Effects of Chemotherapy on Immune Responses in Dogs with Cancer

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Chemotherapy is assumed to be immunosuppressive, but to the authors’ knowledge, the effects of chemotherapy on the immune system of cancer-bearing dogs have not been studied in a prospective manner. Administration of chemotherapeutic agents to mice and humans has variable effects on different components of the immune system. For example, lymphocyte depletion in human patients undergoing chemotherapy has been reported, but the degree of lymphocyte depletion appeared to be dependent on the particular chemotherapy protocol.1–4 Lymphocyte depletion, specifically, depletion of CD4+ T cells, may persist long after completion of chemotherapy.5,6

Not all chemotherapy agents are equally immunosuppressive. For example, cyclophosphamide administered at low doses may actually potentiate humoral immunity and decrease immunologic tolerance.3,4 Doxorubicin and related drugs also have variable effects on adaptive immune responses, with doxorubicin being immunostimulatory and preserving cell-mediated immunity in some studies.8–13 In addition, taxane-based chemotherapeutics in humans also may be immunostimulatory.14 In one study, alterations in immune function were documented to correlate with response to therapy.15

The effects of chemotherapy on humoral immunity may be variable. In dogs, chemotherapy has been documented to have no effect on pre-existing antibody titers, including those to canine distemper virus, parvovirus, and rabies virus.16 In human pediatric oncology patients, pre-existing titers to tetanus, diphtheria, and poliomyelitis antigens were preserved throughout chemotherapy in some, but not all studies.17–19 In some studies, the ability of the humoral immune system to respond to vaccination was restored within 6 months of completing chemotherapy.20–22 The presence of cancer itself may also suppress humoral immunity, as adult human cancer patients had depressed antibody responses to immunization before starting chemotherapy.23

Given the increasing emphasis on multi-modality approaches to cancer, including the use of tumor vaccines and nonspecific immunotherapy, better understanding of the impact of chemotherapy on T- and B-cell responses is essential. This is particularly true in the case of cancer vaccines.24–27

The purpose of the study reported here was to prospectively assess the short- and intermediate-term effects of two commonly used chemotherapy protocols on adaptive immunity in dogs. This evaluation included assessment of numbers and persistence of naïve and memory T and B cells in dogs with cancer and assessment of the impact of chemotherapy on development of humoral responses to de novo vaccination.

Materials and Methods

Inclusion Criteria

Owners of dogs presented to the Colorado State University Veterinary Teaching Hospital (CSU-VTH) were eligible for enrollment in this prospective study. Dogs with cytologically or histopathologically confirmed cancer were included in the study. Dogs were excluded from the study if they had received chemotherapy or steroids within 2 weeks of initiation of chemotherapy. Dogs receiving doxorubicin chemotherapy were...
treated at a dosage of 30 mg/m² of body surface area for a total of 5 treatments administered over a 15-week period. Dogs receiving multi-drug therapy were treated with cyclophosphamide, vincristine, prednisone, doxorubicin, and L-asparaginase (CHOP), according to a described protocol. All chemotherapy administrations and sample collections were performed at the CSU-VTH. Protocols for dogs enrolled in this study were approved by the Animal Care and Use Committee at Colorado State University, and all owners provided informed consent.

**Sample Collection**

Baseline blood samples were collected immediately before initiation of chemotherapy. Subsequent blood samples were obtained at weeks 1 and 3 and months 3 and 6 of treatment. Ten to 15 mL of whole blood was collected into EDTA-containing tubes at each time point for isolation of peripheral blood mononuclear cells (PBMC). Blood samples also were collected from 8 healthy, control dogs owned by CSU-VTH personnel. Blood samples were mixed with phosphate-buffered saline (PBS), then were separated by density gradient centrifugation with commercial separation medium. The separated PBMC were washed and resuspended in cell freezing medium, frozen to −80°C overnight in a controlled-rate cell freezer, then were then transferred to liquid nitrogen for long-term storage. Complete blood counts (CBCs) were obtained at each time point for determination of absolute lymphocyte numbers. All flow cytometric analyses were performed on previously frozen PBMC specimens.

Dogs were vaccinated with 100 μg of keyhole limpet hemocyanin (KLH). The KLH antigen was added to a vaccine adjuvant composed of cationic liposomes and noncoding plasmid DNA immediately before injection, as reported. The vaccines were administered intradermally 24 hours after administration of the first chemotherapy series and again at weeks 3 and 6. Serum was obtained before treatment and at week 3, month 3, and month 6 after initiation of chemotherapy. Serum samples were stored frozen (−80°C) until analysis. A population of 8 clinically normal, age-matched dogs also was vaccinated with KLH and was serologically assessed at the same time points.

**Flow Cytometric Evaluation**

Peripheral blood mononuclear cell specimens were thawed and were distributed at a concentration between 5 × 10⁶ and 10⁶ cells/well in round-bottom, 96-well plates for immunostaining. Cells were stained with directly conjugated antibodies diluted to appropriate dilutions in fluorescence-activated cell sorting (FACS) buffer (PBS with 2% fetal bovine serum and 0.1% sodium azide). The antibodies and fluorochromes used for multi-color flow cytometric analysis included the following: antidog CD4-FITC; antidog CD8-PE; antidog B cell-PE; and cross-reactive antimouse CD44. Cells were washed after antibody incubation, fixed for 30 minutes in 1% paraformaldehyde solution, then stored in FACS buffer at 4°C until flow cytometry was done. Samples were analyzed with a Cyan MLE flow cytometer, and at least 20,000 events were collected for each sample. Analysis gates were set on the live lymphocyte population on the basis of typical forward and side scatter characteristics. The percentage of each lymphocyte subset was determined by flow cytometry, and the absolute numbers are then calculated on the basis of total lymphocyte count determined from the CBC on the same day.

**Serologic Analysis**

The KLH-specific antibody titer was determined by means of ELISA plates coated with KLH at a concentration of 5 μg/mL. Plates were preblocked with nonfat dried milk before incubating serum samples. Serial 2-fold dilutions of serum from each sample were incubated in duplicate wells, starting at a dilution of 1:1,000. Plates were washed, then incubated with goat-antidog IgG conjugated to horseradish peroxidase, then were washed again and incubated with substrate solution. Absorbance was assessed at 405 nm by means of an automated plate reader. End-point antibody titer was calculated.

**Statistical Analysis**

Lymphocyte numbers were logarithmically transformed, after which 2-tailed sample t-tests were used to determine significant differences. Significance was established at α level of < .05. Differences were further assessed using analysis of variance (ANOVA) and Tukey’s multiple comparisons test. Statistical differences between healthy dogs versus dogs with lymphoma, healthy dogs versus dogs with osteosarcoma, and dogs with lymphoma versus dogs with osteosarcoma were determined. Mean (± SD) numbers of CD4⁺ T cells, CD8⁺ T cells, and B cells before and after chemotherapy were calculated for the 3 groups of dogs. Statistical differences were assessed by ANOVA followed by Tukey’s multiple comparisons test. Repeated measures ANOVA was used to further assess changes over time. For statistical analysis of KLH antibody titers, the mean (± SD) end-point titer for each treatment group was calculated. Repeated measures ANOVA with Tukey’s test was used to compare mean ELISA titers between groups at each time point.

**Results**

**Patient population**

Twelve dogs receiving doxorubicin single-agent chemotherapy and 9 dogs receiving a multi-drug chemotherapy protocol for lymphoma were evaluated in this study. In addition, 8 healthy, age-matched dogs were evaluated. Of the 12 dogs treated with doxorubicin only, 9 dogs were treated for osteosarcoma after amputation and 3 dogs were treated for lymphoma. All 9 dogs receiving the multi-drug treatment protocol (CHOP) were treated for lymphoma. The median age of dogs with cancer in this study was 8 (range, 3–13) years. The median age of the 8 control patients was 9 (range, 2–10) years.

**Lymphocyte Numbers and Subsets**

Total lymphocyte numbers and numbers of lymphocytes in subsets (CD4⁺, CD8⁺, and B cells) were compared between healthy, age-matched, control dogs and dogs with cancer (Fig 1). Dogs with osteosarcoma had significantly fewer total lymphocytes (P = .003), compared with healthy, control dogs. In addition, dogs with lymphoma also had significantly fewer total lymphocytes (P = .05) than did healthy, control dogs. All dogs with cancer had significantly fewer CD4⁺ and CD8⁺ lymphocytes (P < .05), compared with healthy dogs. This was also true when the subsets of dogs with lymphoma or dogs with osteosarcoma were compared with control dogs (P = .02 and .03, respectively). However, pretreatment total lymphocyte numbers or numbers of CD4⁺ or CD8⁺ T cells were not significantly different when dogs with lymphoma were compared with dogs with osteosarcoma. The numbers of B cells were not significantly different between dogs with lymphoma and healthy, control dogs or between dogs with lymphoma and dogs with osteosarcoma.
with osteosarcoma and healthy, control dogs. However, dogs with osteosarcoma had significantly fewer ($P < .05$) B cells than did dogs with lymphoma.

### Effects of Chemotherapy on Lymphocyte Numbers

In dogs treated with doxorubicin only, significant differences in numbers of CD4$^+$ T cells or CD8$^+$ T cells were not observed at any time during or after treatment, compared with values before treatment (Fig 2). In dogs treated with CHOP, numbers of CD4$^+$ T cells did not change significantly during the study, compared with values before treatment (Fig 3). Numbers of CD8$^+$ T cells were, however, significantly decreased 1 week after the first treatment ($P = .02$), though the numbers of CD8$^+$ T cells rebounded after week 1, and were not significantly different at any other time point (Fig 3). Significant changes in the percentages of memory T cells, as identified by the CD44$^{hi}$ subpopulations of CD4$^+$ and CD8$^+$ T cells, were not observed between groups (data not shown).

In dogs treated with doxorubicin only, significant changes in B-cell numbers after treatment were not observed (Fig 2). However, in dogs treated with CHOP, B-cell numbers decreased significantly after the first treatment and remained significantly decreased throughout the 6-month period of study ($P < .05$) (Fig 3).

### Serologic Analysis

Dogs treated with either doxorubicin or CHOP and control dogs all mounted significant antibody titer after the 3 vaccinations with KLH (Fig 4). The magnitude of the antibody responses was maximal at postvaccination month (PVM) 3 and decreased by PVM 6 in all 3 groups. Antibody titers to KLH were numerically higher in CHOP-treated, compared with control or doxorubicin-treated dogs, but the differences were not statistically significant. When mean KLH titers at postvaccination week 3, PVM 3, and PVM 6, were compared among all 3 groups, statistically significant differences in antibody titers were not observed.

### Discussion

Several important findings emerged from this study. Administration of doxorubicin single-agent chemotherapy or CHOP multi-agent chemotherapy did not have a significant immunosuppressive effect in terms of inducing a sustained decrease in numbers of CD4$^+$ and CD8$^+$ T cells in dogs during or after chemotherapy. However, administration of CHOP chemotherapy induced a rapid, sustained decrease in B-cell numbers. Administration of either type of chemotherapy also did not appear to significantly impair the ability of dogs to mount antibody responses to a novel antigen (KLH). The lack of T-cell depletion in dogs treated with either doxorubicin or CHOP was surprising in light of the reported immunosuppressive properties of chemotherapy, particularly combination chemotherapy. Though
both of these chemotherapy protocols can induce neutropenia, their effects on lymphocyte numbers or subsets have not been carefully evaluated previously. Therefore, it is important to distinguish between the effects of chemotherapy on innate immunity (eg, neutrophils) versus the effects on adaptive immunity (eg, lymphocytes).

Our results differ in some important aspects from those reported recently from immunophenotyping studies in dogs with lymphoma that were evaluated after the completion of chemotherapy. Dogs with lymphoma previously treated with chemotherapy were reported to have >50% reduction in numbers of total lymphocytes and reductions in most lymphocyte subsets, compared with those in control dogs. However, the dogs with lymphoma were not evaluated before the beginning of chemotherapy, so the starting lymphocyte numbers were unknown. Our results suggest that the decrease in lymphocyte numbers in dogs with lymphoma may be attributed more to the effects of the underlying neoplasm than to the effects of chemotherapy per se. This is in agreement with results of a previous study that suppression of lymphocyte numbers in dogs with lymphoma before treatment was significant.

Though our study did not find significant decreases in CD4+ or CD8+ T cells after administration of single- or multi-agent chemotherapy, the functional status of the T cells in the chemotherapy-treated dogs was not assessed directly. Thus, it is possible that the remaining T cells were functionally impaired. However, the function of CD4+ T cells in our patients treated with chemotherapy appeared to be intact. This conclusion is based on the ability of chemotherapy-treated dogs to mount IgG responses against a novel antigen, a response that is dependent on CD4+ T cells and was preserved in dogs of this study. We cannot exclude the possibility that CD8+ T-cell function was suppressed. However, one might infer clinically that CD8+ T cell function is well preserved in dogs undergoing chemotherapy, since they do not have high incidence of viral, fungal, or intracellular bacterial infections.

Unlike T cells, B cells were found to be susceptible to the effects of multi-agent chemotherapy in this study. It is not clear whether any one drug in particular in the CHOP protocol was responsible for B-cell depletion, though prednisone is a logical candidate. Even though the CHOP chemotherapy induced B-cell depletion, patients treated with CHOP still mounted effective antibody responses to concurrent vaccination. This finding suggests that the memory B cells and plasma cells in lymph nodes may be refractory to the effects of combination chemotherapy, despite the depletion of circulating B cells.

Our results suggest that administration of chemotherapy does not preclude administration of vaccines. This result is particularly relevant when considering the...
concurrent use of tumor immunotherapeutics with chemotherapy.44,35 The ability to administer chemotherapy and vaccinate concurrently would be particularly desirable for the design of clinical trials where the benefit of cancer vaccines could be assessed while patients still were being treated with currently recommended chemotherapy protocols. Moreover, it may be that administration of certain chemotherapy agents can elicit synergistic antitumor activity when combined with tumor vaccines or tumor immunotherapy. For example, this appears to be the case with concurrent administration of cyclophosphamide and tumor vaccines, and this effect has been documented experimentally and clinically.2

One interesting finding to emerge from this study was the fact that dogs with either of 2 common types of cancer (osteosarcoma and lymphoma) had significant depression of CD4+ and CD8+ T-cell numbers before initiation of chemotherapy. Decreased numbers of T cells had been reported previously in dogs with lymphoma but, to our knowledge, has not been reported in dogs with osteosarcoma.33 Though it is generally assumed that canine patients with cancer are immunosuppressed, there is little prior published information on the nature of this immunosuppression in dogs. The mechanisms underlying lymphopenia in dogs with cancer are not known, but could include decreased lymphocyte production attributable to thymic dysfunction, increased peripheral destruction of lymphocytes, or lympholysis as a consequence of stress and increased cortisol production. Development of lymphopenia in human cancer patients is clinically relevant. For example, lymphopenia before beginning therapy has been documented as a negative prognostic indicator for response in humans with cancer.36 A correlation between depressed pretreatment B- and T-cell numbers and remission rates also was made in humans with bladder carcinoma.37 Future studies to correlate clinical outcome with initial lymphocyte numbers appear to be warranted in canine cancer patients.

In conclusion, results of this study suggest that the assumption that chemotherapy is immunosuppressive in dogs may not be true in all instances. However, the small sample size of patients evaluated in this study indicates that these results should be confirmed in a larger study. It would also be helpful to assess other aspects of immune function, including direct assessment of T-cell responses as well as innate immune responses, including neutrophil and monocyte function, after administration of chemotherapy. Assessment of immune function in dogs with other forms of cancer may also be informative, particularly in the case of patients with neoplasms such as multiple myeloma or mast cell tumors that produce immunomodulatory substances.

Footnotes

* Lymphocyte Separation Media (LSM), ICN Biomedicals Inc, Aurora, OH

**Nunclon Cell Freezer, Nalge Nunc International, Rochester, NY

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References


