Lysis of Human Tumor Cell Lines by Canine Complement plus Monoclonal Antiganglioside Antibodies or Natural Canine Xenoantibodies

STUART C. HELFAND,*†⁻¹ JACQUELYN A. HANK,†⁻¹ JACEK GAN,†⁻¹ and PAUL M. SONDEL†⁻¹‡⁻¹§⁻¹

*Department of Medical Sciences, School of Veterinary Medicine, Departments of †Human Oncology, ‡Pediatrics, and §Genetics, School of Medicine, and †University of Wisconsin Comprehensive Cancer Center, University of Wisconsin–Madison, Madison, Wisconsin 53706

Received June 1, 1995; accepted August 13, 1995

Because certain antiganglioside monoclonal antibodies can facilitate antibody-dependent cellular cytotoxicity against GD2-ganglioside-bearing human and canine tumor cells, we wished to determine if clinically relevant antiganglioside monoclonal antibodies (Mabs) could also fix canine complement to lyse tumor cells in vitro. Using flow cytometry, human tumor cell lines (M21 melanoma and OHS osteosarcoma) were shown to highly express ganglioside GD2 and, to a lesser degree, GD3. In ⁵¹Cr release assays, M21 cells were lysed with canine serum, as a source of complement, plus either Mab 14.G2a or its mouse–human chimera, ch14.18, specific for GD2. Heating canine serum abrogated its lytic activity and addition of rabbit complement reconstituted M21 lysis. Similar results were obtained with M21 cells when Mab R24 (against GD3) and canine serum were used. OHS cells were also lysed with canine serum plus Mab 14.G2a and lytic activity was abolished by heating canine serum but reconstituted with rabbit complement. Alone, canine serum or Mabs were not lytic to M21 or OHS cells. Conversely, human neuroblastoma (LAN-5) and K562 erythroleukemia cells were lysed by canine serum alone which was shown by flow cytometry to contain naturally occurring canine IgM antibodies that bound LAN-5 and K562 cells. The lytic activity of canine serum for LAN-5 or K562 cells was abolished by heating and restored by addition of either human or rabbit complement. Thus, human tumor cell lines can be lysed with antiganglioside Mabs through fixation and activation of canine complement-dependent lytic pathways. Canine xenoreagents also mediate complement-dependent cytotoxicity of some human tumor cell lines. Together, these results are significant because they demonstrate an antitumor effect of the canine immune system which is of potential importance for cancer immunotherapy in a promising animal model.

INTRODUCTION

Effector functions of antitumor monoclonal antibodies (Mabs) include antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Both mechanisms are considered relevant for tumor cell destruction in vitro (1–4) and in vivo (5). Some Mabs directed against disialogangliosides GD2 (e.g., Mab 14.G2a, chimeric 14.18) (2, 3) and GD3 (e.g., R24, KM641) (1, 6), antigens expressed on human neuroblastoma and melanoma cells (7–9), are highly effective at mediating both forms of cytotoxicity (1–3, 6, 10, 11). These promising activities of antiganglioside Mabs 14.G2a, ch14.18, and R24 stimulated investigations of their therapeutic use in human cancer patients either alone (6, 12, 13) or in combination with human recombinant interleukin-2 (IL-2) (5, 14).

We recently described a spontaneous canine melanoma model in which canine effector cells mediated ADCC of canine melanoma with murine monoclonal antiganglioside antibody 14.G2a (IgG2a) specific for GD2 or its mouse–human chimera, 14.18, with the mouse variable and human constant domains (15). We determined that human recombinant IL-2 enhanced ADCC of canine melanoma by canine effector cells with these Mabs (15) and human recombinant IL-2 activated the canine immune system in vivo (16). In the current report, we sought to examine additional antitumor lytic mechanisms of this canine model of potential importance for human immunotherapy by determining if canine complement could be activated with antiganglioside antibodies to lyse ganglioside-expressing tumor cell lines. To our knowledge, antitumor properties of canine complement in combination with antibody have not previously been described. Our results indicate that canine complement activity can be targeted against tumor cells with Mabs that bind gangliosides on these cells causing appreciable levels of cytolyis. This additional in vitro mechanism of a canine antitumor immune response with antiganglioside Mabs demonstrates an in vitro canine effector function that may potentially have an in vivo correlate.

¹To whom reprint requests should be addressed at School of Veterinary Medicine, University of Wisconsin–Madison, 2015 Linden Drive West, Madison, WI 53706.
Furthermore, while pursuing these investigations of canine CDC, we observed that canine serum was naturally cytolytic to some human tumor cell lines. We dissected this phenomenon and found that naturally occurring canine antibodies of the IgM class mediated tumor cytotoxicity in conjunction with canine complement. Thus, natural canine IgM antibodies can function as xenoreactive antibodies to lyse human tumor cells in conjunction with canine complement.

Taken together, our results indicate that activated canine complement mediates potent antitumor activity and this mechanism could be additive with ADCC in a canine immunotherapy model targeting tumor gangliosides. Our findings that natural canine antibodies bind some human tumor lines and react with canine complement to lyse these cells extend the spectrum of observations of xenogeneic immunoreactivity of the canine immune system. These results could also be relevant to the study of canine xenotransplantation models, which are currently receiving considerable attention (17–21).

**MATERIALS AND METHODS**

**Cells.** A canine malignant melanoma cell line, CML-10 (originally published as CML-6M) (22), was kindly provided by Dr. Lauren Wolfe (Auburn, AL). We have previously shown that it moderately expresses ganglioside GD2 (15). Malignant human cell lines included M21 (melanoma) (2), a gift from Dr. R. Reisfeld (La Jolla, CA), OHS (osteosarcoma) (23), a gift from Drs. Ø. Bruland (Oslo, Norway) and D. Haines (Saskatoon, Canada), and LAN-5 (neuroblastoma), a gift from Dr. R. Seeger (Los Angeles, CA). M21 and OHS cell lines were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 10 mM Heps buffer, penicillin (100 U/ml), and streptomycin (100 μg/ml). The CML-10 and LAN-5 cell lines were cultured in Liebovitz L-15 medium containing the same supplements. Cultures of CML-10, M21, OHS, and LAN-5 were adherent and cells were detached with 0.54 mM EDTA for use as single cell suspensions in experiments. K562 (human erythroleukemia) cells, from ATCC (Rockville, MD), were grown in suspension in supplemented RPMI 1640.

**Monoclonal antibodies.** Murine Mab 14.G2a (IgG2a isotype), kindly provided by Dr. Reisfeld and the National Cancer Institute (NCI), recognizes disialoganglioside GD2 (3). Its mouse-human chimera, ch 14.18, constructed of the identical murine variable region, human IgG1 constant region, and κ light chains (2), was also a gift from Dr. Reisfeld and the NCI. Murine R24 (IgG3) (24), kindly provided by the NCI, recognizes disialoganglioside GD3 (25). Monoclonal antibody W3/62 (IgG2a), as hybridoma supernatant (ATCC), was used for detection of major histocompatibility (MHC) class I molecules. Monoclonal antibodies against human MHC class II molecules DP (IgG1), DQ (IgG1), and DR (IgG2a) were obtained commercially (Becton Dickinson, San Jose, CA).

**Dogs.** Normal adult male and female mixed breed dogs and young dogs raised in a research colony served as the source of whole blood for serum. All phlebotomy procedures were performed in accordance with a protocol approved by the University of Wisconsin Institutional Animal Care and Use Committee.

**Complement.** Cold serum, separated from freshly drawn dottled whole blood of healthy dogs or human donors, served as the source of canine or human complement, respectively. Rapidly processed, pooled lyophilized rabbit serum with complement activity was purchased commercially (Cappel Research Products, Durham, NC). Human blood donations were performed in accordance with a protocol approved by the Institutional Review Board of the University of Wisconsin Medical School.

**Flow cytometry.** Binding of antibodies to live cells was assessed on a FACScan flow cytometer (Becton Dickinson) using standard indirect immunofluorescent techniques, as described (15). Irrelevant murine monoclonal isotype antibodies (Becton Dickinson) were used as controls and included IgG1 for anti-DP and DQ antibodies, IgG2a for Mabs 14.G2a, W3/62 and anti-DR antibody, and IgG3 for R24. Goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) (Becton Dickinson) was used to detect binding of primary murine Mabs to tumor cells. A murine anti-canine IgG monoclonal antibody (IgG1) conjugated to FITC (Sigma Chemical Co., St. Louis, MO) was used to detect canine IgG bound to tumor cells. Sheep polyclonal IgG against canine IgM (μ chain) conjugated to FITC (The Binding Site Inc., San Diego, CA) was used for detection of canine IgM bound to tumor cells. All incubations lasted 30 min and were carried out at 4°C in the dark. Propidium iodide was used as a live–dead cell discriminator and was added just prior to cell analysis.

**Complement-dependent cytotoxicity assay.** Release of chromium-51 was used to assess lysis of tumor cells mediated by antibody and complement. Single cell suspensions of targets consisting of CML-10, M21, OHS, LAN-5, or K562 were each labeled with 250 μCi 51Cr for 2 hr at 37°C in 5% CO2 atmosphere. These cells were washed in medium and 5 × 103 targets in 50 μl of supplemented RPMI 1640 were placed into round-bottom microwells. Various concentrations of Mabs 14.G2a, ch 14.18, and R24 (also in 50 μl) were added individually to the cell targets and allowed to incubate for 20 min at room temperature. At that time, 100 μl of diluted (1:3) fresh canine, human, or rabbit serum was added to the cells, resulting in a final 1:6 dilution of serum complement in the microwells. Some wells received serum which had undergone prior heat inactivation (56°C × 1 hr) while other wells received heat-
inactivated serum plus fresh serum, also at a final concentration of 1:6 for each. The final volume of each well was adjusted to 200 μl with supplemented RPMI 1640 as necessary. All experimental conditions were performed in quadruplicate. Spontaneous and maximum release of 51Cr from targets was assayed in replicates of eight each. Plates were allowed to incubate for an additional 2 hr at 37°C in 5% CO2 atmosphere. Supernatants were harvested (Skatron, Sterling, VA) at that time and radiolabel released into the supernatant was measured with a gamma counter. The percentage cytotoxicity was calculated using the formula

\[
\text{% of cytotoxicity} = \frac{\text{Exp cpm} - \text{Spon cpm}}{\text{Max cpm} - \text{Spon cpm}} \times 100,
\]

where Exp was the experimental number of counts from target cells incubated with serum (and antibody), Spon was the spontaneously released counts obtained from targets incubated in medium alone, and Max was the maximum counts obtained from targets lysed with a 2% cetrimide detergent solution (Sigma Chemical Co.).

Statistical analysis. A two-way nested model was used to compare the cytotoxic effect of serum (and antibody) on the tumor target. The effect of each individual dog has been included in the model as a block effect. The SAS statistical package was used for analysis and the P value for the comparison was derived from the least-squared mean of the generalized linear model procedure.

**RESULTS**

Expression of Surface Markers Detected on Tumor Lines by Flow Cytometry

M21. Staining of M21 melanoma cells with primary Mabs 14.G2a and R24 resulted in bright fluorescence, indicating high expression of gangliosides GD2 and GD3 on these cells (Figs. 1A and 1B). Expression of GD2 by the M21 line was expected as this cell line was previously selected by subcloning for strong expression of ganglioside GD2 (2).

Canine serum, the source of complement in the cytotoxicity assays, was screened for the presence of preformed, natural canine antibodies that could react with these human tumor cell targets. Sera from six dogs were examined and flow cytometric analyses indicated that binding of canine IgG and IgM to M21 cells was nondetectable (IgG) or minimal (IgM) (Figs. 1C and 1D).

OHS. Human osteosarcoma OHS cells also stained brightly with Mabs 14.G2a and R24 (Figs. 2A and 2B). Fluorescence intensity and overall distribution of cells positive for Mab 14.G2a were greater than those for R24 indicating ganglioside GD2 expression exceeded that of GD3 on these cells. Canine IgG, present in fresh dog serum, did not bind and canine IgM showed only minimal reactivity with the OHS line (Figs. 2C and 2D).

LAN-5. Incubation of LAN-5 human neuroblastoma cells with fresh, chilled canine serum, a prerequisite step for flow cytometric examination, resulted in appreciable cell death (determined by flow cytometry), precluding cellular analysis. Heat-inactivating canine serum prior to its incubation with LAN-5 cells did not result in cell lysis enabling examination of canine immunoglobulin binding to LAN-5 cells (Fig. 3). Both canine IgG and IgM showed strong binding to these cells (Figs. 3A and 3B). The LAN-5 line has previously been shown to express ganglioside GD2 (5). To determine if natural canine immunoglobulins bound ganglioside GD2, a flow cytometry blocking experiment was performed. LAN-5 cells were initially incubated with heat-inactivated canine serum, washed, and then incubated with ch 14.18, the mouse–human chimera of Mab 14.G2a sharing identical reactivity for ganglioside GD2 (2). This was compared to binding of ch 14.18 to LAN-5 cells not preincubated with canine serum (Fig. 3C). Binding of ch 14.18 was not inhibited by initial
Reactivity of natural canine immunoglobulins with human K562 erythroleukemia cells. Indirect flow cytometry was used to detect naturally occurring canine IgG or IgM bound to K562 cells. FITC-conjugated mouse IgG against canine IgG was used to detect cell-bound canine IgG (A). FITC-conjugated polyclonal sheep IgG against canine IgM was used to detect cell-bound canine IgM (B). Data are from a single dog and are representative of results from 5 adult dogs and 10 young dogs.

from the ganglioside GD2 binding site of ch 14.18 (and Mab 14.G2a) on human LAN-5 cells. Furthermore, in one experiment, we individually examined sera from 10 young (i.e., 14-week-old) dogs and obtained the same results. Because canine maternal antibody would be low at 14 weeks of age (26), these data show natural canine antibodies against this human neuroectodermal tumor cell line are present at an early age in the dog. LAN-5 cells were negative for expression of MHC class I and II molecules (data not shown).

K562. To extend our observations of the LAN-5 line and canine serum, human K562 erythroleukemia cells were analyzed for their capacity to bind canine IgG and IgM (Fig. 4). This cell line was negative for expression of gangliosides GD2 and GD3 as determined by flow cytometry (data not shown). Similar to LAN-5, we found incubation of K562 cells with fresh canine serum induced considerable cell death prohibiting flow cyto-

incubation of LAN-5 cells with canine serum. These results, repeatable in four separate experiments with sera from four adult dogs, indicated that natural canine immunoglobulin recognized antigenic sites distinct
metric analysis. Heat-inactivated canine serum was not lethal to K562 cells and allowed binding patterns of canine immunoglobulin to K562 to be determined (Fig. 4). In contrast to LAN-5, canine IgG did not recognize the K562 cells. However, binding of canine IgM to K562 was similar to that observed with the LAN-5 cells. These flow cytometry results were obtained with sera from 5 adult dogs as well as with sera from the same 10 young dogs evaluated against the LAN-5 line. Thus, as with LAN-5, it appears that natural canine antibodies against this human erythroleukemia cell line are present in dogs at an early age. K562 cells were negative for expression of MHC class I and II molecules (data not shown).

Lysis of Tumor Cell Lines Mediated by Canine Complement and Antiganglioside Mabs

We sought to determine the tumoricidal properties of canine complement found in canine serum, in combination with antitumor ganglioside Mabs, as the next step in developing a canine immunotherapy model (15). Initially, we sought to answer this question with the GD2+ canine target previously used to demonstrate canine ADCC (15). However, we did not see tumor killing of the canine CML-10 melanoma target mediated by canine complement or by either rabbit or human complement (not shown). Possible explanations for this included a problem with (canine) complement, a problem with antibody, or resistance of the CML-10 cell line to complement-mediated lysis. Thus, we tried other cell lines with higher expression of gangliosides as targets. We used human tumor cell lines because additional canine tumor lines expressing gangliosides of interest were not available.

The ganglioside-expressing tumor lines M21 and OHS were used as tumor targets. These lines were selected because of their immunoreactivity with antiganglioside Mabs 14.G2a and R24, and lack of reactivity with natural canine antibodies. In a 2-hr 51Cr release assay with the M21 melanoma target, complement-dependent cytotoxicity mediated by complement in canine or rabbit serum, in conjunction with Mabs 14.G2a, ch 14.18, and R24, was compared (Fig. 5). Canine serum, combined with Mab 14.G2a, ch 14.18, or R24, mediated significant cytotoxicity of M21 cells compared to that of canine serum alone. The magnitude of cytotoxicity was comparable to that mediated by rabbit serum with Mab 14.G2a or R24. The combination of canine serum plus ch 14.18 mediated lower levels of cytotoxicity than rabbit serum plus ch 14.18. This suggests that canine complement is more effectively activated for cell lysis by murine monoclonal antibody than by a mouse–human chimeric monoclonal antibody. Heating the dog sera, which inactivates the canine complement, abrogated its lytic activity when combined with Mabs, and the cytolytic capacity of heat-inactivated canine serum (complement) plus Mab 14.G2a was reconstituted by adding rabbit serum (Fig. 5). This suggests that the component of canine serum responsible for tumor lysis with Mab was in fact complement. The cytolytic potential of heat-inactivated canine complement plus R24 was only slightly reconstituted by the addition of rabbit complement. Cytotoxic activity of heat-inactivated human serum plus R24 also failed to be completely reconstituted with rabbit complement (not shown), possibly suggesting that there was something in heat-inactivated serum competing with the lytic activity of R24 and complement.

Similar results were obtained with the OHS osteosarcoma target using the 14.G2a antibody while the cytolytic activity of the R24 and ch 14.18 antibodies plus canine serum as complement source was weak against this target (Fig. 6). Lysis of the target was also minimal using rabbit serum plus R24 combined with heat inactivated canine serum. The low level of cytotoxicity mediated by R24 and complement against this target may reflect the lower expression of the target GD3 antigen (Fig. 2B) available to bind R24 which was below the threshold needed to trigger CDC. This could also explain lack of complement-dependent cytotoxicity observed with the canine CML-10 target.

Lysis of Tumor Cell Lines Mediated by Canine Complement and Natural Canine Antibodies

Our flow cytometry results suggested that fresh canine serum killed the LAN-5 neuroblastoma and K562
target in a 2-hr \(^{51}\text{Cr}\) release assay (Fig. 8). In this system, we again found that heating the serum neutralized its lytic properties for tumor cells. As with LAN-5, adding noncytotoxic rabbit complement to heated dog serum restored the serum’s tumoricidal effects. These data indicate that heat-labile complement in canine serum was a mediator of tumor cell lysis by canine anti-K562 IgM present in both the fresh and heated serum.

**DISCUSSION**

We have previously described a canine model for cancer immunotherapy based on ADCC employing tumor-reactive antiganglioside Mabs. In the current study, we have extended the utility of this model by demonstrating antitumor properties of canine complement in conjunction with antiganglioside Mabs. Several teams have described the importance of CDC as a mechanism for human tumor destruction in vitro (1–3, 10) and CDC is receiving increased attention in immunotherapy (27). Our findings advance those observations by detailing this pathway in a unique large animal model. Because the dog spontaneously develops malignant melanomas that are reactive with these antiganglioside antibodies, the clinical correlates of ganglioside-specific antibody therapy in tumor-bearing dogs is of significance to human cancer immunotherapy.

This study has also helped define the requirements for in vitro antitumor CDC in the dog. Binding of C1q to the Fc end of antigen-bound antibody initiates the classical complement pathway resulting in tumor cytosis. Antibody isotypes IgG1, IgG2a, IgG2b, and IgG3 activate this cascade with human complement (3, 4, 28), and murine IgG2a and its mouse-human chimaera ch 14.18 (human IgG1 Fc end) activate human complement equally well for lysis of M21 cells (2). Little is known about the requirements for activation of canine complement to lyse tumor cells through the classical pathway. Our results indicate that (murine) antibody isotypes IgG2a and IgG3 are effective triggers of canine complement-mediated tumor cell lysis. Human IgG1, on the other hand, is apparently a less potent activator of canine complement for cell lysis as ch 14.18, an antibody with an IgG1 Fc end, mediated only low levels of tumor lysis with canine complement. However, because this antibody is a mouse-human chimera, it is possible that canine complement is not activated by the human IgG1 Fc end but may potentially be activated by murine IgG1. Species-specific differences can account for variability of complement activation with chimeric antibodies (29). One investigator has suggested that IgG1 can activate canine complement (30). Additional experiments will need to be done to answer this question. Finally, although C1q is highly conserved between species (31), the concentration of hemolytic C1 in normal canine serum is only 20% that of human (30) and appreciably less than that of rabbit
serum, which might suggest that lower levels of CDC may be mediated by canine complement compared to human or rabbit. Our results indicate this was not the case because the percentage of tumor cell cytotoxicity mediated by canine complement plus some antiganglioside antibodies was comparable to that of rabbit complement plus the same antibodies.

Which of the cytolytic mechanisms involving antitumor Mab is more important in achieving in vivo antitumor responses is unknown. Antibody-dependent cellular cytotoxicity is triggered by relatively few antibody molecules bound to tumor antigen (2) while CDC depends on large amounts of antibody bound to antigen expressed on the tumor surface (28). Numerous, closely spaced antibody binding sites facilitate close association of bound complement-fixing antibodies enabling cross-linking of C1q (28). Thus, CDC might be of greatest clinical significance for the treatment of tumors.
reactivity with the LAN-5 line and could have contributed to CDC of these cells as well. Thus, while our data show canine natural antibodies react with some xenogeneic tumor lines to trigger CDC, the significance of this finding may relate to analogous reactivity of natural canine antibodies for parenchymal cells of xenotransplants, with delayed organ rejection potentially mediated by CDC directed against the parenchymatous cells proper. Little is known about this possibility in vivo because endothelial cells in the transplant are the first targets to be encountered by natural antibodies in xenotransplants (36). Binding of natural antibodies to the xenograft endothelium causes hyperacute rejection of the transplanted organ (37) prohibiting evaluation of the effects of natural antibodies and complement on the parenchymal cells of the xenograft. Our results could be germane to these topics in the field of xenotransplantation.

The antigen(s) on LAN-5 and K562 tumor lines that were recognized by canine natural antibodies remain unknown. We and others found LAN-5 and K562 cells lack class I and II molecules (38, 39). Natural xenoreactive antibodies are thought to develop in response to chronic, subclinical stimulation of the immune system, normal symbiotic bacterial or viral infection, or stimulation by external macromolecules encountered through ingested food or inhalation (36, 40). Frequently, the stimulating antigens are carbohydrate epitopes (41) present on bacteria of the digestive tract providing continuous, “physiological” mucosal stimulation (40). Regardless of the inducing immunogen(s), our results indicate some of these antibodies are present in healthy dogs from an early age and the epitopes they bind are expressed on some, but not all, human tumor lines.

Thus, we have shown that canine complement reacts with several antiganglioside Mabs which are currently in clinical trials for immunotherapy of human cancer. These interactions promote appreciable lysis of tumor cells, a mechanism of potential importance for the continued development of our spontaneous canine malignant melanoma immunotherapy model. Furthermore, the demonstration that canine complement and natural antibodies mediated high levels of tumor cell lysis in the xenogeneic system provides additional evidence that complement-mediated pathways could offer a promising opportunity to exploit for cancer immunotherapy in the dog.

ACKNOWLEDGMENTS

This work was supported by Physician Scientist Award NIH CA01696 (S.C.H.), NIH UO1-CA61498 (P.M.S.); NIH CA32685 (P.M.S.), UWCCC NIH P30-CA14520, and American Cancer Society IM-678K (P.M.S.). The technical assistance of Robin L. Donner and Keith L. Munson is appreciated. We also thank Wayne R. Borchering and Dr. Ron Schultz for their helpful suggestions and Dr. Schultz for providing the serum from young dogs. We thank Yonghong Yang for statistical analysis.
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