# *In vitro* Canine Distemper Virus Infection of Canine Lymphoid Cells: A Prelude to Oncolytic Therapy for Lymphoma

Steven E. Suter,[1] May B. Chein,[1]
Veronika von Messling,[3] Becky Yip,[1]
Roberto Cattaneo,[4] William Vernau,[2]
Bruce R. Madewell,[1] and Cheryl A. London[1]

Departments of [1]Surgical and Radiological Sciences and [2]Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, California; [3]INRS-Institut Armand-Frappier, Université du Québec, Quebec, Canada; and [4]Molecular Medicine Program, Mayo Clinic, Rochester, Minnesota

## ABSTRACT

*Purpose:* Measles virus (MV) causes the regression of human lymphoma xenografts. The purpose of this study was to determine if canine lymphoid cells could be infected *in vitro* with MV or canine distemper virus (CDV, the canine Morbillivirus equivalent of MV) and determine if *in vitro* viral infection leads to apoptotic cell death.

*Experimental Design:* Reverse transcriptase-PCR was used to examine the expression of both signal lymphocyte activation molecule (CD150) and membrane cofactor molecule (CD46) mRNA. An attenuated CDV expressing enhanced green fluorescent protein was used to infect canine cells *in vitro*. Both flow cytometry and reverse transcriptase-PCR was used to document CDV infection. Cell death was examined using a propidium iodide staining assay and Annexin V binding.

*Results:* Canine lymphoid cell lines and neoplastic B and T lymphocytes collected from dogs with spontaneous lymphoma expressed the Morbillivirus receptor CD150 mRNA. In contrast, only neoplastic lymphocytes expressed detectable levels of CD46 mRNA. Although MV did not infect canine cells, CDV efficiently infected between 40% and 70% of all three canine lymphoid lines tested. More importantly, CDV infected 50% to 90% of neoplastic lymphocytes isolated from dogs with both B and T cell lymphoma. Apoptosis of CDV-infected cell lines was documented.

*Conclusions:* Attenuated CDV may be a useful treatment for canine lymphoma. As such, dogs with lymphoma may represent a biologically relevant large animal model to investigate the feasibility, safety, and efficacy of Morbillivirus therapy in a clinical setting with findings that may have direct applicability in the treatment of human non-Hodgkin's lymphoma.

## INTRODUCTION

The beneficial effects of some bacterial and viral infections on the progress of malignancy have long been observed. The oncolytic viruses studied in some detail in recent years include adenovirus, mumps, measles, bovine enterovirus, Newcastle disease virus, attenuated herpes simplex virus, West Nile virus, and others (1, 2). Attenuated viruses are viewed as novel anticancer agents and clinical testing of several agents is under way (3).

Morbilliviruses are promising anticancer agents for the treatment of non-Hodgkin's lymphoma in human beings and in dogs. Morbilliviruses—including measles virus (MV), rinderpest virus, peste de petits ruminants virus, and canine distemper virus (CDV)—are enveloped viruses in the family *Paramyxoviridae*. They have two membrane glycoproteins—hemagglutinin and fusion. The hemagglutinin envelope glycoprotein binds to the cell surface protein signaling lymphocyte activation molecule (SLAM or CD150; refs. 4–6). Cellular receptors are key determinants of host range and tissue tropism of the Morbilliviruses. In the human, CD150 is expressed on T, B, natural killer, and dendritic cells (7). CD46, also called membrane cofactor protein, is a ubiquitous protein receptor for vaccine strains of MV but not for wild-type strains of the virus (8, 9). During MV infection of human cells, the two envelope glycoproteins are expressed on the surface of the infected cell and induce cell-fusion leading to syncytial formation or apoptosis (10).

Clinical consequences of infection with Morbilliviruses include profound immunosuppression and lymphopenia, and it is the latter effect that has prompted consideration of the use of attenuated MV for treatment of lymphoma. This rationale is supported by clinical reports and laboratory studies. In the clinical setting, regression of human Burkitt's lymphoma, Hodgkin's disease, and acute lymphoblastic leukemia occurred in children after concurrent MV infection (11–17). This effect was noted after both natural MV infection and with the administration of attenuated MV vaccines. In the laboratory, intratumoral injection of an attenuated Edmonston-B-based vaccine strain of MV (MV-Edm) induced regression of large, established human B cell lymphoma xenografts (18). Importantly, because most people are immune to MV, the antitumor effect occurred despite the presence of large amounts of passively transferred anti-MV antibody. Intravenous administration of MV-Edm also slowed tumor progression. Similar

*in vitro* cytopathic effects were seen when both human myeloma cell lines and primary myeloma cells isolated from patients were infected with MV-Edm (19). Both intratumoral and intravenous MV-Edm injections induced potent myeloma xenograft remissions.

These findings led us to hypothesize that attenuated MV or CDV might have oncolytic activity in canine lymphoma. As a prelude to clinical testing of MV and/or CDV as a therapy for canine lymphoma, the aims of this study were to determine if canine cells express CD150 and CD46, to evaluate the efficiency of MV and CDV infection of canine lymphoid cell lines and lymphoblasts isolated from canine lymphoma patients, and to determine if MV or CDV infection leads to cell death.

## MATERIALS AND METHODS

### Cells and Cell Lines

Cell lines included human Jurkat T cells (ATCC TIB-152), marmoset B95a lymphoblastoid cells (20), Vero cells (ATCC CCL-81), and several established canine cell lines, i.e., primitive leukocytic round cell neoplasia, CLL-1390 (CD45+, CD18+, CD11b+, CD34+, CD21−, CD79a−, CD3−), chronic large granular lymphocytic T cell leukemia, CLGL-90 (CD45R+, CD18+, CD3+, TCRαβ+, variably CD8α+, and CD79a−: all kind gifts from Dr. Peter Moore, UC Davis, Davis, CA), and acute B cell lymphoma, 17-71 (21), (CD45+, CD79a+, CD34−, CD3−, TCR αβ−, TCR γδ−, CD21−: kind gift from Dr. Ilene Kurzman, University of Wisconsin, Madison, WI). These canine cell lines are referred to collectively in the text as canine lymphoid cell lines. The canine osteosarcoma cell line, D-17 (ATCC CCL-183), and canine melanoma cell line, DC (ATCC 339301-7), were also used. All cells were maintained in 10% fetal bovine serum-Iscove's modified Dulbecco's medium containing 1% penicillin, streptomycin, fungizone at 37°C/5% CO$_2$. Peripheral blood mononuclear cells and peripheral blood lymphocytes were separated from the whole blood of clinically healthy dogs using gradient centrifugation as previously described (22). Neoplastic lymphocytes were collected by aspiration of enlarged peripheral lymph nodes of dogs with B and T cell lymphoma.

### Flow Cytometry

Cells (1 × 10$^6$) were stained with mouse anti-human CD150 and CD46 monoclonal antibodies and analyzed using flow cytometry. Cells were stained with a phycoerythrin (PE)-conjugated anti-CD46 antibody (E4.3, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and an anti-CD150 antibody (clone IPO-3, Kamiya Biomedical Company, Seattle, WA) followed by a FITC-conjugated goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA). Goat anti-mouse PE-conjugated IgG$_{2a}$ and goat anti-mouse FITC-conjugated IgG antibodies (Santa Cruz Biotechnology) were used to discern nonspecific staining. Cells were analyzed using a Becton Dickinson FACSCalibur (San Jose, CA) machine equipped with an argon laser using standard optics to detect PE and FITC fluorescence. Jurkat cells served as positive cells for expression of CD46 (CD150 negative), whereas marmoset B95a cells served as positive cells for CD150 (CD46 negative).

### Reverse Transcriptase-PCR of CD150, CD46, and Enhanced Green Fluorescent Protein mRNA in Canine Cell Populations

Total cellular RNA was isolated from 5 × 10$^6$ cells using Trizol reagent as recommended by the manufacturer (Life Technologies, Grand Island, NY). cDNA was made as described (23). One microliter of the cDNA reaction was used for a single PCR in a 50 μL reaction containing 1 unit Amplitaq polymerase (PE Biosystems, Frazier, CO) using the manufacturer's recommended buffer conditions. The reaction mixtures were subjected to heating at 95°C for 3 minutes, followed by 35 cycles consisting of 45 seconds at the annealing temperature specific for the primer pair, 1 minute at 72°C, and 1 minute at 95°C.

Reverse transcriptase (RT)-PCR CD150 primers: forward, 5′-AGC TGA GCT GTG GGA CAG GT-3′; and reverse, 5′-GCT CTT GTT CAT CGT CTT GC-3′ were used based on the published canine CD150 sequence (Genbank AF390108) to produce a 125-bp product. Canine-specific CD46 primers were based on the TIGR *Canis familiaris* gene index BM537630: forward, 5′-CCA CAA TGT GAA AAG ATT TAC TGT-3′; and reverse, 5′TTG ACC ACT TTA CAA TGA GGA G-3′, to produce a 225-bp product. To detect enhanced green fluorescent protein (eGFP) mRNA of infected cells, eGFP primers: forward, 5′-CTG AAG TTC ATC TGC ACC ACC-3′; and reverse, 5′-CAT GGC GGA CTT GAA TAA GTC-3′ were used. β-Actin primers: forward, 5′-TGA CCC AGA TCA TGT TTG AGA CC-3′; and reverse, 5′-TCC TGC TTG CTG ATC CAC ATC T-3′, were used to control for the presence of cDNA. All RT-PCR products were electrophoresed on 1% to 1.5% agarose gels with 0.5 μg/mL ethidium bromide.

### Construction and Recovery of a Canine Distemper Virus Genomic Full-length Plasmid Expressing Enhanced Green Fluorescent Protein from an Additional Transcription Unit

To introduce eGFP in an additional transcription unit located at the first position upstream of the N gene in the CDV-Onderstepoort genome, a three-step cloning strategy was pursued. First, the leader sequence and the 5′ untranslated region of the N gene were connected directly to the eGFP gene using overlap extension PCR (24). In a second overlap extension PCR, the untranslated region between the N and P gene was attached to the stop codon of the eGFP gene. The resulting PCR product was digested with *Aat*II and *Psh*AI, and transferred into the full-length plasmid pCDVII that codes for the small plaque-forming variant of the vaccine strain Onderstepoort (25), resulting in pCDVeGFPΔN. To reinsert the N gene, the untranslated region between N and P was connected upstream of its start codon using overlap extension PCR. The resulting PCR product was digested with *Psh*AI and introduced in pCDVeGFPΔN, yielding pCDVeGFPuN.

After sequence verification of the inserted fragments, recombinant viruses were recovered as described previously, using a MVA-T7-based system (25). First eGFP-expressing syncytia were detected around 8 days after transfection, as compared with 6 days in the control transfected with standard virus cDNA. For each virus, three syncytia were picked, transferred onto fresh Vero cells in six-well plates, and expanded into 75 cm$^2$ flasks with 10 mL DMEM supplemented

with 2% FCS. When the cytopathic effect was pronounced, the cells were scraped into the medium and subjected once to freezing and thawing. The cleared supernatants containing the viral vector, called CDV-eGFP in the text, were used for all further analysis.

The construction and propagation of a human Edmonston-B strain MV vector, MV-eGFP-Edm, has been previously described (26).

### Virus Growth Analysis

Vero cells ($5 \times 10^5$ per well) were seeded into 12-well plates and infected at a multiplicity of infection of 0.01 with the respective viruses. All analyses were done in duplicate. After 2 hours of adsorption, the inoculum was removed and the cells washed twice with medium and further incubated at $32°C$. At various times after infection, supernatant and cell-associated virus were recovered separately and stored at $-70°C$. The 50% tissue culture infectious dose ($TCID_{50}$) of the samples was determined by limited dilution.

### Virus Infection Assays

After harvesting, cell lines were washed twice in PBS and counted. Cells ($5 \times 10^6$) were aliquoted to 1.5 mL Eppendorf tubes and centrifuged briefly to remove the PBS. Cells were incubated with either MV-eGFP or CDV-eGFP at a multiplicity of infection of 2.0 in 250 mL Opti-MEM (Life Technologies) for 2 hours at $37°C$. At the end of the incubation period, the viral supernatant was removed by centrifugation, the cells were washed thrice with PBS, and then maintained in 10% fetal bovine serum-Iscove's modified Dulbecco's medium containing 1% penicillin, streptomycin, and fungizone at $37°C/5\%$ $CO_2$. At various time points, the cells were harvested and assayed for eGFP expression using flow cytometry or assayed for eGFP mRNA expression as described above.

Neoplastic lymphocytes were harvested from lymphoma patients via fine needle aspiration of enlarged peripheral lymph nodes. The aspirates were washed twice in PBS and resuspended in ice-cold RBC lysis buffer (Sigma, St. Louis, MO), incubated at $37°C$ for 5 minutes, and washed twice with PBS. After counting, $5 \times 10^6$ cells were incubated with virus as described above.

### Cell Death Assays

A propidium iodide assay was used to determine whether or how efficiently MV or CDV caused cell death. Briefly, after virus infection of $5 \times 105$ cells as described above, the cells were collected after 24, 48, and 72 hours of culture, washed twice with 0.1% glucose-PBS, fixed in 1 mL cold 70% ethanol, and stored at $4°C$. Before flow cytometric analysis, the cells were centrifuged, and 0.5 mL propidium iodide staining solution was added (50 μg/mL propidium iodide and 10 μg/mL RNase in 0.1% glucose-PBS). The fold increase in cell death was calculated by comparing the difference in cell death at specific time points between infected and uninfected cells.

Genomic DNA was also isolated using standard procedures from infected and uninfected cells to document apoptosis. Briefly, all attached and floating cells were harvested and dissolved in 500 μL pyruvate kinase buffer [0.1 mol/L Tris-HCl

(pH 7.8), 0.5% SDS, 0.005 mol/L EDTA, 20 μg/mL proteinase K] at $37°C$ for 4 to 6 hours. The lysates were centrifuged ($13,000 \times g$) for 5 minutes with subsequent supernatant extraction with phenol/chloroform/isoamyl alcohol. The DNA was precipitated using isopropol alcohol at $-20°C$ for at least 1 hour. After washing with 70% ethanol, the pellet was resuspended in TE buffer (pH 8.0), treated with RNase A (Sigma) at $37°C$ for 30 minutes, and electrophoresed on a 1.5% agarose gel with 0.5 μg/mL ethidium bromide.

An Annexin V-PE apoptosis detection kit (BD Biosciences PharMingen, San Diego, CA) was also used to detect early apoptosis of CDV-eGFP-infected cells. After CDV-eGFP infection of various cells, all floating and adherent cells were harvested at the prescribed time points and stained according to the manufacturer's instructions. Cells were analyzed using a Becton Dickinson flow cytometer.

## RESULTS

### Evaluation of Canine Cells for the Expression of CD150 and CD46

Wild-type members of the Morbillivirus genus bind to target cells via CD150, whereas certain attenuated vaccine strains can also bind to CD46. Initially, canine cell lines and peripheral blood lymphocytes from a normal dog were examined for CD150 and CD46 cell surface expression with commercially available mouse anti-human monoclonal antibodies using flow cytometry. In Fig. 1, human Jurkat cells, which are CD46+/CD150−, and marmoset lymphoblastoid B95a cells, which are CD46−/CD150+, were used as staining controls. None of the canine cell lines examined, which included CLL-1390, CLGL-90, and 17-71 cells, or peripheral blood mononuclear cells isolated from a normal dog were stained using these antibodies. Neoplastic lymphocytes isolated from dogs with both B and T cell lymphoma were also not stained (data not shown).

Because mouse anti-human monoclonal antibodies may not cross-react with the canine proteins, total RNA was isolated from a variety of cells and examined for CD150 and CD46 mRNA expression via RT-PCR. In Fig. 2A, the lack of a RT-PCR product with the marmoset B95a cells (which are CD150+) shows the specificity of the reaction for canine CD150. No CD150 expression was seen in non-lymphoid melanoma and osteosarcoma canine cell lines. In contrast, CD150 mRNA expression was seen in all three canine cell lines tested (CLL-1390, CLGL-90, and 17-71). Additionally, CD150 mRNA was detected in peripheral blood mononuclear cells isolated from a normal dog and, more importantly, in neoplastic lymphocytes isolated from the enlarged lymph nodes of dogs with both B cell and T cell lymphoma.

RT-PCR was also used to examine the expression of CD46 mRNA. In Fig. 2B, the lack of a RT-PCR product with the human Jurkat cells (which are CD46+) shows the specificity of the reaction for canine CD46. Similar to the CD150 RT-PCR experiment, canine CD46 mRNA was not detected in the non-lymphoid melanoma and osteosarcoma canine cell lines. In contrast to the CD150 RT-PCR experiment, CD46 mRNA was expressed only in the neoplastic lymphocytes isolated from dogs with B and T cell lymphoma.
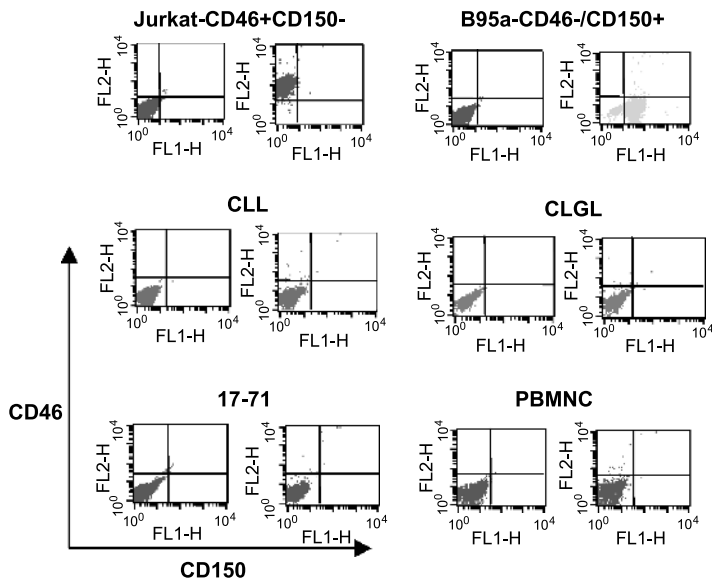
Fig. 1   Canine cell surface expression of CD150 and CD46. Various canine cell lines and cells isolated from normal dogs were stained with commercially available mouse anti-human CD150 and CD46 monoclonal antibodies. Human Jurkat T cells, which are CD46+/CD150−, and marmoset B95a cells, which are CD46−/CD150+, were used as positive staining controls for the CD46 and CD150 antibodies, respectively. PBMNC, peripheral blood mononuclear cells isolated from a normal dog as described in Materials and Methods.

## Measles Virus–Enhanced Green Fluorescent Protein Infection of Canine Cells

Initially, the *in vitro* infection of canine cells with MV-eGFP was examined. Human Jurkat cells and marmoset B95a cells are efficiently infected with MV (20, 27). Figure 3 shows the eGFP RT-PCR results of infected canine cells (*top*); the percentage of selected cell populations that were infected using flow cytometry to detect eGFP fluorescence is also shown (*bottom*). Using RT-PCR, it was possible to detect eGFP mRNA in all the cell populations infected with MV-eGFP. As seen in the bottom of the figure, the efficiency of infection of the canine cells was much lower (1–2% eGFP+) when compared with that achieved in the Jurkat cells (94% eGFP+). The low efficiency of infection was also observed in 17-71 canine lymphoid cells as well as in freshly isolated neoplastic lymphocytes from a dog with T cell lymphoma (data not shown).

## Canine Distemper Virus–Enhanced Green Fluorescent Protein Infection of Canine Cells

We next asked whether canine cells can be infected *in vitro* with CDV-eGFP. Figure 4A shows that, as with the MV-eGFP virus, human Jurkat cells were highly infectable at the same multiplicity of infection of 2.0 (98% eGFP+). The canine lymphoid cell lines 17-71 and CLGL-90 were also efficiently infected (93% eGFP+ and 60% eGFP+, respectively). More importantly, neoplastic lymphocytes isolated from patients with B cell and T cell lymphomas were also efficiently infected with CDV-eGFP (47% and 90% eGFP+, respectively; Fig. 4B). Repeat experiments showed that CDV-eGFP consistently infected neoplastic lymphocytes isolated from dogs with both B cell (*n* = 3) and T cell (*n* = 2) lymphoma (data not shown).

## Propidium Iodide Cell Death Assays after Enhanced Green Fluorescent Protein–Canine Distemper Virus Infection

Cell death resulting from CDV infection has been documented in a variety of *in vitro* and *in vivo* experiments (28–30). A propidium iodide assay was used to document cell death after infection with CDV-eGFP. Figure 5A is a graph generated by comparing the percentage of cell death at specific time points between uninfected cells and CDV-eGFP-infected cells. Five days after CDV-eGFP infection, there was close to a 10-fold increase in cell death of Jurkat cells, with an ∼5-fold increase in cell death of both CLGL-90 and 17-71 cells. Fluorescent micrographs of CDV-eGFP-infected CLGL-90 and 17-71 cells are shown in Fig. 5B. The infected cells were eGFP+ and, more importantly, exhibited some of the characteristic morphologic signs of apoptosis, including cell shrinkage and nuclear fragmentation. It was not possible to document cell death
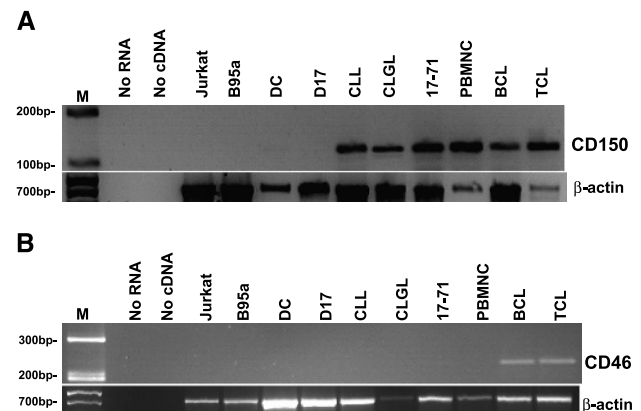


Fig. 2   Expression of message for CD150 and CD46. Total RNA was used for RT-PCR reactions using canine-specific CD150 (*A*) and CD46 (*B*) primers. No RNA, no RNA in the cDNA reactions; No cDNA, no cDNA in the PCR reactions; Jurkat, human Jurkat T cells; B95a, marmoset lymphoblastoid cell line; DC, canine melanoma cell line; D17, canine osteosarcoma cell line; CLL, CLL-1390, canine primitive leukocytic cell line; CLGL, CLGL-90, canine chronic large granular lymphocytic leukemia cell line; 17-71, canine acute B cell lymphoma cell line; PBMNC, peripheral blood mononuclear cells isolated from a normal dog; BCL, neoplastic lymphocytes isolated from the enlarged lymph node of a dog with B cell lymphoma; TCL, neoplastic lymphocytes isolated from the enlarged lymph node of a dog with T cell lymphoma.

*Fig. 3* MV-eGFP infection of normal and neoplastic lymphoid cells. Cell populations were infected with MV-eGFP and assayed 2 days later for eGFP mRNA expression using RT-PCR (*top*). β-Actin primers were used to control for the presence of cDNA. Flow cytometry was used to assay for eGFP expression as an indirect measure of MV-eGFP infection efficiency. LN Asp, neoplastic lymphocytes isolated from a dog with B cell lymphoma; 1st and 3rd wash, 10 μL aliquot of PBS used to wash the cells after incubation with MV-eGFP.
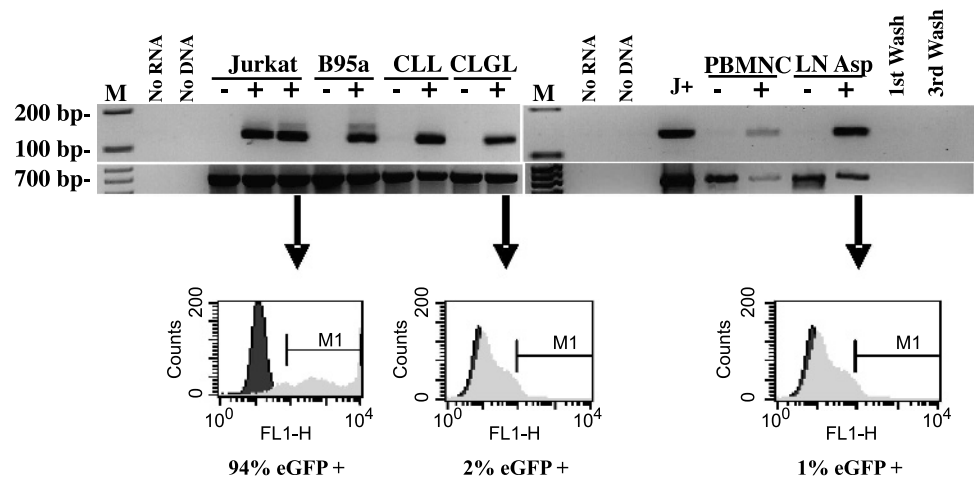
of CDV-eGFP-infected neoplastic lymphocytes isolated from dogs with lymphoma because the optimal culture conditions for extended *in vitro* studies are currently unknown. After only 2 days in culture, ~ 50% of the uninfected neoplastic lymphocytes died, as evidenced by increased propidium iodide staining. The addition of phytohemaglutinnin, concanavalin A, or increases in serum concentrations (up to 20% fetal bovine serum) did not affect survival. Both CDV-eGFP-infected and -uninfected neoplastic lymphocytes exhibited similar cell death profiles (data not shown).

**Apoptotic Assays after Canine Distemper Virus–Enhanced Green Fluorescent Protein Infection**

Apoptosis after CDV infection was documented using two different assays. An Annexin V staining kit (see Materials and Methods) was initially utilized to document early apoptosis by binding to exposed phosphatidylserine, an early event in the apoptotic pathway. In Fig. 6*A* (*top*), ~ 50% of CDV infected 17-71 cells showed increased staining of phosphatidylserine when compared with uninfected cells 2 days after infection. Figure 6*A* (*bottom*) shows CDV-infected 17-71 cells that were



*Fig. 4* CDV-eGFP infection of normal and neoplastic lymphoid cells. *A,* cell populations were infected with CDV-eGFP and assayed 2 days later for eGFP mRNA expression using RT-PCR (*top*). β-Actin primers were used to control for the presence of cDNA. Flow cytometry was used to assay for eGFP expression as an indirect measure of MV infection efficiency. Vero, African green monkey kidney cell line. LN Asp, neoplastic lymphocytes isolated from a dog with B cell lymphoma. *B,* RT-PCR and flow cytometry of CDV-eGFP-infected neoplastic lymphocytes isolated from a dog with T cell lymphoma.

**A** *Jurkatt* / **17-71** / **CLGL**
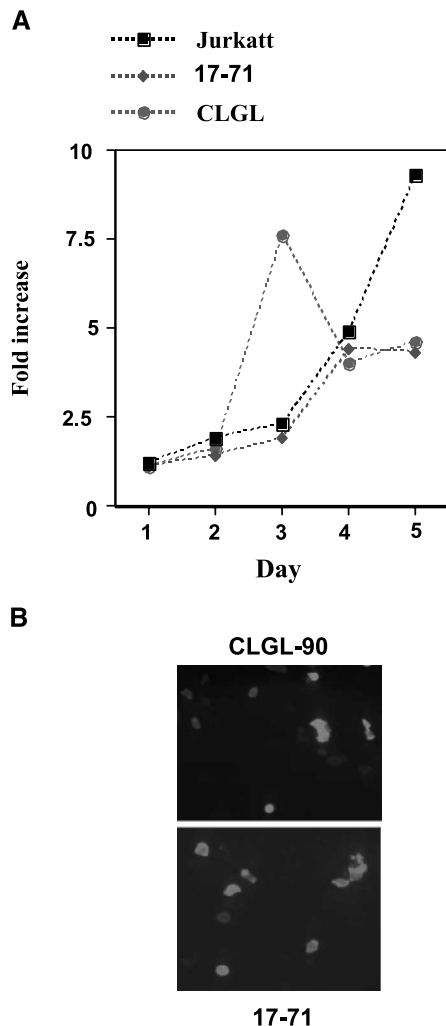
**B**
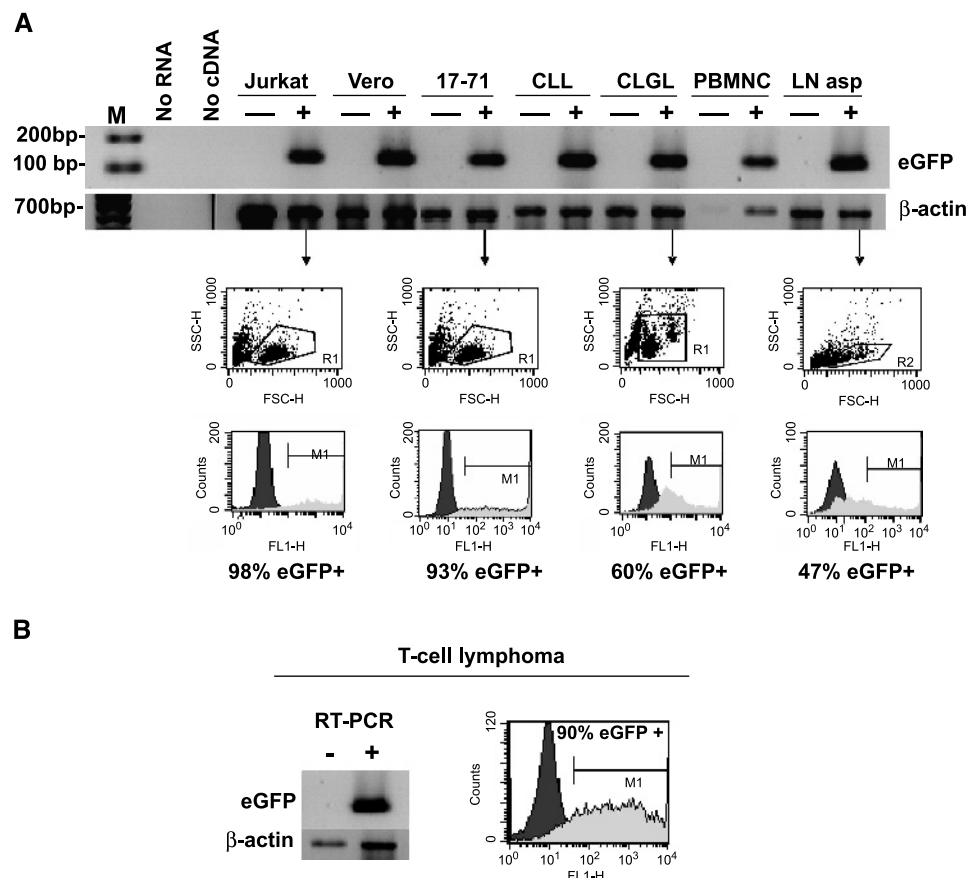
**CLGL-90**

**17-71**

Fig. 5 Propidium iodide cell death assays of CDV-eGFP-infected cells. Human Jurkat T cells and two canine cell lines, 17-71, and CLGL-90, were infected with CDV-eGFP. Cell death was assayed 2 days later with propidium iodide staining followed by FACS analysis. *A*, graph showing fold increase in cell death of CDV-infected cells when compared to uninfected cells; *B*, fluorescent micrographs of CDV-eGFP-infected canine 17-71 and CLGL-90 cells.

stained with both Annexin-PE and 7-AAD. Approximately 35% of the cells were both 7-AAD- and Annexin-PE-positive. Similar results were seen with infected CLGL-90 and Jurkat cells (data not shown).

Apoptosis leads to a classic DNA "ladder pattern" as genomic DNA is degraded into individual ~300 bp nucleosomes. Figure 6*B* shows a typical DNA ladder in CDV-eGFP-infected 17-71 cells 2 days after infection (*lane 2*), although this pattern was not seen in uninfected cells (*lane 1*). Genomic DNA isolated from the other CDV-eGFP-infected canine cell lines and human Jurkat cells also exhibited similar DNA laddering (data not shown).

## DISCUSSION

We have begun to use canine lymphoma as a model to determine the efficacy and feasibility of using Morbilliviruses

as a novel treatment of lymphoma. Commercially available mouse anti-human CD150 and CD46 monoclonal antibodies did not cross-react with canine cells. The results of our RT-PCR experiments clearly showed that canine lymphoid cells express CD150 mRNA, suggesting the presence of the CD150 protein. The tissue tropism of CDV, via binding to canine CD150, is analogous to human MV, which explains the clinical findings of CDV infection-lymphopenia and subsequent immunosuppression.

After adaptation to Vero cells *in vitro*, MV mutations in the hemagglutinin glycoprotein allow the virus to efficiently use CD46 as a receptor (9, 31–33). CD46, a regulator of complement-mediated cell lysis, is expressed on all nucleated cells in humans (34). In contrast to humans, mouse, rat, and guinea pig CD46 is expressed preferentially in the testis (35–38), with evidence in the mouse suggesting localization to the sperm (39). Our RT-PCR experiments did not detect CD46 mRNA in 17-71, CLGL-90, CLL-1390, and nonlymphoid cell lines. Interestingly, we were able to detect CD46 mRNA in neoplastic lymphocytes isolated from both B and T cell lymphoma patients. It is plausible that CDV enters nonlymphoid cells through a receptor other than CD46 (40, 41).

Not surprisingly, the infection efficiency of canine cells with MV-eGFP was low, although infection with CDV-eGFP
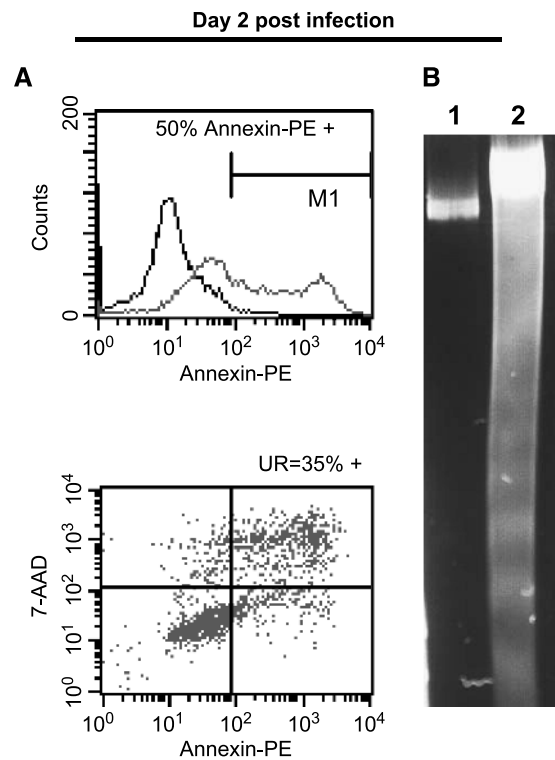


**Day 2 post infection**

**A** **B**

Fig. 6 Apoptosis of lymphoid cells following CDV-eGFP infection. *A*, flow cytometry 2 days after infection. Top, histogram of uninfected (left-hand peak) and infected 17-71 cells. Cells under the M1 gate are Annexin-PE-positive (~50%). Bottom, scattergram of the infected cells, which were stained with both an Annexin-PE antibody and 7-AAD. UR, cells that are both Annexin-PE- and 7-AAD-positive (35%). *B*, agarose gel electrophoresis of genomic DNA isolated from 17-71 cells 2 days after CDV-eGFP infection. Lane 1 is genomic DNA isolated from uninfected cells while lane 2 is genomic DNA isolated from CDV-eGFP-infected cells.

was much higher. It is interesting to note that MV-eGFP did not efficiently infect canine cells, whereas CDV-eGFP did infect close to 95% of human Jurkat cells. MV grows efficiently almost exclusively in primate cells, although high titers can be obtained from growth in Madin-Darby canine kidney cells (42) and Cf2Th cells (25). Different CDV strains grow efficiently in several cell types of different species (42, 43). The underlying molecular interactions that account for these differences have not been elucidated.

Although the major sites of Morbillivirus propagation are lymphoid cells and organs (44), CDV, MV and rinderpest virus can invade the central nervous systems of their hosts. Lymphoid and neuronal cell death is most likely due to apoptosis, rather than necrosis. CDV infection caused apoptosis of Vero cells (30) and activated peripheral blood mononuclear cells *in vitro* (45). *In vivo* apoptosis was seen in the cerebellum and retropharyngeal lymph nodes of both naturally and experimentally CDV infected dogs (28, 29). *In vitro* CDV-eGFP infection of the canine cell lines CLGL-90 and 17-71 led to substantial cell death via apoptosis, as evidenced by the typical "DNA laddering" of genomic DNA and the increased binding of Annexin V (Fig. 6). Although we were able to document CDV-eGFP infection of neoplastic lymphocytes isolated from canine lymphoma patients (Fig. 4), it was not possible to determine if the infection resulted in substantial cell death because the conditions for long-term culture of these cells are currently unknown.

Recent advances in tumor biology, genetics, and virology have provided the knowledge and tools necessary to develop oncolytic adenovirus (ONYX-015 and CV706; refs. 46, 47), reovirus (48), herpesvirus (49, 50), and Newcastle disease virus (51) into effective viral therapies. A live attenuated MV was shown to induce the regression of human lymphoma xenografts in immunodeficient mice (18). A genetically modified MV engineered to enter cells through the CD20 antigen (the target for Rituximab) has also shown promise for the treatment of non-Hodgkin's lymphoma in a xenograft setting (52). One of the major disadvantages of these xenograft experiments is that tumors grown in immunodeficient mice do not mimic the true *in vivo* setting of natural spontaneous tumors. For example, xenograft transplants utilize immortalized cell lines which tend to exhibit exponential growth *in vivo*, whereas natural tumor cell kinetics follow a Gompertzian growth curve. Also, the lack of a functional immune system eliminates important immunologic considerations in the setting of neoplasia such as immune surveillance, induction of antitumor immunity, and importantly for viral oncolytic strategies, the natural development of neutralizing antibodies. For these, and perhaps other reasons, the treatment of tumor xenografts in mice using a variety of anticancer strategies often does not translate into clinical benefit for human cancer patients.

Canine lymphoma is a natural, spontaneous tumor in a large, long-lived animal that is similar phenotypically and biologically to the human variant of non-Hodgkin's lymphoma called diffuse large B cell lymphoma (53). Diffuse, high-grade, B cell lymphoma is one of the most common malignant tumors of middle-aged to older dogs and is the most common hematopoietic tumor of the dog (83%; refs. 54, 55). Death is uniformly the result of recrudescent disease that is nonresponsive to conventional chemotherapeutic drugs, with an overall cure rate below 10% and a 25% 2-year survival (56) compared with a 50% cure rate in human diffuse large B cell lymphoma.

Most dogs and humans are vaccinated regularly against natural CDV and MV infection, respectively. Theoretically, the presence of anti-CDV or MV antibodies may compromise the value of Morbillivirus therapy. However, recent evidence suggests that the therapeutic efficacy of conditionally replicating adenoviruses (57), herpesviruses (58), and reoviruses (59) is not negated by preexisting antiviral antibodies. Additionally, *in vitro* CDV-eGFP infection and/or apoptosis does not guarantee *in vivo* efficacy as evidenced by the finding that although all human tumor cell lines tested were highly susceptible to MV *in vitro*, they were not equally responsive to *in vivo* MV therapy when tested in the xenograft setting (18).

Similar to MV, live attenuated CDV vaccines, derived from the Onderstepoort strain, have been used worldwide for close to 40 years, with millions of doses given yearly in the United States alone with an excellent safety record. The availability of this oncolytic virus, coupled with the striking similarities between canine lymphoma and human non-Hodgkin's lymphoma, offers a unique opportunity to develop much needed alternative treatments for each of these diseases. As such, this disease in dogs represents a biologically relevant large animal model in which to study the utility of novel therapeutics for the treatment of human non-Hodgkin's lymphoma. The experiments presented here show that the canine Morbillivirus, CDV, can infect canine hematologic cell lines and neoplastic lymphocytes isolated from dogs with B and T cell lymphoma. Furthermore, our results show that CDV effectively kills the infected canine cell lines. This provides the rationale for additional *in vitro* and *in vivo* studies of CDV in canine lymphoma with the intention that information gained from these studies will have direct applicability to the future treatment of human patients with non-Hodgkin's lymphoma.

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# Clinical Cancer Research

## *In vitro* Canine Distemper Virus Infection of Canine Lymphoid Cells: A Prelude to Oncolytic Therapy for Lymphoma

Steven E. Suter, May B. Chein, Veronika von Messling, et al.

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