receptor for CDV is located on human chromosome 19 (47). Interestingly, human chromosome 12, which encodes for CD9 (4), was present in addition to chromosome 19 in the permissive cell hybrids. This finding supports the hypothesis that CD9 expressed by these cells could be a cofactor for a second CDV binding molecule encoded by chromosome 19.

CD9 has also been found to be a putative cellular receptor for feline immunodeficiency virus (50), and a different member of the TM4 superfamily, C33 (CD82), was identified to be involved in the syncytium formation by human T-cell leukemia virus type 1 (19). Similar to our findings, neither a direct binding of feline immunodeficiency virus to feline CD9 nor human T-cell leukemia virus type 1 to CD82 was demonstrated, suggesting again indirect mechanisms for these two members of the TM4 family in virus uptake.

It has been reported that several signal transduction pathways are affected by antibodies against CD9 (36, 37, 52, 53), that small G proteins are associated with CD9 (45), and that the binding activity of integrins is regulated by CD9 (28). Therefore, it is possible that CD9 is necessary not only for the surface expression of the CDV binding protein but also for the regulation of the binding activity of such a protein. In addition, interaction of cells with CDV might have similar consequences as anti-CD9 antibodies, resulting in intracellular signal transduction. Such signals induced by CDV binding to cells could play an important role in the pathogenesis of canine distemper. In this context, it is important to know that CDV has been reported to enhance the procoagulant activity of infected macrophages (9).

Interestingly, Feng et al. (14) recently identified a seven-transmembrane protein called fusin as a cofactor for human immunodeficiency virus type 1 (HIV-1) entry in target cells. Fusin is a putative G-protein-coupled receptor, the normal function of which is unknown. Antibodies to fusin blocked HIV-1 Env-mediated cell fusion and infection of CD4-positive human target cells with HIV-1. An additional seven-transmembrane protein, the substance P receptor, was identified several years ago as a factor enhancing the fusion and replication of measles virus (17, 44). The similarity of the structure of CD9 with these proteins and the role of CD9 as a cofactor for CDV infection is striking. These findings suggest that other enveloped viruses entering their target cells by fusion may as well need multispan transmembrane proteins as cofactors for successful entry.

ACKNOWLEDGMENTS

We thank the Deutsche Forschungsgemeinschaft, the Bundesministerium für Forschung und Technologie, the European Community, and the World Health Organization for financial support.

We thank C. Örvell, Laboratory of Clinical Virology, Huddinge, Sweden, and M. Appel, James R. Baker Institute of Animal Health, Ithaca, N.Y., for providing antibodies. We thank I. Lipkin for critically reading the manuscript.

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