DISSERTATION

CHROMOSOMAL ABERRATIONS IN THE TUMOR AND PERIPHERAL BLOOD AND CHANGES IN ABERRATIONS DURING TREATMENT OF CANINE LYMPHOMA

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JENNIFER J. DEVITT ENTITLED CHROMOSOMAL ABERRATIONS IN THE TUMOR AND PERIPHERAL BLOOD AND CHANGES IN ABERRATIONS DURING TREATMENT OF CANINE LYMPHOMA BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

CHROMOSOMAL ABERRATIONS

IN THE TUMOR AND PERIPHERAL BLOOD

AND CHANGES IN ABERRATIONS DURING TREATMENT

OF CANINE LYMPHOMA

Lymphoma is the most frequently diagnosed hematopoietic malignancy in dogs. Untreated, the survival times are approximately one month. Chemotherapy is the current standard of care and can initiate and temporarily maintain remission, with average survival times of one year.

Cytogenetic abnormalities can aid in diagnosing tumors as well as in giving a more accurate prognosis for the specific mutations present. In human lymphoma patients, chromosomal changes from peripheral lymphocytes have been used prognostically and to document response to treatment. Evaluating peripheral lymphocytes instead of tumor cells is less invasive for the patient and technically easier.

Recurrent aberrations have been reported in canine lymphomas. Since this cancer parallels human Non-Hodgkin's Lymphoma which has recurrent chromosomal anomalies that have been correlated with clinical behavior of the tumor and patient survival, it reasons that canine lymphoma would as well. This study was designed to investigate a correspondence between numerical aberrations detected in the tumor and the peripheral blood in dogs with lymphoma. Additionally, the peripheral blood aberrations

were monitored during the course of treatment to document changes seen during remission and at the time of disease recurrence.

Twenty-five dogs with lymphoma had one lymph node excised, a peripheral blood sample drawn, and a bone marrow aspirate performed. A portion of the lymph node was submitted for histopathology and immunophenotyping and another portion was retained for cytogenetic analysis. The peripheral blood sample was cultured for chromosome counting and cytogenetic analysis. The bone marrow aspirate was used for staging purposes.

A significant correspondence between the numerical aberrations in the tumor and the peripheral blood was found with six out of the seven numerical aberrations demonstrating predictive value of the peripheral blood. During the course of treatment, the quality and quantity of aberrations changed, likely due to DNA damaging treatment modalities. Once treatment ended, the frequency of aberrations diminished. A prognostic significance could not be determined using the additional diagnostic information that was garnered such as age, gender, histological classification, breed, immunophenotype, or stage of disease. This was probably due to a limited sample size and is worthy of further investigation.

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CHAPTER I

REVIEW OF THE LITERATURE

Canine lymphoma

Canine lymphoma is one of the most common cancers seen in dogs(1). The annual incidence is estimated to be between 24 to 33 per 100,000 dogs(2). It affects primarily middle aged or older dogs with an average age between 6.3 and 7.7 years of age(1;3-6). It is a progressive and fatal disease. Certain breeds have been shown to be at higher risk of developing lymphoma including Saint Bernards, Scottish Terriers, Airedale Terriers, Bulldogs, Golden Retrievers, Basset Hounds, Labrador Retrievers, Bouvier des Flandres, and Rottweilers(3;7-9). Breeds that have been shown to be at a lower risk include Dachshunds and Pomeranians(6). The prognosis is poor, with dogs that are untreated rarely living beyond two months(10). However, the survival rate of untreated dogs is hard to measure since the time between diagnosis and the actual onset of lymphoma is inconsistent and the possible decision to euthanize at the point of diagnosis compounds it(11). In addition, contemporary incidence and prevalence data is often lacking in pet animal populations.

Typically the first clinical sign an owner recognizes is an enlarged lymph node but some affected dogs also have a history of lethargy, weight loss, and/or anorexia(12). The presenting clinical signs are varied but can include generalized lymphadenopathy, mediastinal or abdominal masses, hepatic or splenic enlargement, weight loss, anorexia,

and anemia(8). The presence of clinical signs in addition to lymphadenopathy at the time of diagnosis has been shown to be a negative prognostic factor(13). There are four forms of the disease which are classified based on their anatomical location: multicentric, alimentary, mediastinal, and extranodal. Multicentric is the most common form, comprising approximately 80% of diagnosed cases. It is found in the lymph nodes, with or without bone marrow and spleen/liver involvement. On clinical presentation, the lymph nodes are generally enlarged and firm but painless. The alimentary form involves focal or disseminated intestinal lesions and the clinical signs include vomiting, abdominal pain, anorexia, diarrhea, and significant weight loss. The mediastinal form involves lymphadenopathy of the cranial mediastinal lymph nodes. The last form is extranodal which consists of variable clinical signs dependent on the organ affected by the disease(14).

Cancer is a disease of unregulated cell growth and the ability of the mutated cells to invade adjacent tissues and/or metastasize to different locations within the body. The initiating event is DNA damage to a normal cell which can lead to a sequential accumulation of additional mutations. Lymphoproliferative diseases are a diverse group of cancers that result from the neoplastic proliferation of a single clone of lymphoid cells. They typically originate from lymphoreticular tissues such as lymph node, bone marrow and spleen, but they can originate from virtually any tissue(12). It is generally a disease that is histopathologically and immunologically heterogeneous(10). A diagnosis of lymphoma is often made with a fine-needle aspirate of an enlarged lymph node. While a definite diagnosis is possible with an aspirate, the morphology (diffuse vs. follicular), histologic grade (high vs. low), or immunophenotype (T-cell vs. B-cell) can not be

determined. In this regard, a tumor biopsy or excision is needed for more complete classification(14).

Lymphoma is a systemic disease and therefore requires a systemic treatment such as chemotherapy. Repeated cycles of multidrug chemotherapy achieve the best results, most of which are based on the CHOP protocol (cyclophosphamide, doxyrubicin, vincristine, prednisone) which was developed for human lymphoma patients. Approximately 60-90% of dogs reach a complete remission in which there is a disappearance of detectable cancer, with a median survival of 6-12 months. Up to 25% of dogs live more than two years(12). Dogs typically tolerate chemotherapy well and achieve a good quality of life during the time of remission(12). Fortuitously, lymphopoietic tissues are exquisitely sensitive to radiation. Therefore, an adjuvant treatment of half body radiation within the chemotherapy protocol is well tolerated and may increase remission length in comparison to chemotherapy as the sole treatment modality(15).

The etiology of canine lymphoma is unclear and probably multifactorial. Hypothesized causes for canine lymphoma include retroviral infection, environmental contaminates, magnetic field exposure, chromosomal abnormalities, and immune dysfunction(8;13;16-19). A retroviral cause in dogs has not been confirmed. Immunosuppression has been associated with a higher risk of lymphoma(12). Also, dogs with owners that applied 2,4-dichlorophenoxyacetic acid, a broadleaf herbicide, to their lawns have been shown to be at higher risk of developing lymphoma(17). One study found an additional risk factor in dogs living in close proximity of electromagnetic fields such as power lines(20).

Since most dogs are diagnosed via cytologic evaluation, there is a lack of information to subcategorize the disease using classification schemes that are inherent to human lymphoma diagnoses. Most lymphoma treatments have been used indiscriminately despite the differences in morphologic subtypes(1;21). Histological subclassification is important not only because it is predictive of response but also to aid in the appropriate chemotherapy selection. It is commonly known that human lymphomas have different rates of response to treatment and this knowledge led to the formation of classification schemes(22). Increased diagnostic procedures in dogs would allow for more accurate prognostic information and the most effective treatment options based on the disease characteristics. The current diagnostics that are available, although rarely included in current standard-of-care protocols, consist of immunophenotyping, proliferation rate (via expression of Ki-67), proliferating cell nuclear antigen, argyrophilic nucleolar organizer regions (AgNORs), and PCR for antigen rearrangement clonality assay (PARR). Only immunophenotyping and AgNORs have demonstrated prognostic significance(3;12). The immunophenotype is correlated with survival time with B-cell having a better prognosis(3;21). The majority of canine lymphomas are B-cell in origin and there is a low occurrence of low grade and follicular disease(23;24). There are a low percentage of T-cell lymphomas in dogs and T-cell is less responsive to treatment(23-25). In both human and canine lymphomas, AgNORs have proven to be prognostically significant. A smaller number of AgNORs per nucleus, a larger mean AgNOR area, and a larger maximal AgNOR area correlates with a longer disease-free interval(3). Other characteristics shown in some studies to lack prognostic significance include age, sex, stage of disease, and body weight(1;4;26). Yet more recent studies have

found the stage of disease to be prognostically valuable(4;27). Consistent information about the behavior and reaction to different treatment protocols of the varied subsets of lymphoma is necessary. The development of meaningful predictive assays could aid in the appropriate treatment selection and provide more accurate prognostic information. Additionally, the different subtypes must be identified in order to study the underlying genetic causes of the disease(28).

Canine lymphoma is similar in clinical signs, histopathology, and treatment response to Non-Hodgkin's Lymphoma (NHL) in human beings(10;29). The adjusted age distribution for both species is comparable. According to the American Cancer Society, the average age of diagnosis for NHL is 60, with half of the patients being over 65 years of age. Likewise, dogs are typically middle-aged to older when they are diagnosed. NHL accounts for 4% of the cancers diagnosed in the United States and is the fifth most commonly diagnosed cancer. In dogs, lymphoma is the third most diagnosed cancer. As with most human cancers, the incidence of NHL is slightly higher in males than females, although in the majority of canine lymphoma studies, a gender discrepancy has not been found(9).

Demographics in human and canine lymphoma

Genetics appears to play a role in both canine lymphoma and NHL. A family history of NHL is a risk factor for people, and in dogs, a familial link has also been found(30;31). Racial and geographical distributions of NHL have been identified. Rates of NHL are higher in Caucasians than African-Americans, with the exception of peripheral T-cell NHL. Geographical differences show that follicular lymphomas occur

more frequently in Western countries. Asia has higher rates of aggressive NHL, T-cell lymphomas, and extranodal disease, but overall, their incidence of NHL is relatively low. The Middle East has higher occurrences of intestinal extra nodal disease and Africa has higher rates of endemic Burkitt's Lymphoma(30). In dogs, differences between the immunophenotypic subtype and different breeds indicates a genetic link(7). The prevalence of a specific immunophenotype is often distributed among closely related breeds. For example, Modiano et al. found that T-cell lymphomas were common in breeds that are members of the oldest domestic dog breeds such as Spitz breeds and many Asian breeds. On the other hand, B-cell lymphomas were found in the Australian Cattle Dog, Chow-chow, Doberman Pinscher, Poodle, and Standard Schnauzer(7).

Both human NHL and canine lymphoma are highly heterogeneous at the clinical and histological level. The common schemes used to classify human lymphomas, the Working Formulation, Kiel Classification, and Rappaport Classification can also be used for canine lymphomas(21). There are different theories as to which scheme works best for canine lymphomas, some believe the Working Formulation is best for the different morphologies found in canine lymphoma whereas others believe that the Kiel Classification system is best because of its emphasis on cytology rather than morphology and more high grade tumor divisions(1;21;27). In the treatment of human lymphoma patients, appropriate classification based on the histological subclassification, immunophenotyping, and cytogenetic analysis has lead to the identification of subtypes that respond well or poorly to traditional treatment(32-35). Clinical staging is vital to human NHL prognosis and treatment planning but in dogs, stage I and II are rarely diagnosed(1). Contrary to human NHL, a distinct follicular architecture is infrequently

found in canine lymphoma, possibly because canine lymphoma progresses to a diffuse and aggressive tumor faster than its human counterparts(21). A retrovirus has been instigated as a cause of a form of human cutaneous T-cell lymphoma but a direct correlation between a viral agent and lymphoma has not been confirmed in dogs(18). As with canine lymphoma etiology, a specific component of many herbicides, phenoxyacetic acid, especially 2,4-dichlorophenoxyacetic acid, has been implicated as a causative agent in human NHL. Interestingly, Hodgkin's Lymphoma (HL) is not routinely recognized in dogs(8).

Chromosomal abnormalities in human and canine lymphoma

Human lymphomas have been found to have specific chromosomal abnormalites, some of which have been correlated with clinical presentation, treatment response, and prognosis(22). Research has shown that human NHL tumors are typically diploid, or near diploid, just as is found in canine lymphoma(2;36). High-grade human lymphomas are more apt to be aneuploid than those of a lower grade. Currently, little is known about correlations between ploidy and histological or immunological classification and the prognosis in canine lymphoma(2). However, the specific immunophenotype of NHLs in humans is vital to classification because it is specific to biological behavior and response to treatment. Hence, the human lymphoma classification schemes use immunophenotype data for classification purposes(23). T-cell lymphoma is associated with a poorer prognosis in both humans and dogs(3;11;37). Variations in survival times in human lymphoma patients led researchers to develop histological classification schemes that could identify optimal treatment strategies(24). Most canine lymphomas are not

distinguished between morphologic subtypes(1;9). However, a study by Ponce et al. showed prognostic differences between morphologic subtypes of canine lymphoma which demonstrated that there were intrinsic biological attributes and clinical behaviors inherent to different subtypes. They found that each subtype had a specific clinical presentation, just as is found in human patients. Using just a pure diagnosis of lymphoma or just a B-cell vs. T-cell would not allow for prognostic range among lymphomas and would not identify dogs for which the standard protocol of choice is not ideal(24). In veterinary medicine, a wide variety of treatment options exist. A rigid standard-of-care protocol does not exist for canine lymphoma as many owners must choose a suboptimal treatment plan due to financial constraints.

Chromosomal aberrations have been reported in canine lymphoma tumors but there have not been enough cases to validate a diagnostic or prognostic importance. In the largest cytogenetic study of canine lymphoma to date, Hahn et al. studied 61 cases and identified the most common chromosomal aberrations. The majority of the cases demonstrated aneupolidy (43/61). Strikingly, the most common aberration observed, trisomy of canine chromosome (CFA) 13 (15/61), also demonstrated prognostic significance in that dogs with this abnormality had a significantly longer first remission and survival time(10). Likewise, Thomas et al. found trisomy 13 to be the most common aberration in her study of 25 canine lymphoma samples (12/25). CFA13 is evolutionarily linked to two sites on human chromosome (HSA) 8 and HSA4. HSA8 contains the oncogene c-myc and HSA4 contains the c-kit oncogene(38). C-myc is activated in intermediate and high-grade human NHLs(39). Thomas also found trisomy CFA31 (8/25). CFA31 has synteny with HSA21 which is amplified in many human cancers,

although not specifically NHL. A monosomy of CFA11 was observed in over 10% of patients (3/25). Each dog with lymphoma had a mean number of three chromosomal aberrations(38). Monosomy of CFA11 is more common in T-cell lymphoma, trisomy of CFA31 is more common in B-cell, but trisomy of CFA13 was common in both B- and T-cell(38). Monosomy of CFA14 was more common in B-cell and also occurred in 100% of Golden Retrievers in Modiano's study (7/7) but only in 4/31 of other breeds(7).

The instigating event in canine lymphoma is likely a chromosomal segregation mistake which leads to gene amplification. Genetic changes may be assisted by a gross chromosomal change and may be cytogenetically evident. Numerical aberrations are common in human tumors and they probably occur due to an error in chromosomal disjunction (anaphase lag)(10). In canine tumors, numerical aberrations occur more frequently than structural changes like deletions, translocations, and rearrangements(10;38;40-45). Canine chromosomes appear to be more stable than human chromosomes with one hypothesis being that canine DNA has very little heterochromatin. In human beings, structural changes frequently occur near centromeric regions which are rich in heterochromatin(40). The dog genome is 2.4 Gb as compared with 2.9 Gb for the human genome, however, the dog genome has a lower amount of repetitive sequences (31%) whereas the human genome contains 46%(46).

Extravasation of tumor cells is well documented in a variety of human tumors(47-51). A study on human neuroblastoma found that 75% of the 12 patients examined had circulating tumor cells in their peripheral blood at the time of diagnosis. During treatment, the percent of patients with circulating tumor cells dropped to 34%. They also found tumor cells were present in the peripheral blood independent of bone

marrow involvement(52). A study in human prostate cancer studied the feasibility of using a predictive assay for the detection of prostate-specific antigen (PSA) mRNA in circulating tumor cells. The popular method of measuring serum PSA protein levels is limited to patients with an elevated serum PSA. Their results found that patients with localized disease had evidence of tumor cells in their peripheral blood and those with progressive metastatic disease had even higher numbers of circulating tumor cells(53). M'Kacher et al. observed chromosomal abnormalities in extravasted tumor cells in the peripheral blood of human Hodgkin's lymphoma patients. They found that there was a higher frequency of chromosomal abnormalities in the HL patients at the time of diagnosis than in the normal control population. The number of abnormalities increased slightly as the HL patients underwent chemotherapy and increased more significantly immediately following radiation therapy treatment. A decrease in the number of abnormalities was noted after the commencement of treatment with the most significant decline occurring within the first six months post-treatment and a more gradual decline after the six month mark(50).

Studies have also found peripheral blood involvement in canine lymphoma. One study observed that 28% of dogs with lymphoma had peripheral blood involvement(54). A similar study showed that 57% of canine lymphoma patients had peripheral blood involvement(55). And a third study found 65% had circulating tumor cells in the peripheral blood(8). Evaluating chromosomal changes from peripheral blood offers a huge advantage over evaluating cells obtained from the tumor. First, drawing blood is a minimally invasive procedure. Second, harvesting and processing peripheral lymphocytes for cytogenetic evaluation is faster and provides a higher yield of successful

samples than evaluating cells obtained from the tumor. Finally, blood samples can be packaged and sent via overnight shipping for processing. This means that the procedure could be performed routinely in clinical settings, and sent off-site for evaluation.

Cytogenetics

The field of cytogenetics began in the 1880s when an Austrian cytologist, Walther Flemming, studied the role of chromosomes and cell division. He published the first illustration of a human chromosome in 1882. Shortly afterwards, chromosomes were identified as the unit of heredity by Theodor Boveri. He found that each individual chromosome contained different genetic information. He studied sea urchin eggs that contained abnormal chromosome numbers and observed that tumors developed in these abnormal eggs. He postulated that chromosomal changes via abnormal segregation of chromosomes during cell division could lead to cancer development(56). This was the beginning of the notion that genetic instability was a major contributor to oncogenesis.

The modern age of cytogenetics was ushered in seventy years later when T.C. Hsu discovered that a hypotonic solution generated better chromosome spreads which made it possible to count chromosome numbers(57). Then, in 1956, the correct number of human chromosomes was elucidated by Joe Hin Tijo and Albert Levan. During the next two decades, the techniques for cell culture and fixation improved allowing for detection of karyotype abnormalities which were found to be specific for certain disease syndromes. Banding techniques achieved with different stains provided a means to observe chromosomal abnormalities such as translocations and break points. Researchers began to rapidly discover chromosomal anomalies that proved to be specific components

of genetic diseases and cancer. Yet classical cytogenetics proved to be tedious and labor intensive. It requires a high mitotic rate, good chromosome morphology, and generous amounts of time for analysis which necessitated the advent of molecular cytogenetics.

Molecular cytogenetics helped alleviate these problems by increasing the efficiency, preciseness, and sensitivity of classical cytogenetics. The technique termed fluorescence *in situ* hybridization (FISH) takes advantage of DNA's double strandedness and ability to denature and rehybridize. In this method, a short segment of DNA is developed as a probe by attaching a fluorescent tag directly, or indirectly using a reporter molecule that will later be conjugated with a fluorescent tag. Both the target DNA and the probe segment DNA are denatured and allowed to hybridize back together with the homologous sequences pairing up, allowing for recognition of the segment in both metaphase spreads and interphase nuclei when viewed under a fluorescent microscope(58).

Lymphocytes are not typically dividing in adult mammals so to obtain cells in metaphase for cytogenetic analysis, the cells must be stimulated with a mitogen such as pokeweed, concanavalin A, or phytohemagglutinin. Pokeweed has been shown to stimulate both B- and T-cells whereas concanavalin A and phytohemagglutinin target primarily the T-cells. The benefits of using peripheral lymphocytes is that they can be collected non-invasively, they are inexpensive to culture and the culture time is reasonably short, they generate a high mitotic index, and they yield a good quality of metaphase spreads. Additionally, they have the durability to withstand a delayed culture set up, meaning that they could be mailed in their sodium heparin collection tube and set up in culture upon arrival. Peripheral blood is produced in the bone marrow, although

lymph nodes contribute lymphocytes and monocytes, as well. If the bone marrow fails, the spleen can produce lymphocytes. The mononuclear leukocytes, or lymphocytes, are used for cytogenetic analyses. In normal blood circulation, about 20-40% of the white blood cells are lymphocytes, while the rest are neutrophils, eosinophils, basophils, and monocytes. Approximately 55-75% of the lymphocytes are T-cells (thymus-dependent cells for immunity) and 15-30% are B-cells (bursa-dependent cells for humoral immunity or antibody production). Differences between T- and B-cells can not be determined using a microscope(59).

A major benefit in using FISH technology for some applications is that interphase cells can be cytogenetically analyzed, in addition to the traditional metaphase chromosomes. "FISH technology is simple and robust, and its phenomenal contribution to the cancer field largely relies on its applicability to interphase cells(58)." Scoring of interphase cells is dependent on the successfully probe hybridization to the appropriate DNA sequence within the cell. The presence of background noise will make scoring of interphase cells inaccurate. The signals need to be of similar brightness and different focal planes must be examined to allow for the spatial positioning of the chromosomal DNA. The cell cycle must be accounted for in that if the cell is in G2, then two smaller signals that are in close proximity may be present that represents the two sister chromatids. These two smaller signals must be scored as one signal since they originate from one chromosome. The total number of cells that must be scored is a reflection of the sample limitations and the context in which the results will show. For samples in which quality cells are hard to produce, a limited number of abnormally scored cells may

suffice. For samples which generate quality cells, a scoring of 200 nuclei is optimal(58;60).

Canine chromosomes are notorious for being one of the most difficult mammalian karyotypes to study(61). Their chromosomes are numerous, have a similar size distribution, and have similar banding patterns. Not only do dogs possess a high diploid number of chromosomes (2n=78) but all 38 autosomes are acrocentric. The sex chromosomes are metacentric(46;62-64). The X chromosome is approximately 137 Mb which is similar in size to canine chromosome one, the largest autosome. The Y chromosome is approximately 27 kb and is the smallest of all canine chromosomes(64).

The advent of molecular cytogenetics has facilitated the examination of canine tumors. Prior findings in human NHL have proven that differences between different subsets of lymphoma exist and have different biological behaviors. Larger scale studies in canine tumor cytogenetics are needed elucidate trends in recurrent chromosomal aberrations. Ideally this would lead to improved methods of assessing prognosis for this extremely common canine disease.

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CHAPTER II

CORRELATIONS BETWEEN NUMERICAL CHROMOSOMAL ABERRATIONS IN THE LYMPH NODE AND PERIPHERAL BLOOD IN CANINE LYMPHOMA

INTRODUCTION

Lymphoma is one of the most common neoplasms seen in the dog and can be located in any lymph node, organ, and/or bone marrow(1;2). Lymphoma is a locally and systemically aggressive tumor, with a survival time of 30 days if untreated. It is similar in clinical signs, histopathology, and treatment response to Non-Hodgkin's Lymphoma in human beings(3;4). Chemotherapy can successfully initiate and temporarily maintain remission. However, despite intense effort within the veterinary oncologic research community to improve treatment outcome, long term survival remains poor, with median survival between 12 and 18 months(2). One factor that may be contributing to the failure to gain a more durable remission is the lack of predicative assays that can classify patients into clinically relevant subpopulations. In the treatment of human lymphoma patients, appropriate classification based on histological subclassification, immunophenotyping, and cytogenetic analysis has lead to the identification of subtypes that respond well or poorly to traditional treatment(5-7). These predictors of response can then be used to select subgroups with poor prognoses for more aggressive or novel protocols. In veterinary medicine, immunophenotyping is commonly used to distinguish between B- and T-cell lymphomas but there is considerable heterogeneity of

treatment response within the two groups. Histological subclassifications have been developed but their lack of clinical impact is evidenced by the fact that many treatment facilities do not require histology as part of routine staging. Previous cytogenetic research on canine lymphomas has determined that patients with tumors bearing trisomy 13 as their primary aberration have a better prognosis than patients with tumors bearing other aberrations(3;8).

From a clinical standpoint, being able to classify subsets of canine lymphoma quickly, reliably, and non-invasively would allow clinicians to select the most appropriate treatment for the specific tumor type. Our laboratory has determined that chromosomal aberrations are detectable in lymphocytes from the peripheral blood in previously untreated lymphoma patients. Evaluating chromosomal abnormalities in the peripheral blood offers a huge advantage over evaluating cells obtained from the tumor. First, drawing blood is a minimally invasive procedure. Second, harvesting and processing peripheral lymphocytes from cytogenetic evaluation is faster and provides a higher yield of successful samples than evaluating cells obtained from the tumor. Finally, blood samples can be packaged and sent via overnight shipping for processing. This would allow for the procedure to be performed routinely in clinical settings and sent off-site for evaluation.

A correlation between the chromosomal aberrations was found in the tumor and the peripheral blood of canine lymphoma patients. The most likely origin of the aberrant peripheral blood lymphocytes is due to extravasation of tumor cells from the lymph nodes. The positive results garnered in this study indicate promise for use as a diagnostic tool using the peripheral blood of canine lymphoma patients.

MATERIALS AND METHODS

Experimental Design

Twenty-five dogs with lymphoma who had not received any treatment for lymphoma were identified by the Oncology Service of the Colorado State University Veterinary Medical Center (CSU-VMC). Patients underwent routine staging procedures required by the Oncology Service prior to treatment. Upon admission to the study, a protocol coordinator supervised a lymph node excision, bone marrow aspirate, and collection of a peripheral blood sample. Dogs were pre-medicated and anesthetized with continuous monitoring in accordance with Animal Care and Use (ACUC) standards. A portion of the excised lymph node was submitted to the Colorado State University Diagnostic Laboratory for histology and immunophenotyping. Slides from the bone marrow aspirate were cytologically evaluated for tumor spread. A portion of the lymph node and the peripheral blood samples were cultured using standard cytogenetic protocols. Slides were made of each sample and prepared for chromosome counting and fluorescence *in situ* hybridization (FISH) analysis. Statistical analysis was performed to establish a correlation in the numerical aberrations found in the tumor and the peripheral blood. The aberrations were also correlated with staging criteria and survival. Ten clinically normal dogs and five dogs with solid tumors were used as controls. Each control dog had at least one peripheral blood sample analyzed with both chromosome counting and FISH.

Animal Care and Use

Approval from the Colorado State University Animal Care and Use Committee (ACUC) was obtained prior to patient accrual. A copy of the approved ACUC form is found in the Appendices.

Peripheral blood

Each control dog (n=15) had at least one peripheral blood sample collected using aseptic technique into a lithium heparin tube. Twenty-five dogs with lymphoma had blood drawn at the time of initial diagnosis. Blood was cultured with 1 ml of blood in 10 ml of RPMI-1640 medium supplemented with 20% fetal bovine serum, 4mmol/L L-glutamine, 200U/ml penicillin, and 0.2 mg/ml streptomycin, and 10 μg/ml pokeweed mitogen. Cultures were incubated at 37°C with 5% CO_2 for 96 hours with 100 ng/ml colcemid added during the last hour of incubation. To harvest the lymphocytes, samples were centrifuged at 1000 revolutions per minute (rpm) for 8 minutes. The supernatants were discarded and 8 ml of 37°C 75 mM potassium chloride (KCl) was added drop wise while gently vortexing the cells. The samples were incubated at 37°C for 30 minutes. Approximately 500 μl of 3:1 methanol:acetic acid fixative was added to the samples, gently mixed, and centrifuged at 1000 rpm for 8 minutes. The supernatants were discarded, the pellets were resuspended, and 8 ml of fixative was added drop wise while gently vortexing. Samples were then centrifuged at 1000 rpm for 8 minutes. The fixation step was repeated at least two more times, depending on the quality and cell density of the final samples. Slides were made by dropping 20 μl of the sample onto a slide wetted with fixative and allowed to air dry.

Node excision and bone marrow

Each dog had a tumor excision performed at the time of diagnosis. The dogs were pre-anesthetized using regimens tailored to each patient's unique needs. Common drugs used alone or in combination were atropine (0.4mg/kg), acepromazine (0.01-0.2mg/kg), fetanyl (0.01mg/kg), Valium (0.2mg/kg), oxymorphone (0.05-0.1mg/kg), generally given subcutaneously 30-40 minutes prior to induction. Anesthesia was induced with propofol (6-8mg/kg) intravenously and maintained with isoflorane in oxygen after endotracheal intubation. A palpable popliteal lymph node was excised through a skin incision approximately 4-6 cm in length, dependent on lymph node size, after blunt dissection to free it from surrounding tissue. Any vessels were ligated and subcutaneous tissue and skin was closed in a routine manner. A section of the lymph node was apportioned for cytogenetic analysis and another section was submitted to the CSU-VMC Diagnostic Laboratory for histology and immunophenotyping via flow cytometry. Concurrently, a bone marrow aspirate was performed aseptically on the humerus using a Jamshidi 11 gauge needle. Bone marrow aspirate slides were submitted to CSU-VMC Clincial Pathology for cytologic evaluation of tumor spread.

The section of lymph node allotted for cytogenetic studies was agitated using a sterile surgical blade in RPMI-1640 medium supplemented with 20% fetal bovine serum, 4 mmol/L L-glutamine, 200 U/ml penicillin, and 0.2 mg/ml streptomycin. Cultures were set up with a final cell concentration of 0.75 x 10^6/ml in 10 ml of RPMI-1640 medium and incubated at 37°C, 5% CO_2 for 16 hours. For the final hour of incubation, 100 ng/ml colcemid was added. To harvest the cells, samples were centrifuged at 1000 rpm for 8 minutes. The supernatants were discarded and 8 ml of 37°C 75mM KCl was added drop

wise while gently vortexing the cells. The samples were incubated at 37°C for 30 minutes. Approximately 500 µl of 3:1 methanol:acetic acid fixative was added to the samples, gently mixed, and centrifuged at 1000 rpm for 8 minutes. The supernatants were discarded, the pellets were resuspended, and 8 ml of fixative was added drop wise while gently vortexing. Samples were then centrifuged at 1000 rpm for 8 minutes. The fixation step was repeated at least two more times, depending on the quality and cell density of the final samples. Slides were made by dropping 20 µl of the sample onto a slide wetted with fixative and allowed to air dry.

A limited number of dogs (n=7) had bone marrow cultures performed from an aspirate. The aspirate was added to 10 ml of RPMI-1640 medium supplemented with 20% fetal bovine serum, 4 mmol/L L-glutamine, 200 U/ml penicillin, and 0.2 mg/ml streptomycin and 10 µg/ml pokeweed mitogen. They were incubated at 37°C, 5% CO_2 for 96 hours. For the final hour of incubation, 100 ng/ml colcemid was added. To harvest the cells, samples were centrifuged at 1000 rpm for 8 minutes. The supernatants were discarded and 8 ml of 37°C 75 mM KCl was added drop wise while gently vortexing the cells. The samples were incubated at 37°C for 30 minutes. Approximately 500 µl of 3:1 methanol:acetic acid fixative was added to the samples, gently mixed, and centrifuged at 1000 rpm for 8 minutes. The supernatants were discarded, the pellets were resuspended, and 8 ml of fixative was added drop wise while gently vortexing. Samples were then centrifuged at 1000 rpm for 8 minutes. The fixation step was repeated at least two more times, depending on the cleanliness of the final samples. Slides were made by dropping 20 µl of the sample onto a slide wetted with fixative and allowed to air dry.

Development of BAC probes (DNA extraction through labeling)

Bacterial Artificial Chromosomes (BAC) clones representing segments of canine chromosomes 11, 13, 14, and 31 were inoculated into 2.5 ml of LB broth supplemented with 30μg/ml chloramphenical. The tubes were set in a shaker at 225 rpm at 37°C for at least four hours. Single colony preparations were made onto 20 ml Luria-Bertani (LB) broth plates with 20 μg/ml of chloramphenicol. The inoculated plates were placed upside down at 37°C, without CO_2 or water, for sixteen hours. Individual colonies for BAC isolation were chosen using a sterile instrument. These colonies were then plated onto another agar plate and grown overnight in a 37°C incubator. Part of each colony was then placed in an eppendorf tube with 15 μl of PCR water and vortexed for five minutes. Each sample was then amplified in the PCR machine. A 1% agarose gel was run to check for the sequence-tagged site (STS) marker of the BAC. The chosen BAC colonies were placed in 5 ml of LB broth and grown overnight in a 37°C shaker. Cultures were then centrifuged at 4000 rpm for five minutes. The bacteria was resuspended in 1 ml of 9:1 LB broth:glycerol. To extract the BAC DNA, 3 μl of bacteria was grown in 250 ml LB broth.

DNA extraction was performed using a Qiagen Plasmid Purification Midi Kit. Bacterial cells were harvested by centrifugation at 6000 rpm for 20 minutes and the supernatant discarded. Pellets were resuspended in 25 ml P1 Buffer containing RNase. Then 25 ml of P2 Buffer was added, gently mixed by inversion, and incubated at room temperature for 5 minutes. Then 25 ml of chilled P3 buffer was added, gently mixed by inversion, and incubated on ice for 30 minutes. Samples were centrifuged at 11.5 rpm for 30 minutes at 4°C. The supernatant containing the DNA was transferred to a new tube

and centrifuged at 11.5 rpm for 15 minutes at 4°C. The DNA was precipitated with 0.7 volumes of room temperature isopropanol. Samples were centrifuged at 10000 rpm for 30 minutes at 4°C. The supernatant was carefully removed and the pellet was dissolved in 500 µl TE buffer, pH 8.0.

To purify the DNA, QBT Buffer was added up to a final volume of 12 ml. The Qiagen tip 100 was equilibrated by applying 10 ml QBT Buffer and allowing the column to empty by gravity flow. The DNA solution was applied to the Qiagen tip column and entered the resin by gravity flow. The tip was washed twice with 15 ml QC Buffer. The DNA was eluted with 8 ml QF Buffer and the DNA- containing supernatant was retained. The DNA was precipitated by adding 0.7 volumes of room temperature 2-isopropanol and centrifuged at 15 x g for 30 minutes at 4°C. The supernatant was decanted and the DNA pellet was washed with 1.5 ml room temperature 70% ethanol and centrifuged at 15 x g for 15 minutes at 4°C. The pellet was air dried for 10 minutes and dissolved in 200 µl TE buffer. The DNA concentration was determined using a NanoDrop system with a desired concentration of 400ng.

The probes were fluorescently direct labeled using a nick translation kit. Bacterial DNA was combined with 5 µl dH_2O, 2 µl buffer, 6 µl dNTPs (A,G,C), 1.3 µl dTTP, 0.75 µl labeled dUTP, and 2 µl DNase/polymerase in an eppendorf tube. Then the tube was placed in a thermal cycler at 15°C for 3 hours and 65 °C for 10 minutes.

Canine COT-1 DNA

Canine COT-1 DNA was prepared to block repetitive sequences in the canine genome. Ten milliliters of whole canine blood in sodium heparinized tubes was collected

from several dogs of various breeds, both male and female. The blood was added to 30 ml of lysis buffer, gently mixed, and incubated for 30 minutes on ice. The sample was then centrifuged at 1200 rpm for 10 minutes at 4°C. The supernatant was removed; 10 ml of lysis buffer was added to the resuspended pellet and was centrifuged at 1200 rpm for 10 minutes at 4°C. The supernatant was removed; the pellet was resuspended in 5 ml SE buffer, and centrifuged at 1200 rpm for 10 minutes at 4°C. The supernatant was removed; 5 ml of SE buffer was added to resuspend the pellet. Then 40 µl of proteinase K (10mg/ml) and 250 µl 20% SDS was added, gently mixed, and incubated overnight in a 37°C water bath. Next, 5 ml of SE buffer and 10 ml of phenol were added and shaken by hand for 10 minutes. The samples were centrifuged at 3000 rpm for 5 minutes at 10°C. The DNA-containing supernatant was retained. A 10 ml volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and shaken by hand for 10 minutes and then centrifuged at 3000 rpm for 5 minutes at 10°C. The supernatant was retained, 10 ml of chloroform:isoamyl alcohol (24:1) was added, shaken by hand for 10 minutes, and centrifuged at 3000 rpm for 5 minutes at 10°C. To the supernatant, 300 µl 3M sodium acetate, pH 5.2, and 10 ml isopropanol was added, shaken gently to precipitate the DNA, and the DNA was captured using a sterile glass pipette. The DNA was washed in 70% ethanol and dissolved in 0.5 ml TE buffer overnight at 4°C. The DNA was sonicated for a total of 2.5 minutes in 30 second bursts at 50% duty cycle and setting 5 output control. The DNA was precipitated in 0.5 ml aliquots with 50 µl 3M sodium acetate and 1.65 ml 100% ethanol and placed in a -80 freezer overnight. The tubes were centrifuged at maximum speed in a micro centrifuge for 30 minutes. The supernatant was discarded and the tubes were dried upside down in a warm room for 15

minutes. The DNA was resuspended with 100 µl PCR water and the concentration was measured on a NanoDrop system with an ideal concentration of approximately 40 ng.

Fluorescence *in situ* hybridization

To prepare slides for FISH analysis, 20µl hybridization mix, 4 µl canine COT-1 DNA, fluorescently labeled probes, and sterile water were combined in an eppendorf tube with a final volume of 30 µl. The probe mixture was pipetted onto each sample slide and a 22 X 40 coverslip was applied. The edges of the coverslip were sealed using rubber cement. The slides and probes were codenatured in a PCR machine at 80°C for 5 minutes and 37°C for at least 5 minutes. The slides were incubated at 37°C for at least 12 hours. The slides were washed in the following series of 45.5°C coplin jar washes: 50% formamide in 2XSSC pH 5.8, 2XSSC pH 7.4, and 2XSSC pH 7.4 + 0.1% igepal CA-630. Slides were counterstained with 60 µl DAPI/AF and coverslipped (Figure 2.1).

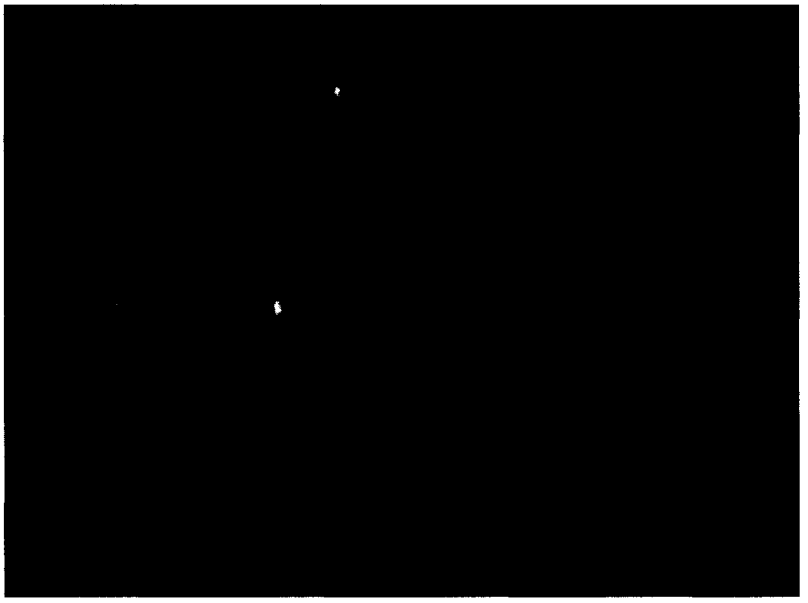

Figure 2.1: BAC probes for canine chromosomes 11 (red), 13 (yellow), 14 (pink), and 31 (green) in a normal canine metaphase cell.

Scoring of samples

Each FISH slide had 200 cells evaluated using a Nikon fluorescent microscope and corresponding MetaMorph™ cytogenetic software. Both metaphase and interphase cells were scored for numerical chromosomal aberrations of the four chromosomes of interest.

Disease Staging

Veterinary oncologists staged each patient according to the World Health Organization Staging Criteria for Canine Lymphoma (Table 2.1). In addition to the Roman numeral, each stage is designated as "a" or "b." The letter "a" follows the Roman numeral if the dog is without systemic signs of illness and a "b" follows if clinical signs are present.

Bone Marrow Cytology

Bone marrow aspirate slides were submitted to the CSU-VMC Clinical Pathology Laboratory for staging of the disease. Additionally, histology slides were read by a veterinary pathologist and classified by the Working Formulation (Table 2.2).

Immunophenotyping

A portion of each excised lymph node was submitted to the CSU-VMC Diagnostic Laboratory for immunophenotyping. Samples were stained for antibodies

Table 2.1: World Health Organization Staging Criteria for Canine Lymphoma.

disease stage	symptoms
I	one lymph node involved
II	two lymph nodes involved (same side of the diaphragm)
III	multiple lymph nodes involved
IV	liver and spleen involved
V	bone marrow involvement

Table 2.2: National Cancer Institute's Working Formulation for Non-Hodgkin's Lymphoma.

low grade	intermediate grade	high grade
small lymphocytic	follicular large cell	large cell immunoblastic
follicular small-cleaved cell	diffuse small cleaved cell	lymphoblastic
follicular mixed small-cleaved and large cell	diffuse mixed small and large cell	small non-cleaved cell
	diffuse large cell	

CD3, CD4, CD8, CD5, CD14, CD21, and CD34 to determine whether the tumor was of B-cell or T-cell origin.

Medical records

Following all cytogenetic analyses, each patient's record was examined to garner relevant clinical information. Age at diagnosis, presenting clinical signs, breed, stage of disease, response to treatment, and survival times were all noted.

Statistics

Frequencies and relative frequencies of aberrations in the tumor and the peripheral blood were measured to test the predictive validity of the blood aberrations. Survivorship functions were calculated using Kaplan-Meier Analysis for a variety of parameters including: histological classification, age at diagnosis, gender, immunophenotype, breed, and bone marrow aspirate results. In addition, log-rank and Wilcoxon tests were performed on each parameter. A series of Fisher's exact tests was conducted to investigate any associations between each aberration (measured in the tumor) and the following variables: cell type, histology (high vs. intermediate), breed (golden retriever vs. other) and age at diagnosis.

RESULTS

Histology

The tumors were classified according to the National Cancer Institute's Working Formulation for Non-Hodgkin's Lymphoma. This classification scheme divides the

tumors into low, intermediate, and high grade. Of the 20 dogs with histological grading, none of the dogs had low grade tumors, 8 dogs (40%) had tumors of intermediate grade, and 12 dogs (60%) had high grade tumors (Table 2.3). Dogs with intermediate grade disease had a median survival time of 332 days whereas dogs with high grade disease had a shorter median survival (232 days). Six morphological subgroups were identified with between one and seven representatives in each group. The two dogs with diffuse small cleaved cell (intermediate grade) had a median survival time of 322 days. Seven dogs had large cell immunoblastic lymphoma (high grade) and had a median survival of 322 days. Five dogs had diffuse large cell lymphomas (intermediate grade) and had a median survival of 226 days. Three dogs had lymphoblastic (high grade) and had a median survival of 162 days. (Table 2.4/Figures 2.2 & 2.3) The log-rank test and Wilcoxon test p-values were insignificant.

Immunophenotype

A section of each lymph node was stained for specific antibodies to determine the immunophenotype. Of the 20 dogs with immunophenotype data, 15 (75%) had tumors of B-cell origin and 5 (25%) had T-cell lymphoma (Table 2.5). The T-cell patients had a median survival time of 332 days whereas the B-cell patients had a median survival time of 322 days (Table 2.6/ Figure 2.4). The log-rank test and Wilcoxon test p-values were insignificant.

Table 2.3: Histology results classified using NCI Working Formulation.

grade	cell type	# of dogs
low	small lymphocytic	0
	follicular small-cleaved cell	0
	follicular mixed small-cleaved and large cell	0
intermediate	follicular large cell	0
	diffuse small cleaved cell	2
	diffuse mixed small and large cell	1
	diffuse large cell	5
high	large cell immunoblastic	7
	lymphoblastic	3
	small non-cleaved cell	2

Table 2.4: Median survival times by histologic categories.

histological subcategory	median survival time (days)
intermediate	332
high	232
diffuse small cleaved cell	-
diffuse mixed small and large cell	332
diffuse large cell	226
large cell immunoblastic	322
lymphoblastic	162
small non-cleaved cell	-

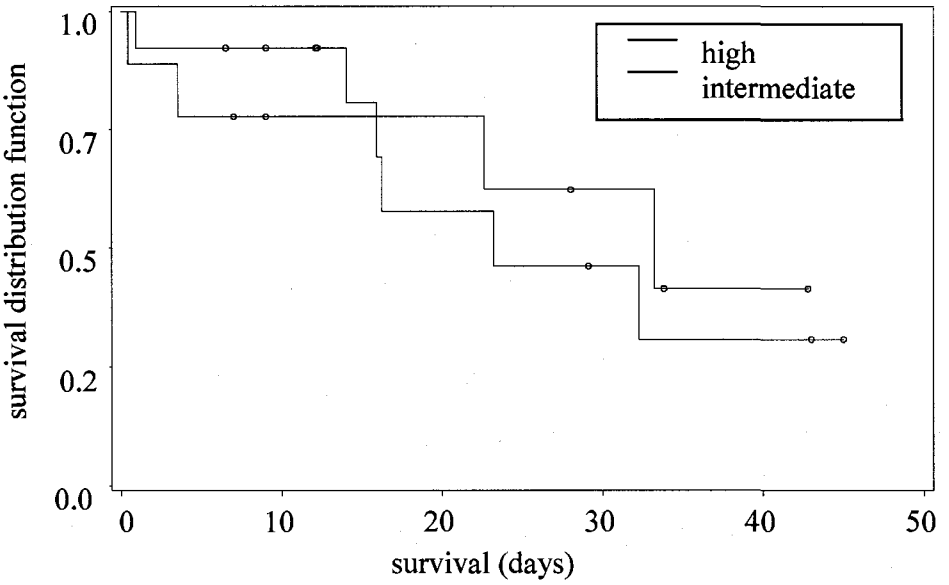

Figure 2.2: Kaplan-Meier survivorship functions by histologic grade (high grade vs. intermediate).

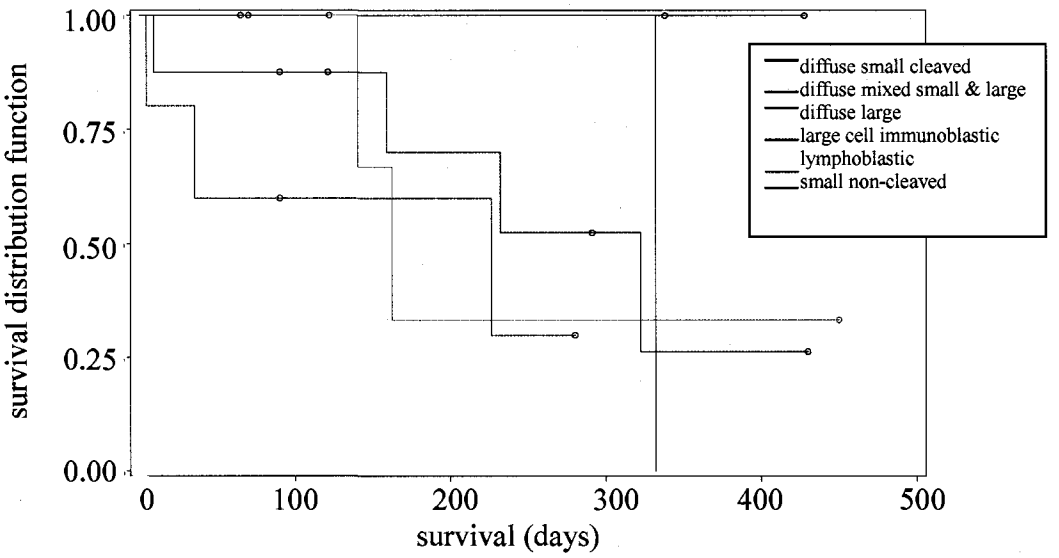

Figure 2.3: Kaplan-Meier survivorship functions by Working Formulation subcategories.

Table 2.5: Distribution of lymphoma immunophenotypes.

immunophenotype	# of dogs
B-cell	15
T-cell	5

Stage of disease

Clinicians staged the lymphoma patients according to the World Health Organization classification scheme for canine lymphoma. Eight dogs were staged as IIIa, three dogs were stage IVa, two dogs were stage IVb, and four dogs were stage Va (Table 2.7). Three dogs were designated as stage V without a substage specified. The median survival differences were insignificant (Table 2.6/ Figures 2.5 & 2.6). Of the 24 dogs with bone marrow aspirates performed, seven dogs had evidence of disease involvement in the bone marrow. The median survival differences were insignificant (Table 2.6/Figure 2.7). The random bone marrow aspirates from dogs with various disease stages (n=7) that were cultured and analyzed cytogenetically yielded no aberrations.

Breed

A total of 12 breeds plus 6 dogs of mixed breed were represented in the group of 25 dogs with lymphoma. Eight out of 25 (32%) were Golden Retrievers (Table 2.8). The Golden Retrievers had a longer median survival time (322 days) as compared to all other breeds combined (232 days) (Table 2.6/ Figure 2.8). The log-rank test and Wilcoxon test p-values were insignificant.

Age

At the time of diagnosis of lymphoma, 11 dogs were younger than 8 years of age (44%) while 14 (56%) were 8 years old or older. Dogs that were diagnosed prior to age 8 had a median survival time of 322 days whereas dogs that were older 8 years or older had

Table 2.6: Median survival times for different variables.

variable		median survival time (days)
stage	IIIa, IVa, Va	322
	IIIb, IVb, Vb	162
stage	IIIa, IIIb, IVa, IVb	322
	Va, Vb	-
age at diagnosis	age <8 years	322
	age ≥8 years	226
gender	female	-
	male	226
breed	other	232
	Golden Retriever	322
cell type	T-cell	332
	B-cell	322
bone marrow	negative	322
	positive	-

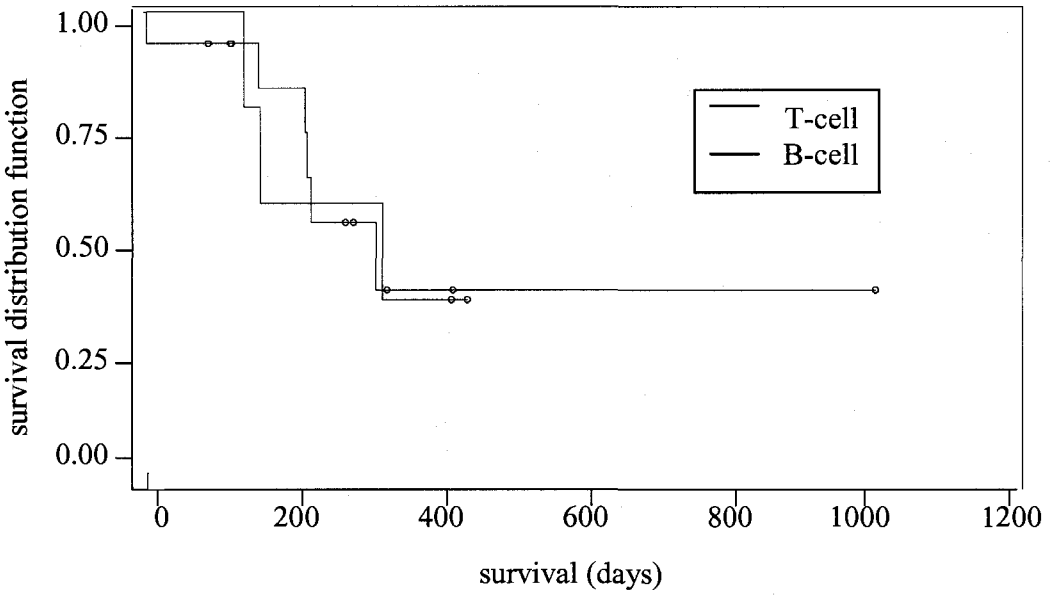

Figure 2.4: Kaplan-Meier survivorship functions by immunophenotype.

Table 2.7: Distribution of lymphoma stages.

disease stage	# of dogs
IIIa	8
IIIb	0
IVa	3
IVb	2
Va	4
Vb	0

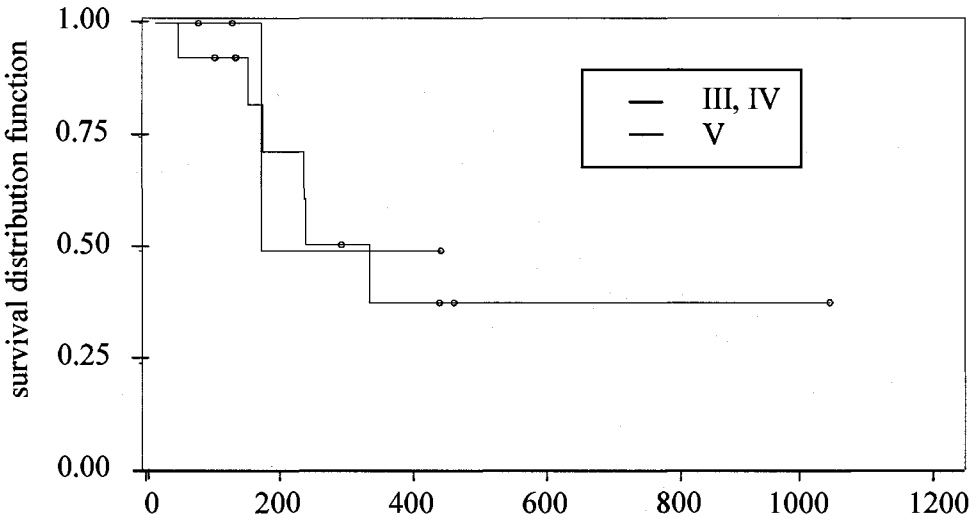

Figure 2.5: Kaplan-Meier survivorship functions by disease stage. (III/IV vs. V).

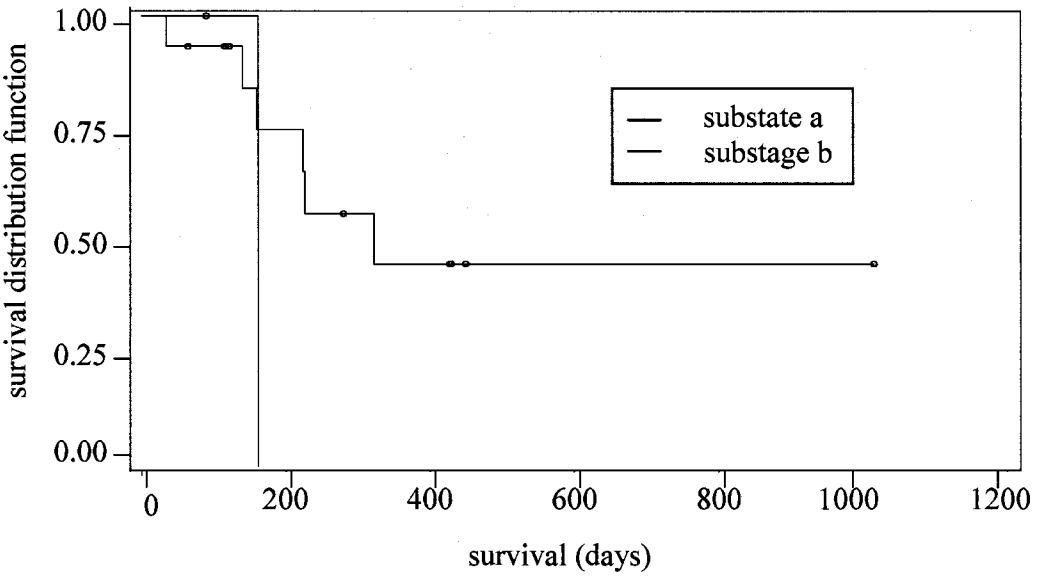

Figure 2.6: Kaplan-Meier survivorship functions by disease stage. Stage a= without systemic signs of illness; Stage b= with systemic signs of illness.

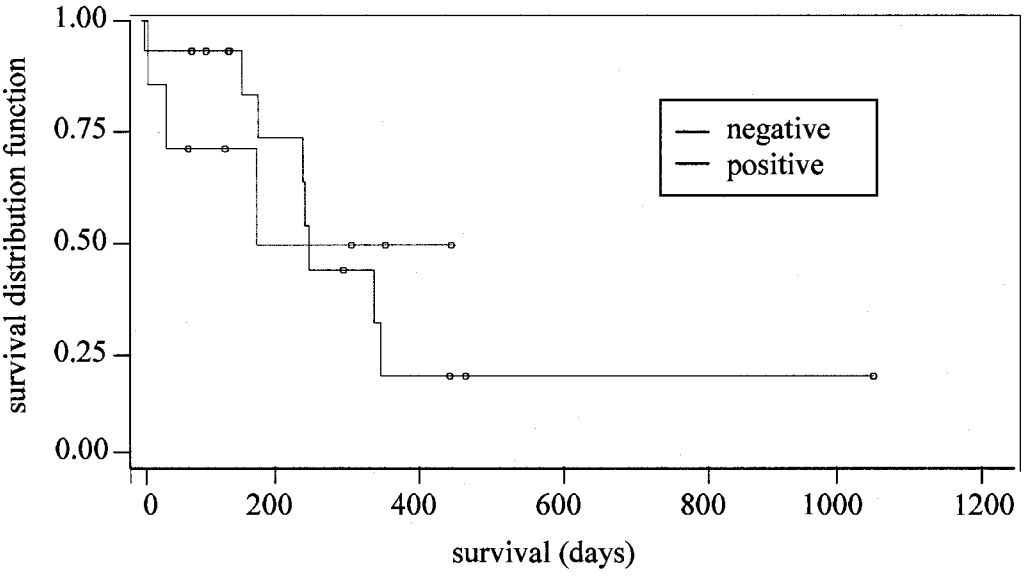

Figure 2.7: Kaplan-Meier survivorship functions by bone marrow cytology.

Table 2.8: Breed distribution among lymphoma dogs.

breed	# of dogs
Jack Russell Terrier	1
Miniature Schnauzer	1
Border Collie	1
Scottish Terrier	1
Boxer	1
Golden Retriever	8
Australian Shepherd	1
Rhodesian Ridgeback	1
Bernese Mountain Dog	1
Doberman Pinscher	1
Bouvier des Flandres	1
Weimaraner	1
mixed breed	6
TOTAL	25

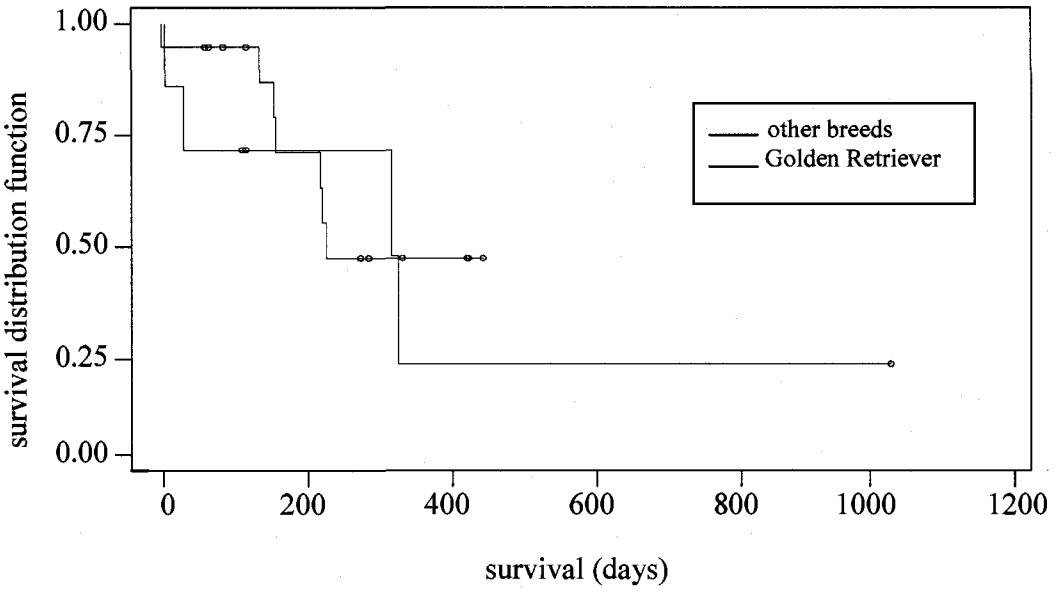

Figure 2.8: Kaplan-Meier survivorship functions by breed.

a median survival time of 226 days. (Table 2.6/ Figure 2.9). The log-rank test and Wilcoxon test p-values were insignificant.

Gender

Out of the 25 dogs with lymphoma, 8 were female and 17 were male. Based on the log-rank test, there was a significant difference between the strata of gender (p=0.0225). Based on the Wilcoxon test, the p-value for gender was 0.1376. The difference between the two tests for gender can be explained in that compared to the log-rank test, the Wilcoxon test places more weight on early survival times, where no differences were observed (Table 2.6/ Figure 2.10).

Clinical records

Careful inspection of each dog's clinical records was performed. Information about remissions, chemotherapy and radiation therapy schedules, bloodwork, and time of death were documented. Two dogs were noted to be leukemic at one time point: Dog 6 at week 22 of treatment and Dog 18 at the time of diagnosis.

Tumor vs. peripheral blood

Seven numerical aberrations were scored in both the lymph node and concurrent peripheral blood sample (Table 2.9 & 2.10). Two hundred cells, both metaphase and interphase, were scored for each sample. A cutoff of 1% was established to determine the presence of an aberration meaning at least two cells demonstrating the aberration were required for inclusion in the statistical data.

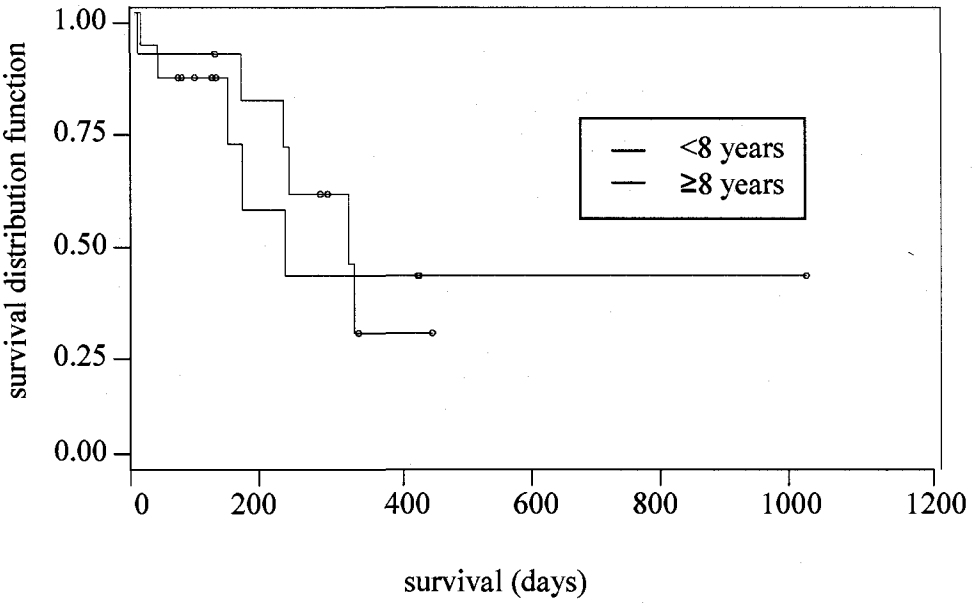

Figure 2.9: Kaplan-Meier survivorship functions by age at time of diagnosis.

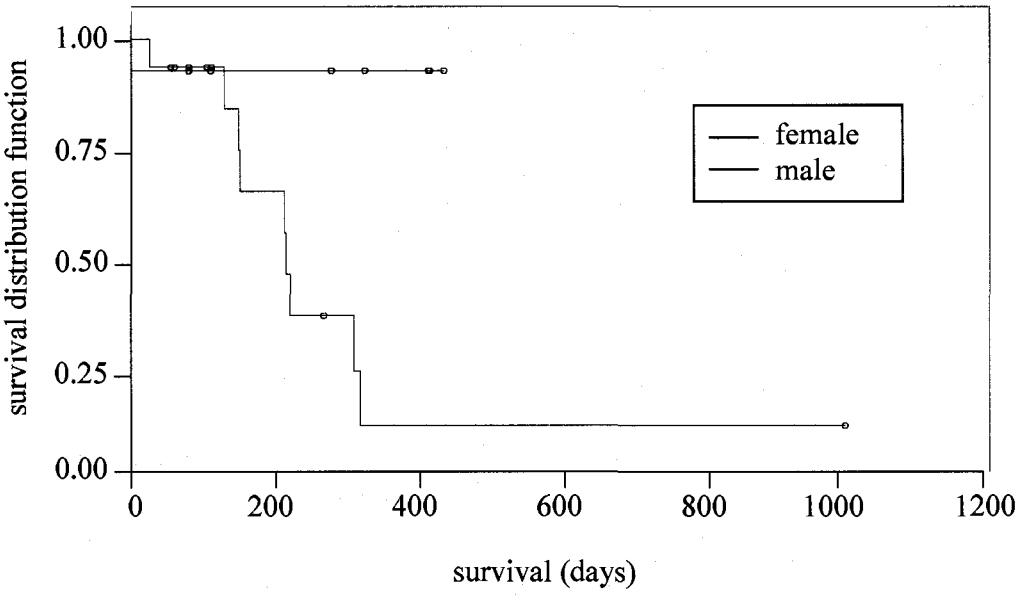

Figure 2.10: Kaplan-Meier survivorship functions by gender.

Monosomy of CFA11 was detected in both the lymph node and peripheral blood in ten dogs. It was detected in one dog in the lymph node but not the blood sample and in one dog in the blood but not the lymph node. The probability that monosomy of CFA11 found in the blood corresponds to the detection of monosomy CFA11 in the lymph node was 0.91. The probability that the absence of monosomy of CFA11 in the blood corresponds to its absence in the lymph node is 0.93. There is an overall agreement of 0.92 for monosomy of CFA11.

Trisomy of CFA11 was detected in both the lymph node and peripheral blood in five dogs. It was detected in two dogs in the lymph node but not the blood sample and in one dog in the blood but not the lymph node. The probability that trisomy of CFA11 found in the blood corresponds to the detection of trisomy CFA11 in the lymph node was 0.83. The probability that the absence of trisomy of CFA11 in the blood corresponds to its absence in the lymph node is 0.89. There is an overall agreement of 0.88 for trisomy of CFA11.

Table 2.9: Comparisons between aberrations seen in tumor and peripheral blood. (+) denotes presence of the aberration, (-) denotes absence of the aberration. Examples of disagreement between tumor and blood are circled.

dog #		mono11	tri11	mono13	tri13	mono14	mono31	tri31
1	node	-	-	-	-	-	-	+
	blood	-	-	-	-	-	-	-
2	node	+	+	-	+	+	-	-
	blood	+	+	-	+	+	-	+
3	node	-	-	+	-	-	-	-
	blood	-	-	-	-	-	-	-
4	node	+	+	+	+	+	+	+
	blood	+	+	+	+	+	+	+
5	node	+	-	-	-	-	-	-
	blood	-	-	-	-	-	-	-
6	node	+	+	+	+	+	+	-
	blood	+	+	+	+	+	+	+
7	node	-	-	-	-	-	+	+
	blood	-	-	-	-	-	-	-
8	node	-	-	-	-	-	-	-
	blood	-	-	-	-	-	-	-
9	node	+	-	-	+	-	+	-
	blood	+	-	-	+	-	-	-
10	node	-	-	-	-	-	-	-
	blood	-	-	-	-	-	-	-
11	node	-	-	-	+	-	-	-
	blood	-	-	-	+	-	-	+
12	node	-	-	-	-	-	-	-
	blood	-	-	-	-	-	-	-
13	node	+	-	-	+	-	-	+
	blood	+	-	+	-	-	-	+
14	node	+	-	-	-	-	-	-
	blood	+	-	-	-	-	-	-
15	node	-	-	-	-	-	-	+
	blood	-	-	-	-	-	-	-
16	node	-	-	-	+	-	-	-
	blood	-	-	-	+	-	-	-
17	node	-	-	-	-	-	+	+
	blood	+	-	-	-	-	-	-
18	node	+	-	-	+	-	-	+
	blood	+	-	-	+	-	-	+
19	node	+	-	+	+	-	-	-
	blood	+	-	+	+	-	-	+
20	node	-	-	-	+	-	-	-
	blood	-	-	-	+	-	-	-
21	node	+	-	-	-	-	-	-
	blood	+	-	+	-	-	-	-
22	node	+	+	-	+	-	-	+
	blood	+	-	-	-	-	-	+
23	node	-	-	-	-	-	-	-
	blood	-	-	-	-	-	-	-
24	node	-	-	-	-	-	-	-
	blood	-	-	-	-	-	-	-
25	node	-	+	-	+	+	-	-
	blood	-	-	-	+	-	-	-

Table 2.10: Frequencies and relative frequencies of aberrations and measures of blood test validity (total n=25). **OA** = Overall agreement = ($n_{Node=0,\ Blood=0}$ + $n_{Node=1,\ Blood=1}$) / 25 **Se** = Sensitivity = ability of the blood test to detect presence of aberrations **Sp** = Specificity = ability of the blood test to detect absence of aberrations **PPV** (positive predictive value) = probability that the presence of an aberration found in the blood corresponds to the presence of an aberration in the node **NPV** (negative predictive value) = probability that the absence if an aberration found in the blood corresponds to the absence of an aberration in the node (PPV and NPV depend on the prevalence of the aberration in the node (% node=1); the higher % node=1, the greater the PPV).

Aberration		Node (–) Blood (-)	Node (–) Blood (+)	Node (–) total	Node (+) Blood (-)	Node (+) Blood (+)	Node (+) total	OA	Se	Sp	PPV	NPV	% node (+)
mono11	n	13	1	14	1	10	11	0.92	0.91	0.93	0.91	0.93	44
	%	92.86											
tri11	n	17	1	18	2	5	7	0.88	0.71	0.94	0.83	0.89	28
	%	94.44	5.56		28.57	71.43							
mono13	n	20	0	20	2	3	5	0.92	0.60	1.00	1.00	0.91	20
	%	100	0		40	60							
tri13	n	11	0	11	2	12	14	0.92	0.86	1.00	1.00	0.85	56
	%	100	0		14.29	85.71							
mono14	n	20	0	20	2	3	5	0.92	0.60	1.00	1.00	0.91	20
	%	100	0		40	60							
mono31	n	19	1	20	2	3	5	0.88	0.60	0.95	0.75	0.90	20
	%	95	5		40	60							
tri31	n	13	2	15	6	4	10	0.68	0.40	0.87	0.67	0.68	40
	%	86.67	13.33		60	40							

Monosomy of CFA13 was detected in both the lymph node and peripheral blood in three dogs. It was detected in two dogs in the lymph node but not the blood sample. The probability that monosomy of CFA13 found in the blood corresponds to the detection of monosomy CFA13 in the lymph node was 1.00. The probability that the absence of monosomy of CFA13 in the blood corresponds to its absence in the lymph node is 0.91. There is an overall agreement of 0.92 for monosomy of CFA13.

Trisomy of CFA13 was detected in both the lymph node and peripheral blood in 12 dogs (Figure 2.11). It was detected in two dogs in the lymph node but not the blood sample. The probability that trisomy of CFA13 found in the blood corresponds to the detection of trisomy CFA13 in the lymph node was 1.00. The probability that the absence of trisomy of CFA13 in the blood corresponds to its absence in the lymph node is 0.85. There is an overall agreement of 0.92 for trisomy of CFA13.

Monosomy of CFA14 was detected in both the lymph node and peripheral blood in three dogs. It was detected in two dogs in the lymph node but not the blood sample. The probability that monosomy of CFA14 found in the blood corresponds to the detection of monosomy CFA14 in the lymph node was 1.00. The probability that the absence of monosomy of CFA14 in the blood corresponds to its absence in the lymph node is 0.91. There is an overall agreement of 0.92 for monosomy of CFA14.

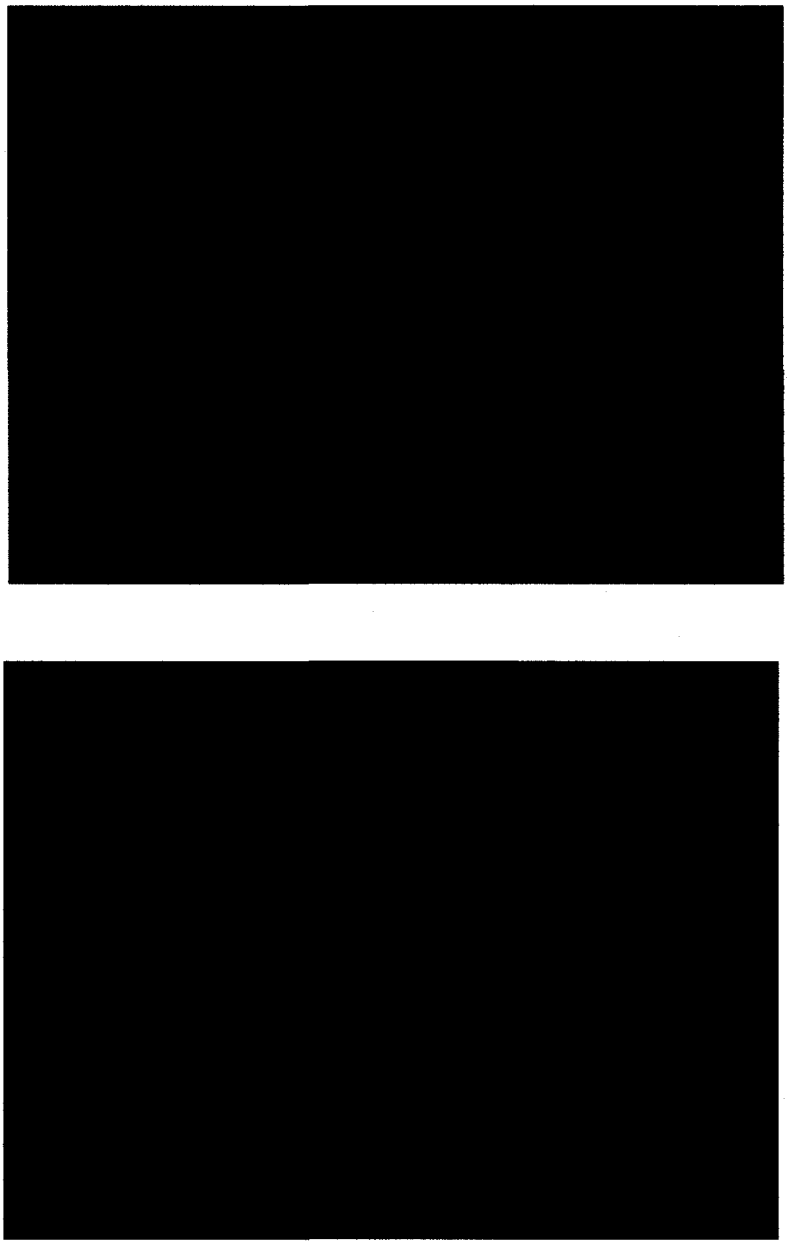

Figure 2.11: Trisomy 13 in a tumor lymphocyte (top) and peripheral blood lymphocyte (bottom) from Dog 11. Note the blood sample has both an interphase and metaphase cell displaying the aberration.

Monosomy of CFA31 was detected in both the lymph node and peripheral blood in three dogs. It was detected in two dogs in the lymph node but not the blood sample and in one dog in the blood but not the lymph node. The probability that monosomy of CFA31 found in the blood corresponds to the detection of monosomy CFA31 in the lymph node was 0.75. The probability that the absence of monosomy of CFA31 in the blood corresponds to its absence in the lymph node is 0.90. There is an overall agreement of 0.88 for monosomy of CFA31.

Trisomy of CFA31 was detected in both the lymph node and peripheral blood in four dogs (Figure 2.12). It was detected in six dogs in the lymph node but not the blood sample and in two dogs in the blood but not the lymph node. The probability that trisomy of CFA31 found in the blood corresponds to the detection of trisomy CFA31 in the lymph node was 0.67. The probability that the absence of trisomy of CFA31 in the blood corresponds to its absence in the lymph node is 0.68. There is an overall agreement of 0.68 for trisomy of CFA31.

FISH data for control dogs

Each control dog had a peripheral blood sample cytogenetically analyzed using four BAC-derived canine chromosome probes. Two hundred cells, both metaphase and interphase, were scored. Only a single monosomy was detected in two of the normal dogs (CFA11 in Dog 2 and CFA13 in Dog 6) and in one solid tumor dog (CFA13 in Dog 1).

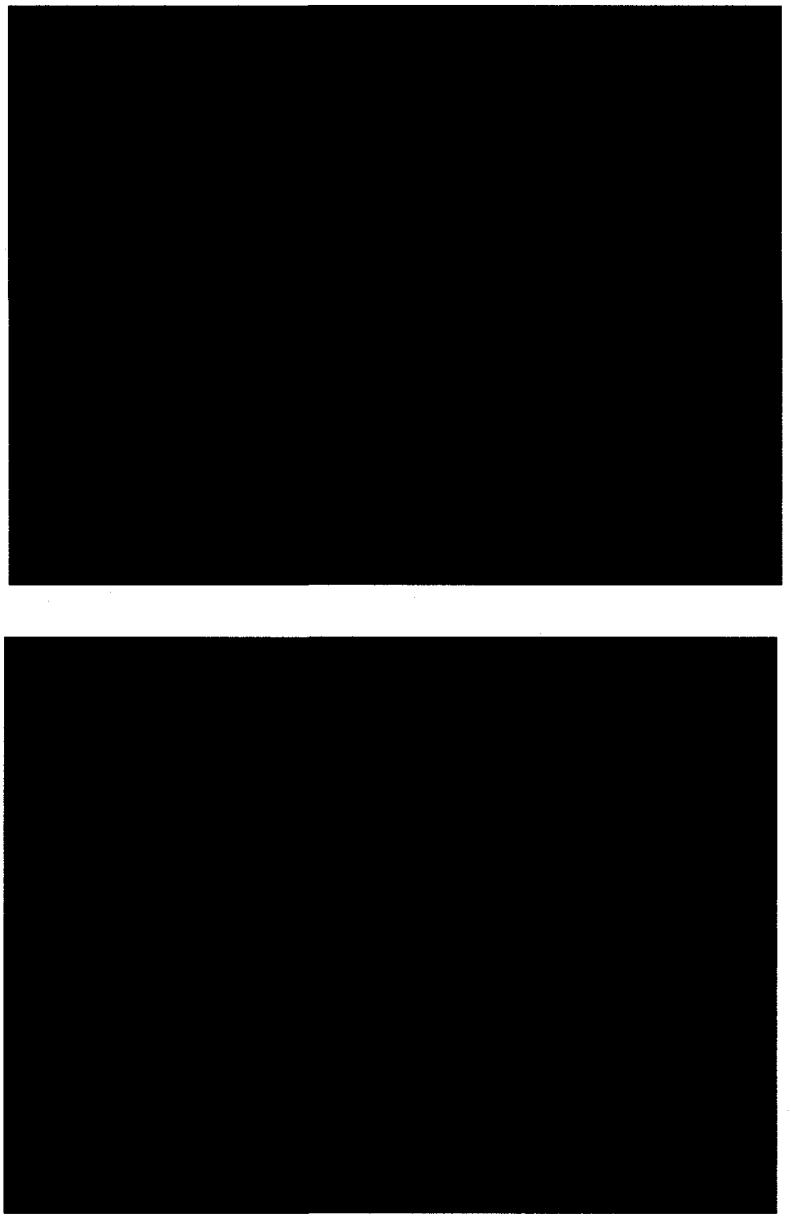

Figure 2.12: Trisomy 31 in a tumor lymphocyte (top) and peripheral blood lymphocyte (bottom) from Dog 22.

DISCUSSION

Histology

Correlations between histologic subtype and clinical behavior of cancers are well-recognized in human medicine. The Working Formulation is a common human lymphoma scheme that is easily amenable to canine lymphomas(9). All of the dogs in this study with histologic data were classified as either intermediate or high grade according to the Working Formulation. None of the dogs had low grade tumors. In human lymphomas, low grade tumors have the best prognosis however low grade lymphomas are rarely diagnosed in dogs. As expected, the dogs with intermediate grade tumors had a longer median survival time than the dogs with high grade lymphoma, although it was not statistically significant. Each case of intermediate and high grade tumors was further divided by cell type. A lack of sufficient case numbers makes it difficult to ascertain any distinctions between the cell types.

Immunophenotype

The immunophenotype of canine lymphomas has been shown to be prognostically significant(10;11). Tumors of B-cell origin have demonstrated a longer survival time and better overall prognosis than those of T-cell origin. In this study, 75% of the dogs had B-cell tumors while 25% were B-cell. This is in agreement with previous reports of a predominance of B-cell lymphomas in dogs(8;12-14). However, this study opposes the findings of other studies in that the median survival time for the T-cell lymphomas was longer than the B-cell tumors. This discrepancy is most likely due to a lack of sufficient case numbers.

Stage of disease

Uncertain results have been obtained in finding an association between stage of disease and prognosis. Many studies have determined that disease stage is not prognostically significant(15-17). However, other studies have reported that a higher clinical stage, particularly with b substage of disease, have a worse prognosis(18-20). In this particular study, all of the dogs were diagnosed as having advanced disease and none of the dogs had stage I or II. This is in accordance with other reports and is likely due to lymphoma detection being reliant on the owner or veterinarian noticing an enlarged lymph node or other subtle clinical signs, which are usually not evident until the disease has progressed an advanced stage(15;16;21). To look for correlations between staging and survival in this project, the stage of disease was analyzed first by dividing the groups by Roman numeral (III and IV vs. IV) and second by substage of disease (presence or absence of clinical signs of disease). No significant differences were detected which may be a result of low case numbers; very few of the dogs were categorized as subset b. In addition, the assignment of a disease stage is very subjective and based on the individual clinician's determination.

Breed

Certain canine breeds have been shown to be at higher risk of developing lymphoma including Saint Bernards, Scottish Terriers, Airedale Terriers, Bulldogs, Golden Retrievers, Basset Hounds, Labrador Retrievers, Bouvier des Flandres, and Rottweilers(10;21-23). In this study, 8 of the 25 dogs with lymphoma were Golden Retrievers. Six dogs were of mixed breed and the remainder represented 11 other breeds.

As found in other reports, the Golden Retrievers in this study had lymphomas of both T- and B-cell origin(22;24). Modiano et al. noticed certain numerical aberrations that segregated by specific breed. Specifically, he identified monosomy of CFA14 in 100% (7/7) of the Golden Retrievers but in only 13% of the dogs of other breeds(22). Contrarily, this study found only two out of eight Golden Retrievers with monosomy of CFA14 and in 18% of the other breeds (3/17). This inconsistency could be due to a lack of case numbers in both studies.

Age and gender

In the majority of the literature, the dog's gender and age at the time of diagnosis are not prognostic indicators(3;15;16;18-20). Yet one study found a longer remission and survival times in females(15). This study demonstrated a significant difference between genders with the females faring better than the males. This may be due in part to a larger proportion of male dogs in the study and the fact that six out of the eight females are still alive. No significant differences in the age at diagnosis were found in this study which supports previous works.

Tumor vs. peripheral blood

The crux of this research endeavor involved trying to determine whether the numerical aberrations that are found in the tumor are also present in the peripheral blood of dogs with lymphoma. Recurrent aberrations have been reported in canine lymphoma. Since this cancer parallels human Non-Hodgkin's Lymphoma, which has recurrent chromosomal anomalies that have been correlated with clinical behavior of the tumor and

patient survival, it reasons that canine lymphoma would as well. The current method for cytogentically analyzing tumors involves a lymph node biopsy or excision. This technique has two drawbacks: first, a biopsy or excision is painful for the animal and costly for the owner, and secondly, once the tumor is in remission, there is no tumor to biopsy which makes it impossible to monitor the patient prior to recurrence. M'Kacher et al. discovered the presence of aberrant peripheral blood lymphocytes in human Hodgkin's lymphoma patients(25). Despite the fact that canine lymphoma more closely mirrors NHL than HL, it still reasons that aberrant lymphocytes would be present in canine lymphoma since they are both hematopoietic diseases with large tumor burdens.

Four canine chromosomes of interest were selected based upon work by Thomas et al. describing the four most common numerical aberrations ascertained in canine lymphoma tumors. These aberrations included: monosomy of CFA11, trisomy of CFA13, monosomy of CFA14, and trisomy of CFA31(8). Due to the difficulty experienced in developing whole chromosome probes for dogs, a different technique was employed to create single locus probes from bacterial artificial chromosomes. This method labeled approximately 200kb of canine DNA on each chromosome of interest. Fortuitously, it has been well documented that the chromosomal aberrations detected in canine tumors are overwhelmingly numerical rather than structural(8;26-30). A structural aberration involves duplication or deletion of chromosomal material, inversions of the direction of genetic material, or translocations in which genetic material is exchanged between chromosomes. A numerical aberration occurs when an entire extra chromosome is present in the karyotype. Due to tendency toward numerical aberrations in canine tumor cells, the use of single locus probes instead of whole chromosome probes

was feasible. The second most frequent aberrations were centric fusions, which are fairly straightforward to recognize in metaphase cells since the canine autosomes are all acrocentric and a centric fusion would appear as a bi-armed chromosome(31;32). The reason for the overabundance of numerical aberrations in canine tumors is unknown but one possible explanation is that canine DNA has very little heterochromatin. Structural aberrations in human chromosomes often occur near the centromere which is rich in heterochromatin(27).

Each dog with lymphoma had a tumor and peripheral blood sample from its initial time point analyzed cytogenetically with the four single locus probes. Two hundred cells of good cytogenetic quality were scored from each sample. A minimum number of 2 cells (1%) with a particular aberration per sample were required to be accepted as a viable aberration for statistical analyses to account for possible misinterpreted aberrations. This criterion was established after analyzing statistical data using 1 (0.5%), 2 (1%), and 4 (2%) aberrant cells. The statistical results showed little variation between the different percentages, but it was decided that the appearance of one aberration per 200 cells could be erroneous but the presence of two of the same aberration was more likely to be a true representation of the sample. The vast majority of scored cells were interphase, particularly in the tumor sample as adequate mitotic indexes for tumors are hard to achieve. The benefit of using interphase cells is a more definite analysis. In metaphase cells, it is possible that chromosomes could be lost during slide preparation, which could appear as a monosomy when in fact that chromosome was misplaced in a location away from its cell of origin. Originally, the objective was to score the aforementioned four chromosome aberrations however, an unanticipated result occurred:

additional aberrations were detected with the probes. Specifically, not only were monosomies of CFA11 discovered, but also trisomies of CFA11. Likewise, monosomies of CFA13 and CFA31 were found. For CFA14, the only aberrations identified were monosomies. Thus, seven numerical aberrations were be discerned instead of the original target of four aberrations. Although these repetitive aberrations are not common, they have been reported in canine lymphoma.

The comparison between the tumoral and peripheral blood aberrations revealed a distinct correspondence. Occasionally the aberration was found in either the tumor or the blood but not both. In the case in which the aberration was detected in the tumor but not the blood, it is possible that 200 cells was not a large enough sample size. When the aberration was found in the blood but not the tumor, one explanation could be due to tumor heterogeneity. During the lymph node excision, only a small portion is prepared for culture so perhaps that region of the tumor processed does not contain that aberration but it was still detected in the blood. Other issues could be problems with the probe hybridization or background debris on the slide that is inadvertently labeled.

The overall agreements among each aberration was convincing in six out of seven of the aberrations. Trisomy of CFA31 had an overall agreement which was significantly lower than the other anomalies. The cytogenetic analyses of the peripheral blood could only predict the presence of the aberration in the tumor 68% of the time which was significantly lower than the predictive values for the other six aberrations. In future studies, perhaps chromosomes other than CFA31 would warrant more attention (Table 10).

Trisomy of CFA13 has been reported as a predictor of longer remission and survival times(3). It has also been described as the most common numerical aberration found in canine lymphoma(3;8). This study was in accordance with predominance of trisomy 13. In addition, the majority of the samples that displayed this aberration had a much higher percentage of cells within the sample with trisomy 13 than was found in any of the other aberrations. Prognostic significance was not found with any of the aberrations in this study, probably due to a small sample size.

Control dogs

The ten normal dogs and five dogs with solid tumors in the control populations were also analyzed cytogenetically with the four single locus probes. A single monosomy was detected in two of the normal (CFA11 in Dog 2 and CFA13 in Dog 6) and one of the solid tumor dogs (CFA13 in Dog 1). Two of the three monosomies were identified in metaphase cells which could have missing chromosomes lost during the slide preparation technique. Another explanation could be a problem in hybridization or merely a random aberrant cell. Since 200 cells were analyzed and only one aberration was found at any one time in the control dog samples, the percentage is lower than the 1% cutoff chosen for statistical study.

GENERAL DISCUSSION

This is the first study to evaluate the correspondence of chromosomal aberrations in the tumor and peripheral blood in canine lymphoma. The positive results garnered indicate promise for use as a diagnostic tool. Further research needs to be conducted to

determine the prognostic significance of the additional aberrations found in canine lymphoma. It is highly likely that an abundance of lymphoma subsets exist in canine lymphoma, just as in human NHL. Once that is established, a peripheral blood sample from a dog diagnosed with lymphoma could be cytogenetically analyzed to better aid the clinician and owner toward an appropriate treatment protocol that is ideal for the particular subset of lymphoma. This method utilizing peripheral blood is superior to relying on tumor tissues given that blood samples could be transported from a veterinary clinic to a laboratory with cytogenetic capabilities, unlike tumor samples. Additional research needs to be undertaken to explore the role of chromosomal changes during the course of treatment which could be performed using peripheral blood. This could lead to a predictive assay for response to treatment and recurrence.

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CHAPTER III

NUMERICAL CHROMOSOMAL CHANGES OVER TIME IN CANINE LYMPHOMA

INTRODUCTION

Canine lymphoma is the most common hematopoietic malignancy diagnosed in dogs. If left untreated, the mean survival time is only one month. Yet even with treatment, long term survival remains poor, with median survival between 12 and 18 months(1). Once a dog has completed its course of chemotherapy and is in remission, the current course of action is to await the return of clinical signs of disease before beginning a rescue chemotherapy protocol. Developing a method of monitoring the patient's subclinical response during treatment offers the potential for medical intervention prior to the reappearance of clinical signs at the time of relapse.

A relationship between cytogenetic changes in peripheral blood lymphocytes and tumors in human lymphoma patients has been established(2-4). Not only was predictive information obtained, but response to therapy could also be monitored(2). Peripheral changes in chromosomes could provide a unique window for determining the patient's response to treatment. Chromosome counts can identify numerical changes, such as chromosome deletions or duplications, which are a hallmark of canine lymphoma(5;6). This study determined the presence of circulating tumor lymphocytes in the peripheral blood of canine lymphoma patients using chromosome counting techniques. Once this

was established, molecular cytogenetic methods were used to determine which chromosomes were implicated in each patient's specific subset of lymphoma.

As mentioned in Chapter II, evaluating peripheral lymphocytes instead of tumor is advantageous for many reasons. This study expounded on the discovery that the aberrations found in the peripheral lymphocytes of dogs with lymphoma were correlated with the aberrations in the tumor. The peripheral blood from fourteen of the twenty-five dogs from the previous study were followed during the course of lymphoma treatment with chromosome counts and/or FISH analysis using four single locus probes. Meaningful cytogenetic changes from lymphocytes could become a powerful clinical tool in the treatment of canine lymphoma. Developing methods for early screening and predicting response to therapy would have a major clinical impact in this important disease.

METHODS & MATERIALS

Experimental Design

Dogs with lymphoma who had not received any treatment for lymphoma were identified by the Oncology Service of the Colorado State University Veterinary Medical Center (CSU-VMC). Patients underwent routine staging procedures required by the Oncology Service prior to treatment. Upon admission to the study, a protocol coordinator supervised a lymph node excision, bone marrow aspirate, and collection of a peripheral blood sample. The lymph node and bone marrow samples were used in the previous study to determine correlations between aberrations in the tumor and peripheral blood and evidence of bone marrow involvement. Dogs were pre-medicated and anesthetized

with continuous monitoring in accordance with Animal Care and Use (ACUC) standards. A one milliliter (ml) peripheral blood sample was collected monthly during the course of treatment and cultured using standard cytogenetic protocols. Slides were made from each timepoint sample and prepared for chromosome counting and/or fluorescence *in situ* hybridization (FISH) analysis. Ten clinically normal dogs and five dogs with solid tumors were used as controls. Each control dog had at least one peripheral blood sample analyzed with both chromosome counting and FISH.

Animal Care and Use

Approval from the Colorado State University Animal Care and Use Committee (ACUC) was obtained prior to patient accrual. A copy of the approved ACUC form is found in the Appendices.

Peripheral blood

Each control dog had at least one peripheral blood sample collected using aseptic technique into a lithium heparin tube. Fourteen dogs with lymphoma had blood drawn at the time of initial diagnosis and at monthly intervals during treatment. Blood was cultured and fixed as documented in Chapter II.

Development of BAC probes (DNA extraction through labeling)

Bacterial Artificial Chromosomes (BAC) clones representing segments of canine chromosomes 11, 13, 14, and 31 were developed into fluorescently labeled probes using the protocol documented in Chapter II.

Canine COT-1 DNA

Canine COT-1 DNA was prepared to block repetitive sequences in the canine genome and generated as documented in Chapter II.

Fluorescence *in situ* hybridization

The slides were prepared for FISH analysis as documented in Chapter II.

Chromosome counting

Slides destined for chromosome counting were accessed for reasonable mitotic indices and good chromosome spreading. They were stained with DAPI/AF and visualized using a Zeiss fluorescent microscope and corresponding MetaSystems cytogenetic software. A modal number of twenty metaphase spreads were counted for each peripheral blood sample.

Scoring of samples

Each FISH slide had 200 cells evaluated using a Nikon fluorescent microscope and corresponding MetaMorph cytogenetic software. Both metaphase and interphase cells were scored.

Medical records

Following all cytogenetic analyses, each patient's record was examined to garner relevant clinical information including remission status during treatment and survival times.

RESULTS

Chromosome Counts

A total of 10 normal dogs, 5 solid tumor dogs, and 14 lymphoma dogs had chromosome counts performed at various time points. The number of metaphase cells that were counted ranged from 8 to 24 with a modal number of 20 cells. For the ten normal control dogs, the mean chromosome counts ranged from 77.56 to 78 (Table 3.1/ Figures 3.1 & 3.2). The range of chromosome numbers was 75 to 78; however, the modal number was 78 for all 10 normal dogs (Table 3.2). In the five solid tumor control dogs, the average number of chromosomes ranged from 77.67 to 78 (Table 3.1/ Figure 3.3). The range of chromosome numbers was 75 to 79; however, the modal number was 78 for all solid tumor dogs (Table 3.2). Chromosome counts were performed on 14 lymphoma patients. Eight of 14 had sequential counts conducted during the course of treatment. The modal number for all counts on the dogs with lymphoma ranged between 76 and 80 (Table 3.3). Through the course of treatment, the distribution of the lymphoma patients' average chromosome counts varied, possibly due to various chemotherapeutic drugs and radiation therapy-induced numerical aberrations, as well as changes associated with disease progression. Upon completion of therapy, the majority of the lymphoma dogs experienced a stabilization of numerical chromosomal aberrations as the averages approached normal diploid numbers (Figure 3.4). To compare the lymphoma dogs with

Table 3.1: Mean number of chromosomes at each time point for normal and solid tumor control dogs.

	dog #	timepoint 1	timepoint 2	timepoint 3	timepoint 4	timepoint 5	timepoint 6	timepoint 7
normal dogs	1	77.93	77.87	77.89	-	-	-	-
	2	77.56	77.71	78	77.94	77.94	-	-
	3	78	77.95	-	-	-	-	-
	4	78	77.92	-	-	-	-	-
	5	78	78	-	-	-	-	-
	6	78	77.89	-	-	-	-	-
	7	77.89	78	-	-	-	-	-
	8	78	78	78	77.94	78	-	-
	9	78	-	-	-	-	-	-
	10	78	78	78	78	78	78	78
solid tumor dogs	1	77.67	78	-	-	-	-	-
	2	77.76	-	-	-	-	-	-
	3	78	-	-	-	-	-	-
	4	78	78	-	-	-	-	-
	5	78	-	-	-	-	-	-

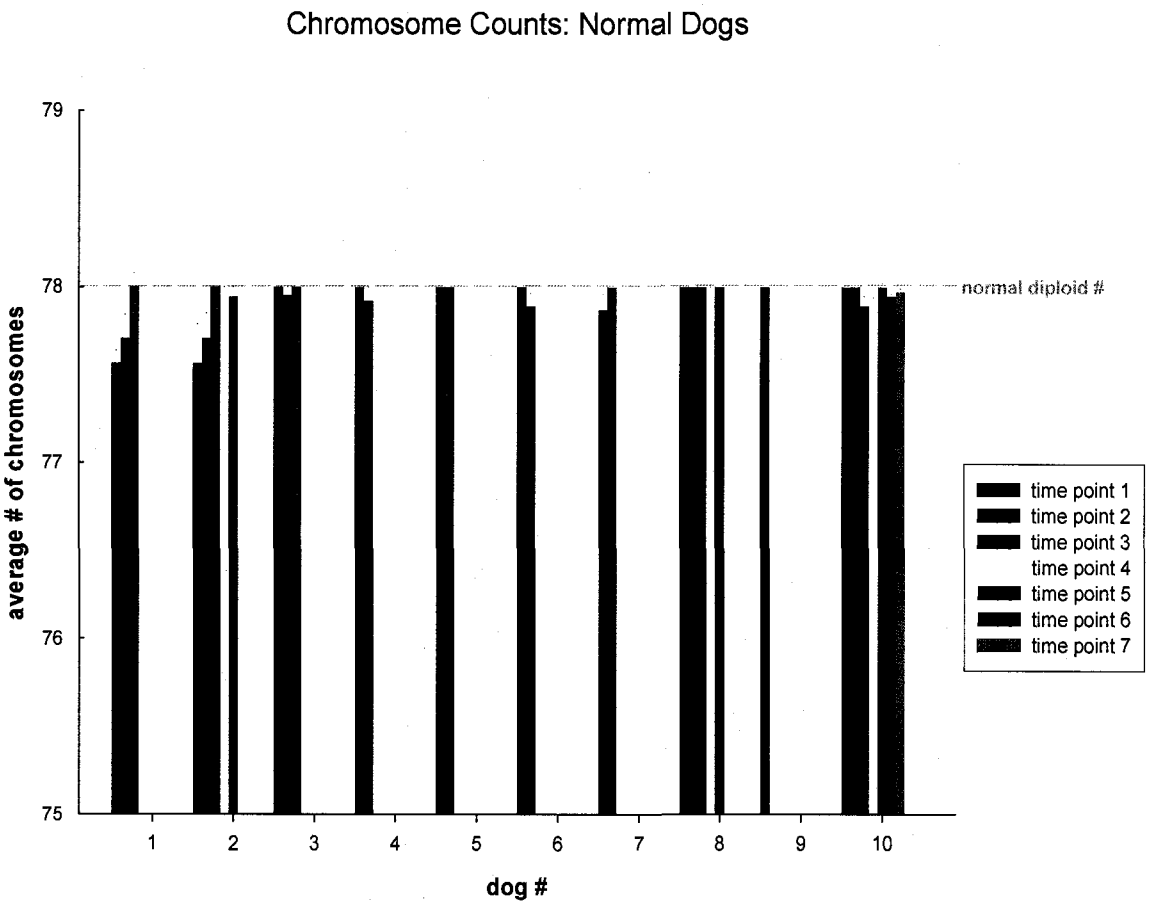


Figure 3.1: Mean number of chromosome counts for normal control dogs at various time points.

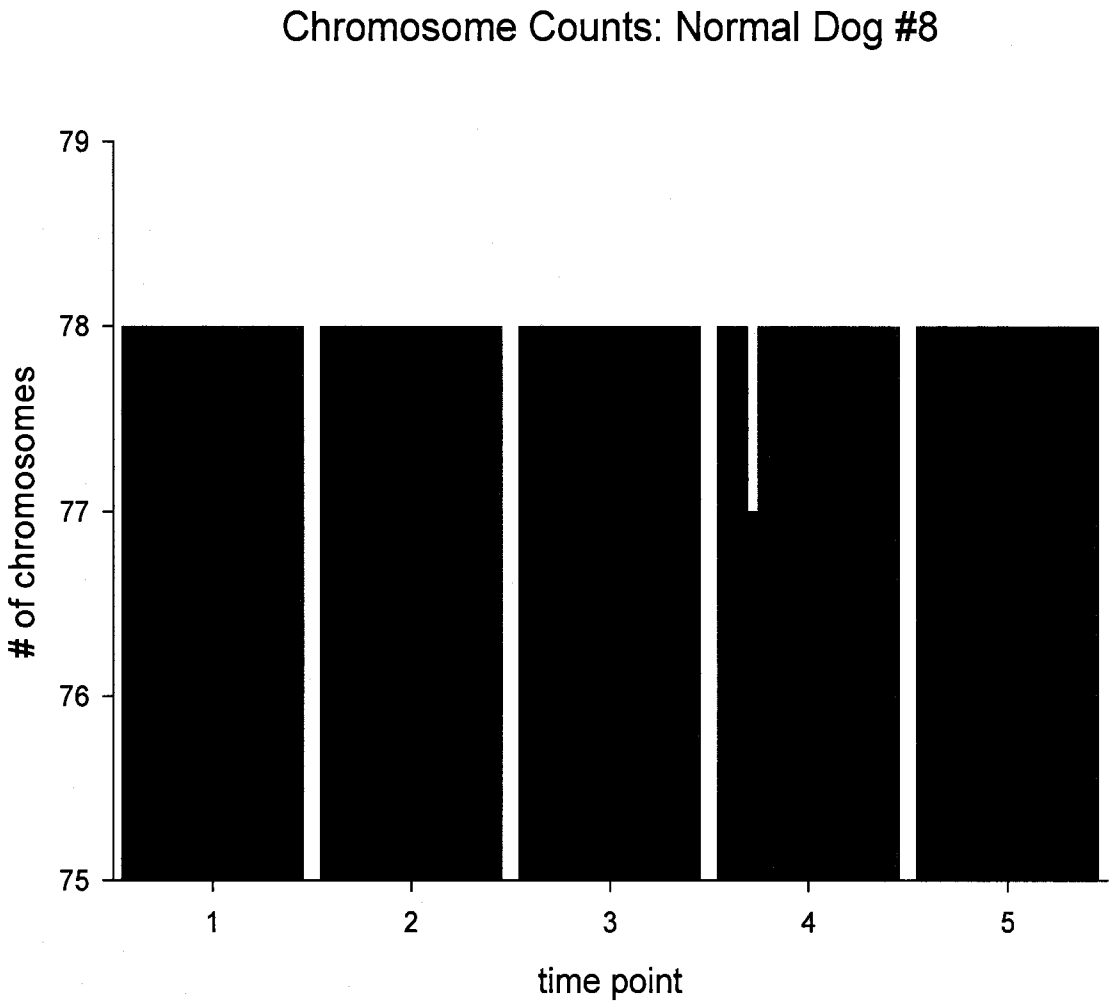


Figure 3.2: Actual number of chromosome counts for one normal control dog at five different time points.

Table 3.2: Distribution of chromosome counts for normal and solid tumor control dogs.

	dog #	time point	# cells scored	75	76	77	78	79	range	mode
normal dogs	1	1	14			1	13		77-78	78
		2	15		1		14		76-78	78
		3	18			2	16		77-78	78
	2	1	16	1	1	2	12		75-78	78
		2	17		1	3	13		76-78	78
		3	20				20		78	78
		4	17			1	16		77-78	78
		5	17			1	16		77-78	78
	3	1	14				14		78	78
		2	21			1	20		77-78	78
		3	18				18		78	78
	4	1	12				12		78	78
		2	12			1	11		77-78	78
	5	1	11				11		78	78
		2	19				19		78	78
	6	1	12				12		78	78
		2	18			2	16		77-78	78
	7	1	15			2	13		77-78	78
		2	19				19		78	78
	8	1	18				18		78	78
		2	18				18		78	78
		3	18				18		78	78
		4	18			1	17		77-78	78
		5	18				18		78	78
	9	1	18				18		78	78
	10	1	14				14		78	78
		2	16				16		78	78
		3	16				16		78	78
		4	14				14		78	78
		5	16				16		78	78
		6	16				16		78	78
		7	15				15		78	78
solid tumor	1	1	16	1			14	1	75-79	78
		2	17				17		78	78
	2	1	13		1	1	11		76-78	78
	3	1	17				17		78	78
	4	1	18				18		78	78
		2	16				16		78	78
	5	1	17				17		78	78

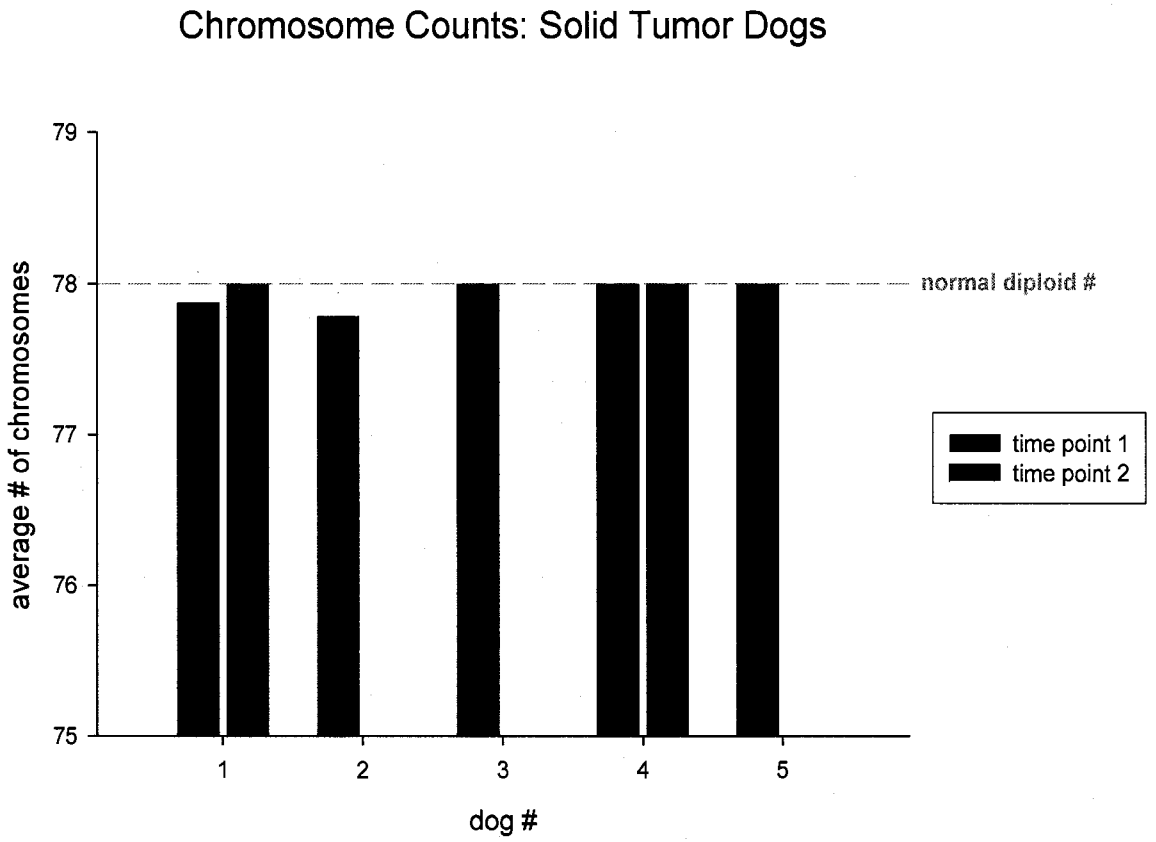


Figure 3.3: Mean number of chromosome counts for solid tumor control dogs at various time points.

Table 3.3: Distribution of chromosome counts for dogs with lymphoma.

dog	time point	# cells scored	< 72	72	73	74	75	76	77	78	79	80	81	82	83	84	> 84	% abn	% high	% low	range	mode
1	1	20	3	1		1		2		4	1	4	3				1	80	45	35	66-85	78,80
	10	20						1	2	17								15	0	15	76-78	78
	39	20						2	4	13	1							35	5	30	76-79	78
2	1	20								13	5	1		1				35	35	0	78-82	78
	6	20								18	2							10	10	0	78-79	78
	19	20	1					1	6	9	3							55	15	40	68-79	78
	30									11	3	5		1				45	45	0	78-82	78
4	1	24					2		4	11	6	1						54	29	25	75-80	78
	8	21	2		2	1	2	1	3	8			1	1				61	9	52	70-82	78
	47	20				2		1	5		2	2		4	1	1	2	100	60	40	74-89	77
5	1	20					1	1	3	9	1			1	1	1	2	55	30	25	75-89	78
	9	20	1		1		2	1	1	9	4					1		55	25	30	71-84	78
	30								2	15	3							25	15	10	77-79	78
6	1	20								7	5	5	3					65	65	0	78-81	78
	13	20				1			1	10	2	5				1		50	40	10	74-84	78
	22	20				1				10	6	1	1			1		50	45	5	74-84	78
8	1	20						2	6	12								40	0	40	76-78	78
	6	12							6	6								50	0	50	77-78	77,78
	11	9				3	4	1	1									100	0	100	74-77	75
	49	12				1			8	2	1							83	8	75	74-79	77
	50	20	2	1			3	5	5	3	1							85	5	80	70-79	76,77
	52	13	2			1		1	8	1								92	0	92	70-78	77
11	1	20							2	9	6	2	1					55	45	10	77-81	78
12	1	20						1		6	4	1	3	4		1		70	65	5	76-84	78
13	1	20							1	8	5	6						60	55	5	77-80	78
	6	20			2		3			9	1	1	1	1		1	1	55	30	25	73-89	78
	10	20	1				2		1	10	2			3		1		50	30	20	63-84	78
	13	20	7		1	3	1	1	1	4		1						80	5	75	62-80	78
	17	14								3	4	1	1	1	2	2		79	79	0	78-84	79
	20	20		1				2	1	10	2	3		1				50	30	20	72-82	78
14	1	20								8	5	5	2					60	60	0	78-81	78
	5	8						2	2	3	1							62	12	50	76-78	78
	7	11							3	4	4							64	36	27	77-79	78,79
	8	11					2		1	5	3							55	28	27	75-79	78
	11	12							1	4	7							67	58	9	77-79	79
	16	20							2	16	2							20	10	10	77-79	78
	22	20							2	13	3	1		1				35	25	10	77-82	78
	29	20							1	6	4	5	2	2				70	65	5	77-82	78
19	1	20			1	1	1	1	3	7	5	1						65	30	35	73-80	78
20	1	17								14	1	1		1				15	15	0	78-82	78
22	1	20					2	1	1	14	1						1	30	10	20	75-86	78
25	1	20								12	7	1						40	40	0	78-80	78

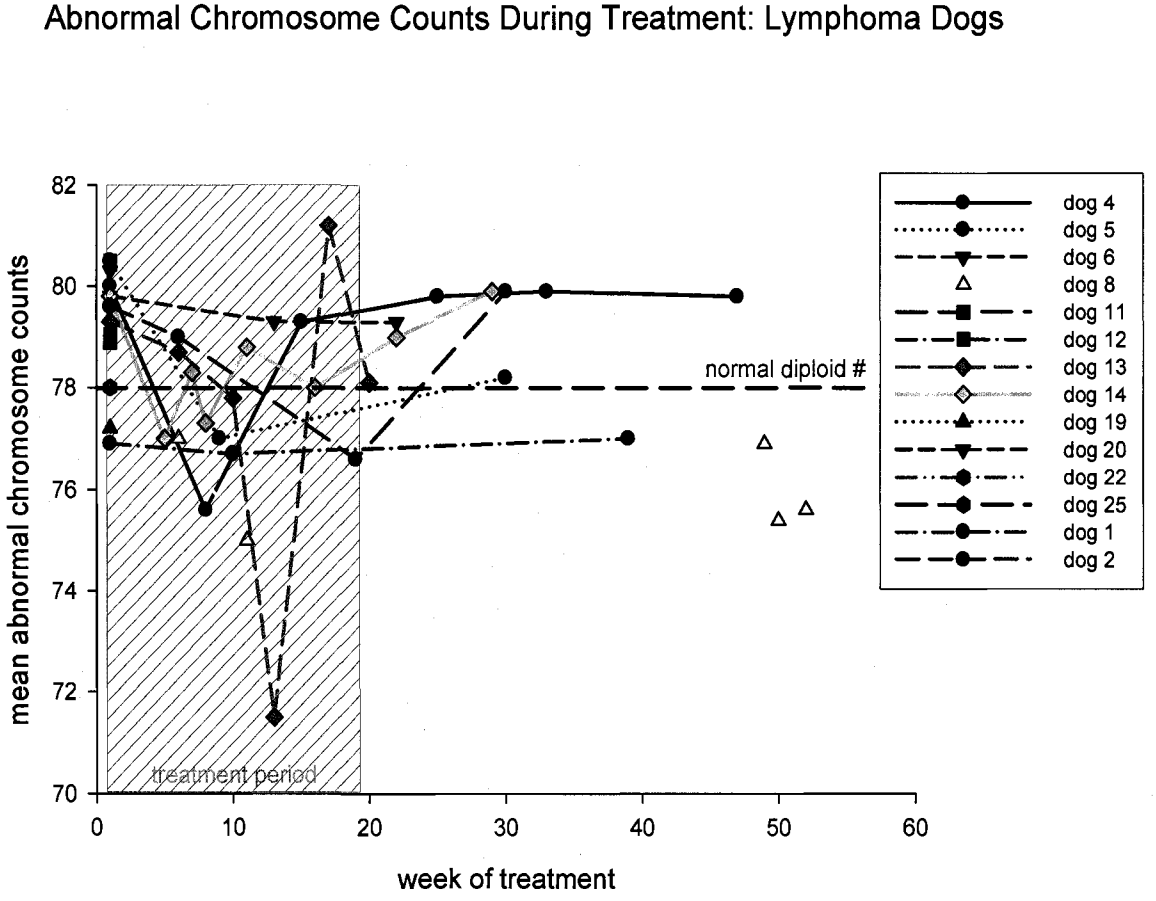


Figure 3.4: Mean abnormal chromosome counts during the course of lymphoma treatment.

the control dogs, the average number of chromosomes was used for the control dogs since their modal numbers were all 78. For the lymphoma patients, only the hyper- or hypoploid cells were averaged. The percentage of abnormal cells in the lymphoma dogs ranged from 10 to 100, with an average percentage of 55.3 (Table 3.3). Both the normal and solid tumor control dogs' chromosome numbers which remained normal at all time points whereas the counts in lymphoma dogs varied, sometimes even oscillating between hyper- and hypoploidy (Figure 3.5). The modal number(s) of chromosomes were compared as the dogs progressed through their course of disease. The distribution of counts changed at each time point, although the modal number was usually 78 (Figures 3.7-3.14).

Clinical records

Careful inspection of each dog's clinical records was performed. Information about remissions, chemotherapy and radiation therapy schedules, bloodwork, and time of death were documented. Two dogs were noted to be leukemic at one time point: Dog 6 at week 22 of treatment and Dog 18 at the time of diagnosis.

FISH data for control dogs

Each control dog had at least one peripheral blood sample cytogenetically analyzed using four BAC-derived canine chromosome probes. Two hundred cells, both metaphase and interphase, were scored. Only a single monosomy was detected in one time point from four of the normal dogs and in one time point from one solid tumor dog (Table 3.4).

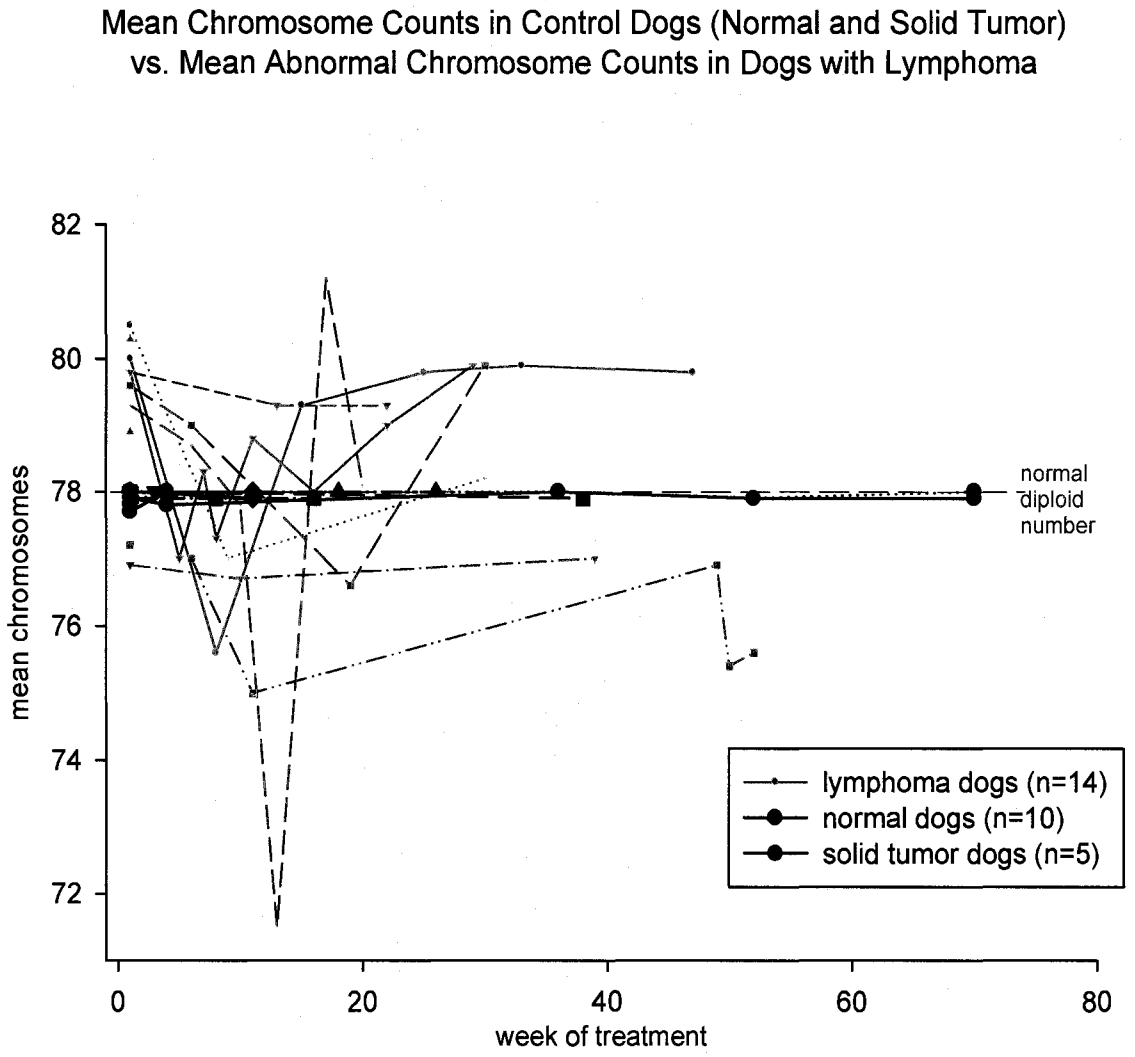


Figure 3.5: Mean of abnormal chromosome counts during the course of lymphoma treatment as compared to the mean of chromosome counts for control dogs (normal and solid tumor).

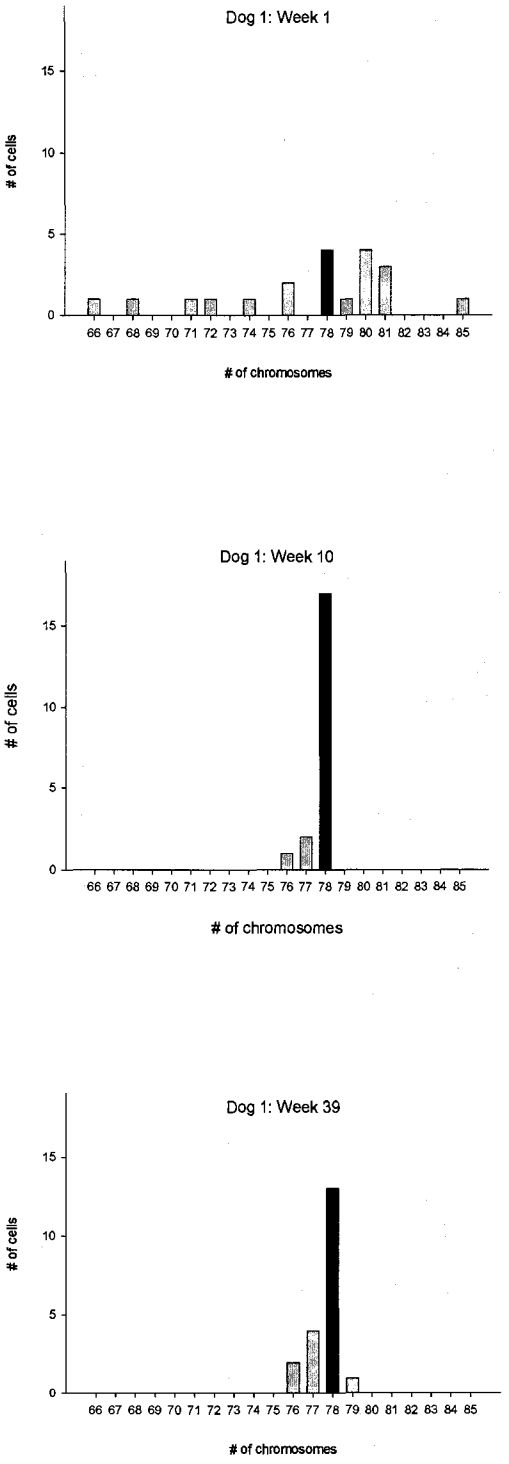


Figure 3.6: Distribution of chromosome counts over time for Dog 1 with normal diploid number of 78 highlighted in black.

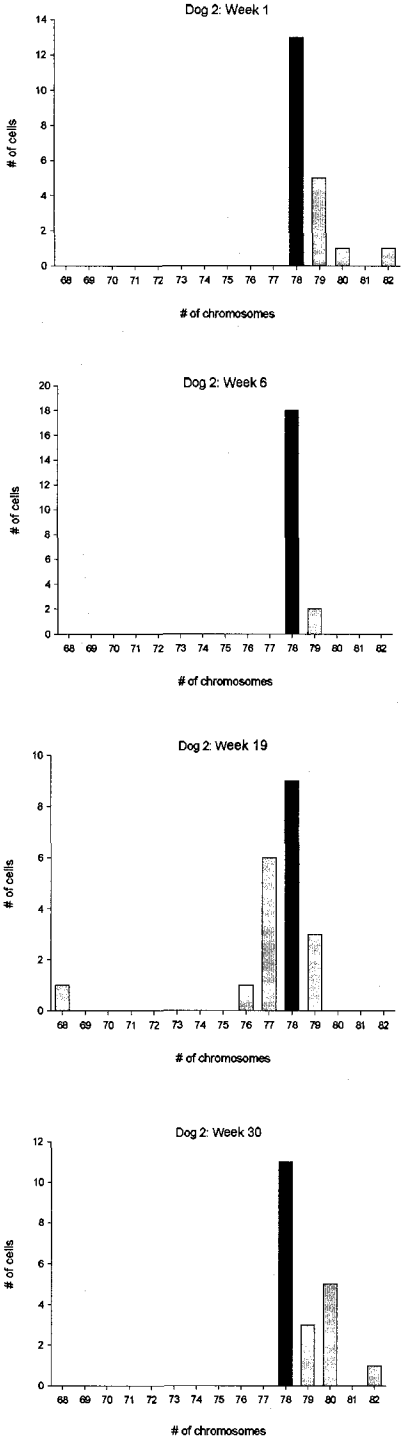


Figure 3.7: Distribution of chromosome counts over time for Dog 2 with normal diploid number of 78 highlighted in black.

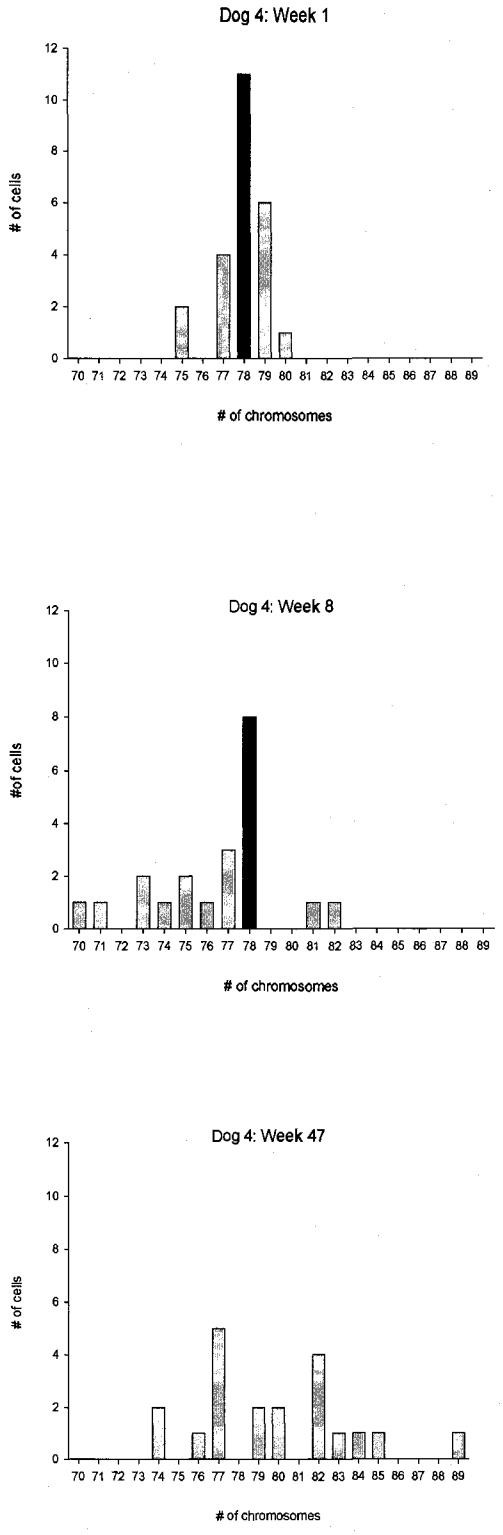
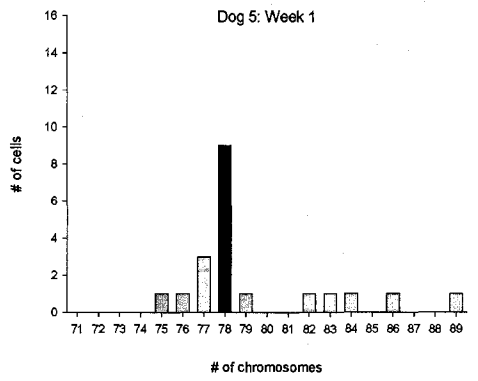
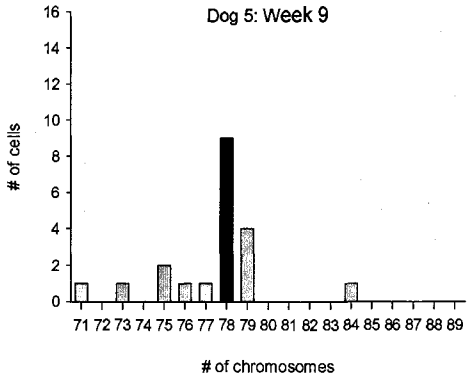


Figure 3.8: Distribution of chromosome counts over time for Dog 4 with normal diploid number of 78 highlighted in black.





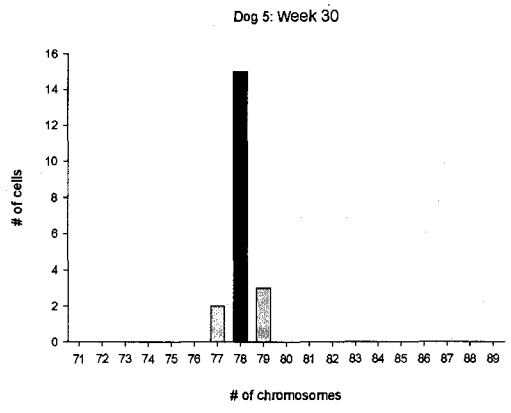
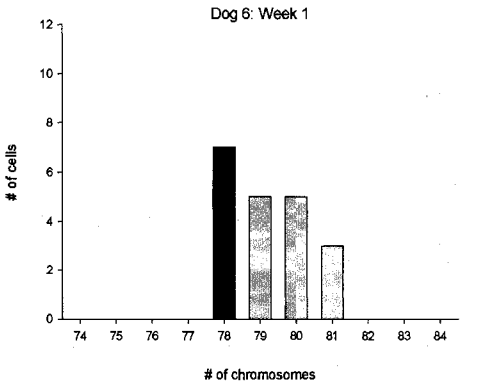
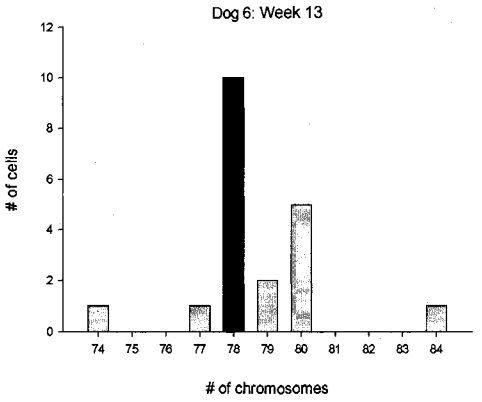


Figure 3.9: Distribution of chromosome counts over time for Dog 5 with normal diploid number of 78 highlighted in black.





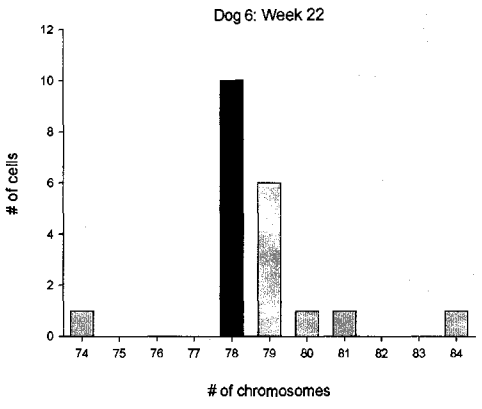


Figure 3.10: Distribution of chromosome counts over time for Dog 6 with normal diploid number of 78 highlighted in black.

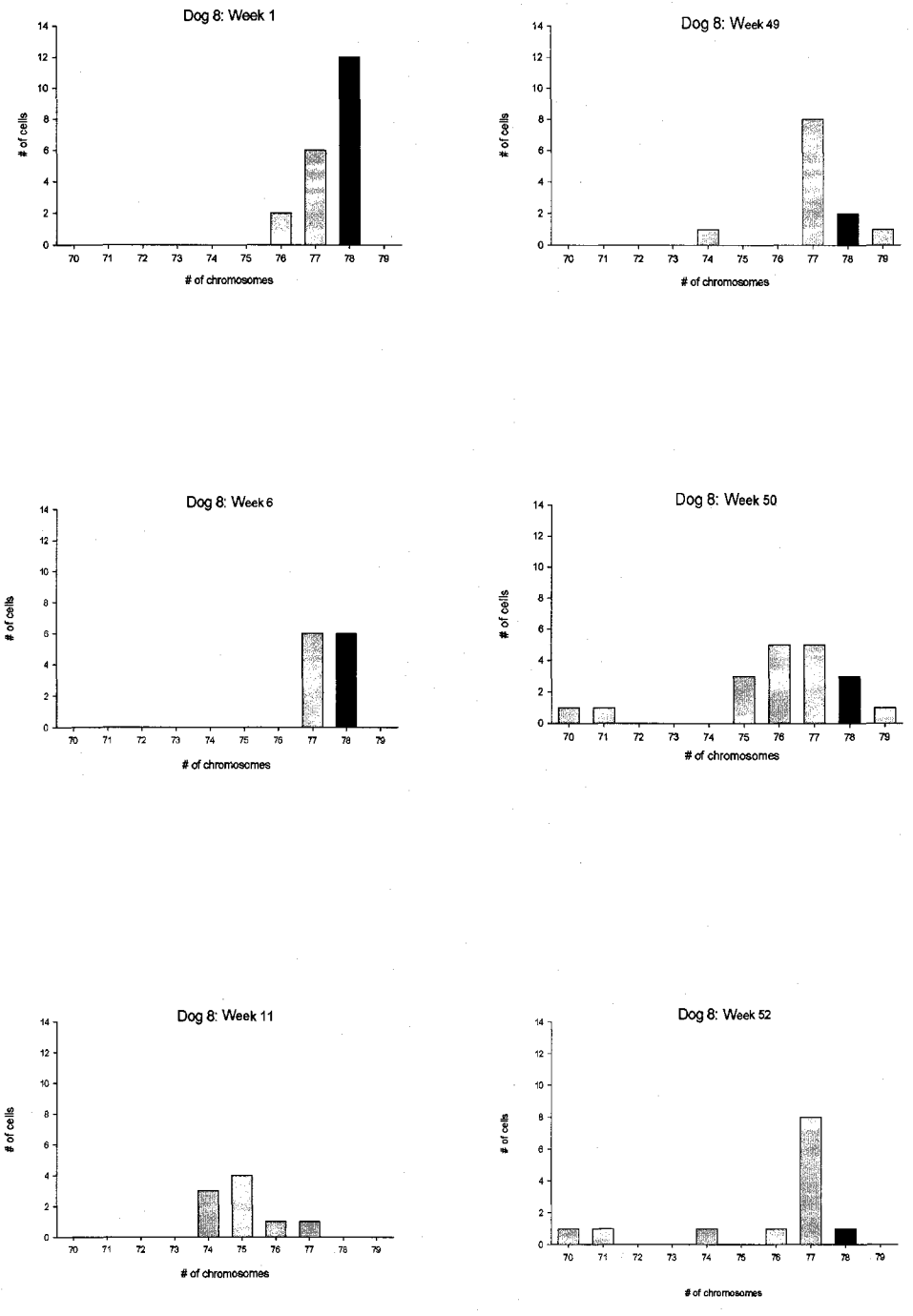


Figure 3.11: Distribution of chromosome counts over time for Dog 8 with normal diploid number of 78 highlighted in black.

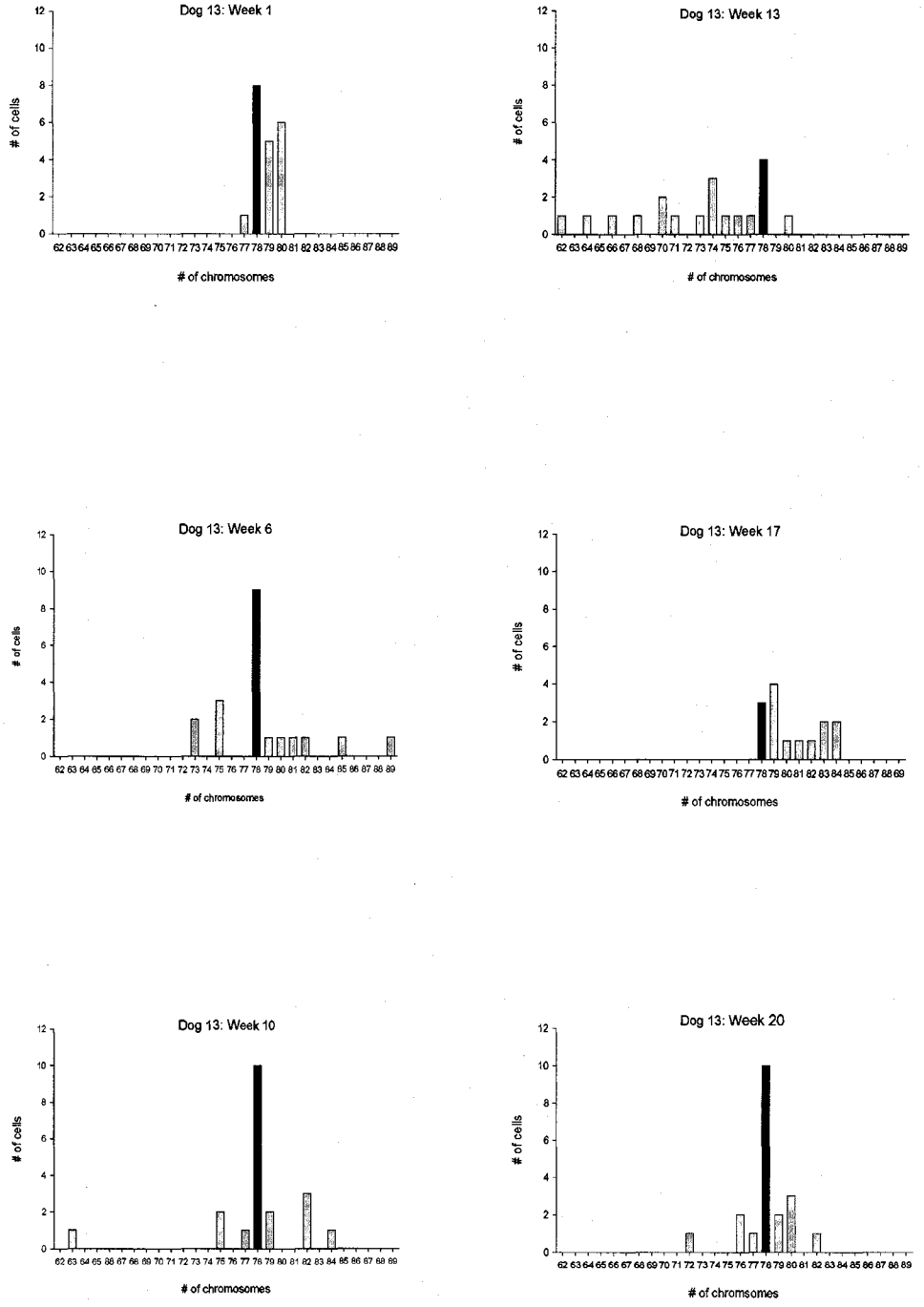


Figure 3.12: Distribution of chromosome counts over time for Dog 13 with normal diploid number of 78 highlighted in black.

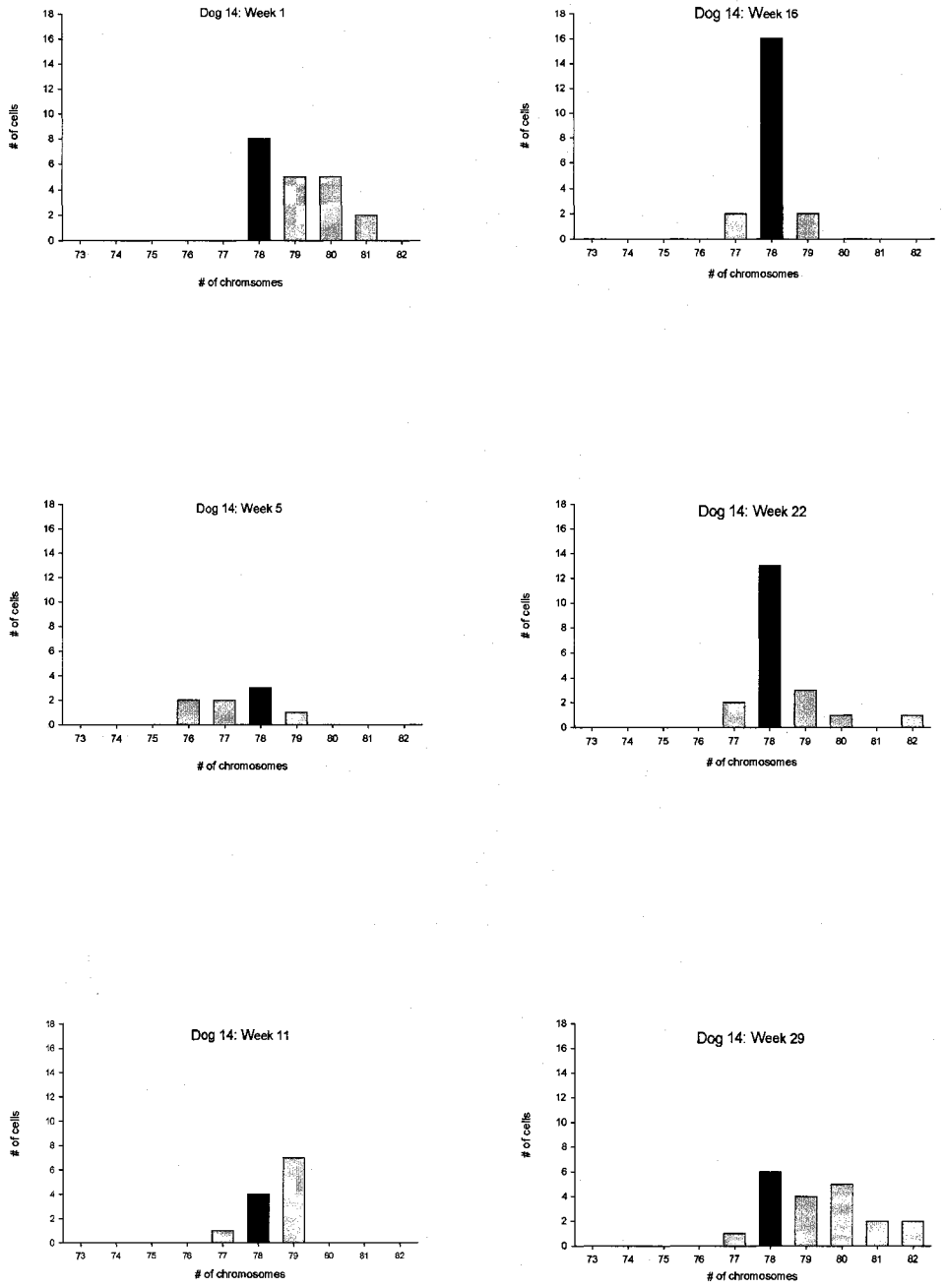


Figure 3.13: Distribution of chromosome counts over time for Dog 14 with normal diploid number of 78 highlighted in black.

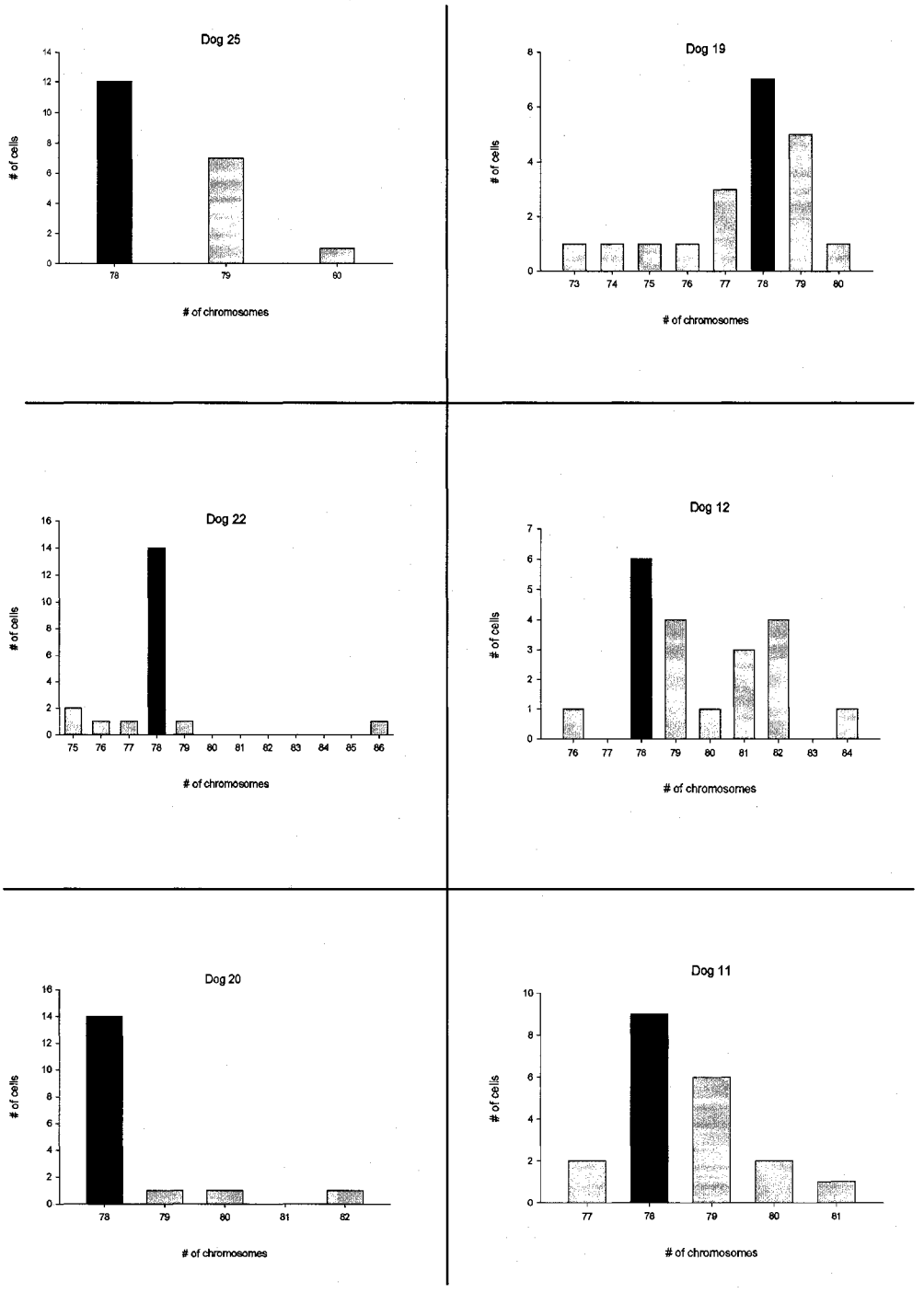


Figure 3.14: Distribution of chromosome counts at presentation for six different dogs with normal diploid number of 78 highlighted in black.

Table 3.4: Chromosomal aberrations detected in control dogs.

	dog #	timepoint 1	timepoint 2	timepoint 3	timepoint 4	timepoint 5
normal dogs	1	0	0	0	-	-
	2	1 mono11	0	1 mono11	0	0
	3	0	0	0	-	-
	4	0	0	-	-	-
	5	0	0	-	-	-
	6	1 mono13	0	-	-	-
	7	0	0	-	-	-
	8	0	0	0	1 mono31	0
	9	0	-	-	-	-
	10	0	0	-	-	-
solid tumor dogs	1	1 mono13	0	-	-	-
	2	0	-	-	-	-
	3	0	-	-	-	-
	4	0	0	-	-	-
	5	0	-	-	-	-

FISH data for dogs during lymphoma treatment

Ten lymphoma dogs had multiple blood samples cytogenetically analyzed during their course of treatment (Table 3.55). Each dog had the percentage of numerical aberrations graphed over time which was compared with the changes in chromosome numbers over time. A trend emerged with the numerical aberrations in that the abnormalities were often high at the initial time point but became closer to normal as they progressed through treatment. However, frequently the percentage of aberrant cells increased following the commencement of treatment but prior to disease recurrence. Dog 8 did not have any cytogenetic abnormalities that could be detected with the four chromosome probes, however his chromosome counts were slightly hypoploid at the start of treatment and became increasingly more hypoploid until week 50 (Figures 3.15-3.24).

Table 3.5: Numerical chromosomal aberrations in the initial lymph node and peripheral blood sample and subsequent blood samples of lymphoma patients.

dog	aberration	tumor	initial blood	time point 2	time point 3	time point 4	time point 5
1	mono11	0	0	4	0	7	-
	tri11	0	0	0	0	0	-
	mono13	0	0	0	0	0	-
	tri13	0	0	2	0	0	-
	mono14	0	0	0	0	0	-
	mono31	0	0	0	0	2	-
	tri31	4	0	0	0	6	-
2	mono11	15	8	7	0	0	6
	tri11	12	4	7	0	0	0
	mono13	0	1	0	0	0	0
	tri13	12	10	0	3	0	4
	mono14	12	4	0	1	0	0
	mono31	0	1	0	0	0	0
	tri31	1	2	0	1	0	0
3	mono11	0	0	0	0	-	-
	tri11	0	0	0	0	-	-
	mono13	2	0	0	0	-	-
	tri13	0	0	0	0	-	-
	mono14	0	0	0	0	-	-
	mono31	0	0	0	0	-	-
	tri31	1	0	0	0	-	-
4	mono11	20	3	0	0	7	8
	tri11	6	3	0	7	7	5
	mono13	15	13	8	0	7	0
	tri13	3	3	8	3	3	10
	mono14	8	10	3	0	0	7
	mono31	6	9	0	3	0	0
	tri31	20	3	0	0	0	0
5	mono11	3	0	2	0	0	-
	tri11	0	0	0	1	1	-
	mono13	0	0	0	0	0	-
	tri13	0	0	0	0	1	-
	mono14	0	0	0	0	1	-
	mono31	0	0	0	0	0	-
	tri31	0	0	0	0	0	-
6	mono11	10	3	3	1	2	-
	tri11	3	3	0	1	0	-
	mono13	6	8	6	0	0	-
	tri13	8	15	0	5	4	-

	mono14	3	2	6	0	0	-
	mono31	2	4	1	1	0	-
	tri31	3	4	3	3	5	-
7	mono11	0	0	0	0	0	0
	tri11	0	0	0	0	0	0
	mono13	0	0	0	0	0	3
	tri13	162	18	0	0	0	3
	mono14	0	0	0	0	0	6
	mono31	3	3	0	0	0	6
	tri31	3	0	0	0	0	0
8	mono11	0	0	0	0	0	-
	tri11	0	0	0	0	0	-
	mono13	0	0	0	0	0	-
	tri13	0	0	0	0	0	-
	mono14	0	0	0	0	0	-
	mono31	0	0	0	0	0	-
	tri31	0	0	0	0	0	-
9	mono11	15	3	-	-	-	-
	tri11	0	0	-	-	-	-
	mono13	0	0	-	-	-	-
	tri13	9	2	-	-	-	-
	mono14	0	0	-	-	-	-
	mono31	6	0	-	-	-	-
	tri31	0	0	-	-	-	-
10	mono11	0	0	-	-	-	-
	tri11	0	0	-	-	-	-
	mono13	0	0	-	-	-	-
	tri13	0	0	-	-	-	-
	mono14	0	0	-	-	-	-
	mono31	0	0	-	-	-	-
	tri31	0	0	-	-	-	-
11	mono11	0	0	-	-	-	-
	tri11	0	0	-	-	-	-
	mono13	0	1	-	-	-	-
	tri13	69	15	-	-	-	-
	mono14	0	0	-	-	-	-
	mono31	0	0	-	-	-	-
	tri31	10	6	-	-	-	-
12	mono11	1	0	-	-	-	-
	tri11	0	0	-	-	-	-
	mono13	0	0	-	-	-	-
	tri13	0	0	-	-	-	-
	mono14	0	0	-	-	-	-
	mono31	0	0	-	-	-	-
	tri31	0	0	-	-	-	-
13	mono11	10	3	0	0	0	0

	tri11	8	3	0	0	0	0
	mono13	0	6	0	8	0	0
	tri13	6	0	0	6	0	0
	mono14	0	0	0	0	0	0
	mono31	0	3	3	0	0	0
	tri31	6	0	0	0	0	0
14	mono11	20	11	0	0	0	5
	tri11	0	0	0	0	0	0
	mono13	0	0	0	0	0	0
	tri13	0	0	0	0	0	0
	mono14	0	0	0	0	0	0
	mono31	0	0	0	0	0	0
	tri31	0	0	0	0	0	0
15	mono11	0	0	0	0	-	-
	tri11	0	0	0	0	-	-
	mono13	0	0	0	0	-	-
	tri13	4	0	0	0	-	-
	mono14	0	0	0	0	-	-
	mono31	0	0	0	0	-	-
	tri31	6	0	0	0	-	-
16	mono11	0	0	-	-	-	-
	tri11	0	0	-	-	-	-
	mono13	1	0	-	-	-	-
	tri13	171	7	-	-	-	-
	mono14	1	0	-	-	-	-
	mono31	0	0	-	-	-	-
	tri31	0	0	-	-	-	-
17	mono11	0	14	0	-	-	-
	tri11	10	14	0	-	-	-
	mono13	0	0	0	-	-	-
	tri13	0	0	0	-	-	-
	mono14	0	0	0	-	-	-
	mono31	3	0	7	-	-	-
	tri31	3	0	0	-	-	-
18	mono11	7	5	0	-	-	-
	tri11	0	0	0	-	-	-
	mono13	0	0	0	-	-	-
	tri13	172	37	0	-	-	-
	mono14	0	0	0	-	-	-
	mono31	0	0	7	-	-	-
	tri31	3	2	0	-	-	-
19	mono11	10	12	-	-	-	-
	tri11	0	4	-	-	-	-
	mono13	12	2	-	-	-	-
	tri13	10	16	-	-	-	-
	mono14	0	0	-	-	-	-

	mono31	0	0	-	-	-	-
	tri31	3	0	-	-	-	-
20	mono11	0	0	-	-	-	-
	tri11	0	0	-	-	-	-
	mono13	0	0	-	-	-	-
	tri13	153	17	-	-	-	-
	mono14	0	0	-	-	-	-
	mono31	0	0	-	-	-	-
	tri31	0	0	-	-	-	-
21	mono11	6	3	-	-	-	-
	tri11	0	0	-	-	-	-
	mono13	2	0	-	-	-	-
	tri13	0	0	-	-	-	-
	mono14	0	0	-	-	-	-
	mono31	0	0	-	-	-	-
	tri31	0	0	-	-	-	-
22	mono11	7	3	-	-	-	-
	tri11	3	0	-	-	-	-
	mono13	0	0	-	-	-	-
	tri13	12	0	-	-	-	-
	mono14	0	0	-	-	-	-
	mono31	0	0	-	-	-	-
	tri31	20	15	-	-	-	-
23	mono11	0	0	-	-	-	-
	tri11	0	0	-	-	-	-
	mono13	0	0	-	-	-	-
	tri13	0	0	-	-	-	-
	mono14	0	0	-	-	-	-
	mono31	0	0	-	-	-	-
	tri31	0	0	-	-	-	-
24	mono11	0	0	-	-	-	-
	tri11	0	0	-	-	-	-
	mono13	0	0	-	-	-	-
	tri13	0	0	-	-	-	-
	mono14	0	0	-	-	-	-
	mono31	0	0	-	-	-	-
	tri31	0	0	-	-	-	-
25	mono11	0	0	-	-	-	-
	tri11	3	0	-	-	-	-
	mono13	0	0	-	-	-	-
	tri13	186	79	-	-	-	-
	mono14	6	0	-	-	-	-
	mono31	0	0	-	-	-	-
	tri31	0	0	-	-	-	-

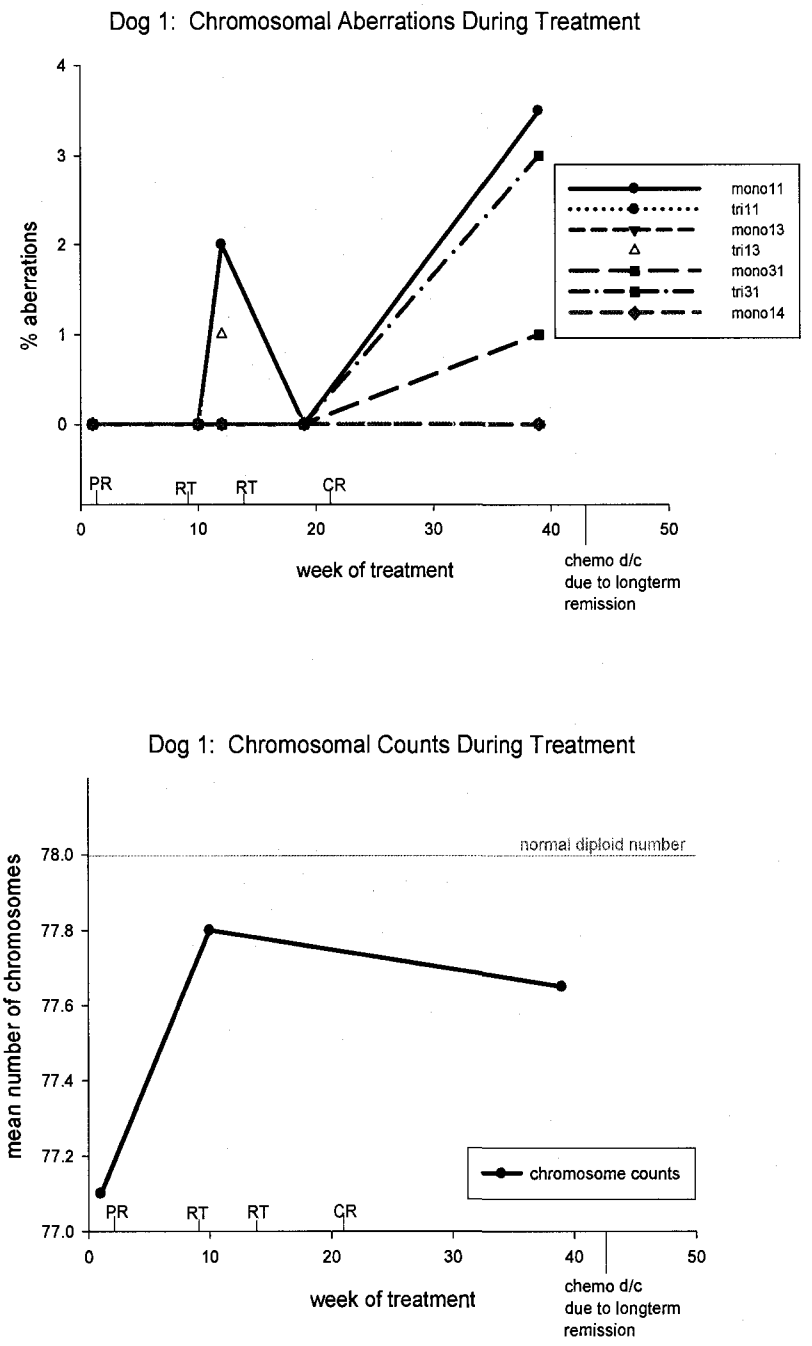


Figure 3.15: Comparison of percentage of numerical aberrations and mean chromosome counts during treatment for Dog 1. Mono=monosomy, tri=trisomy, CR=clinical remission, PR=partial remission, RT=radiation therapy.

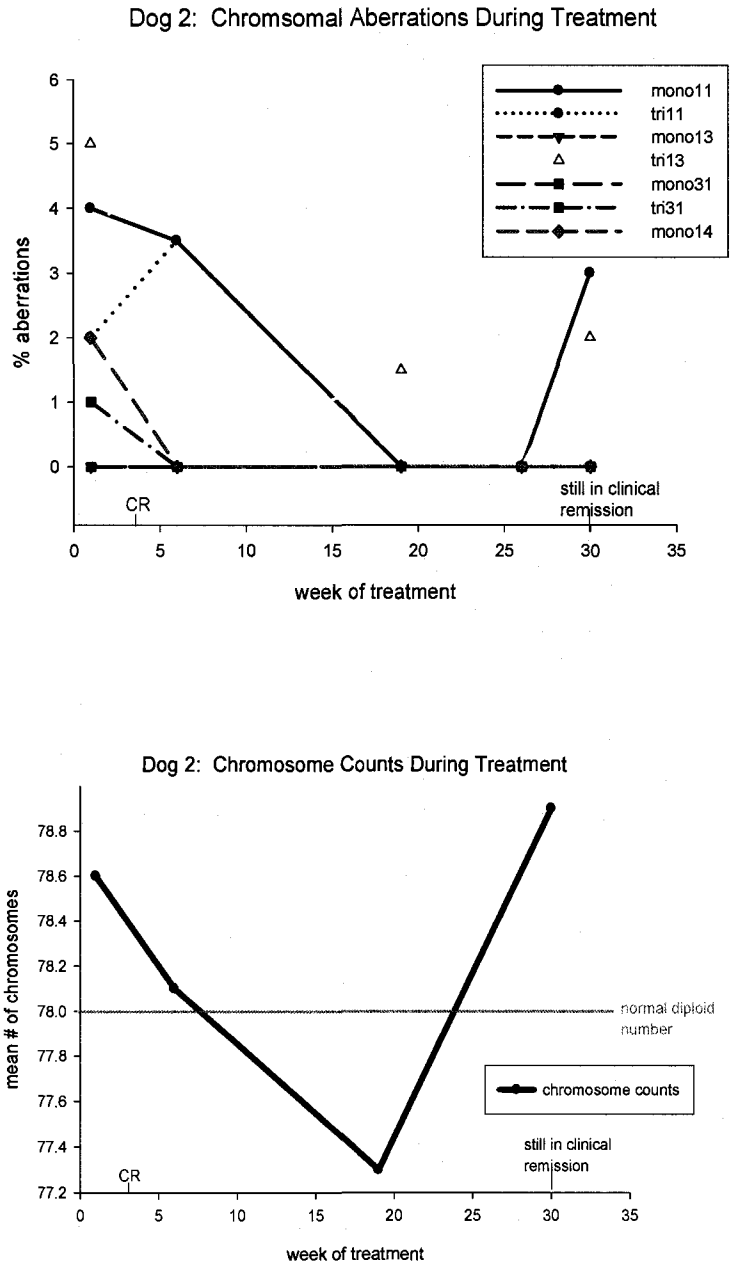
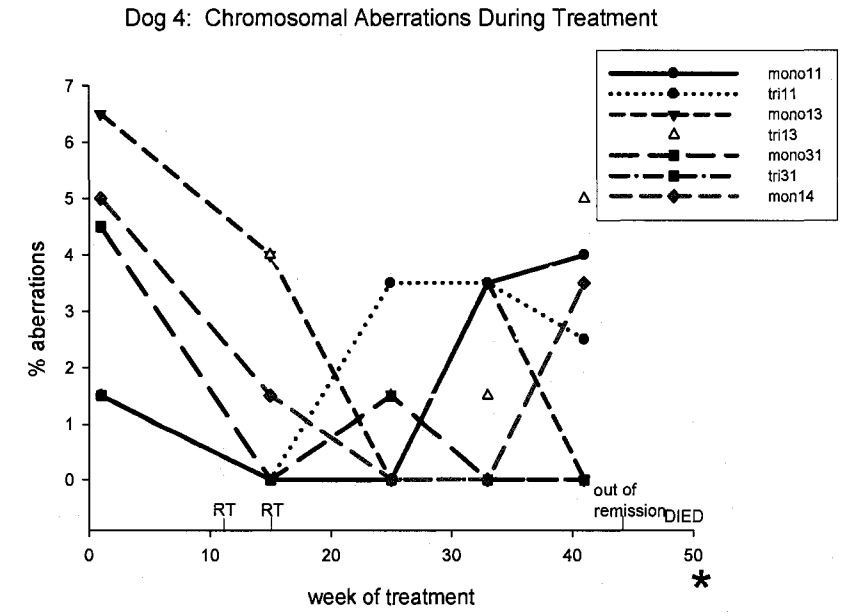


Figure 3.16: Comparison of percentage of numerical aberrations and mean chromosome counts during treatment for Dog 2. Mono=monosomy, tri=trisomy, CR=clinical remission, PR=partial remission, RT=radiation therapy.



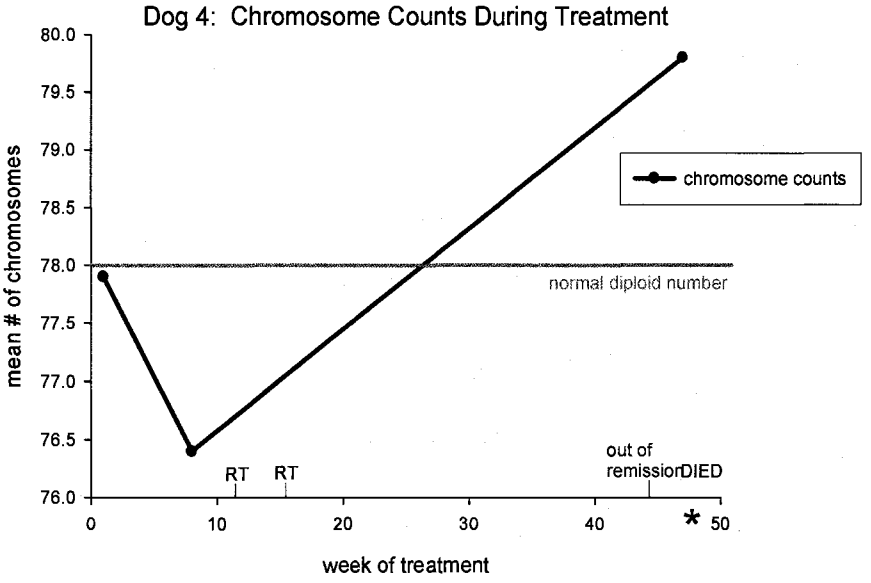
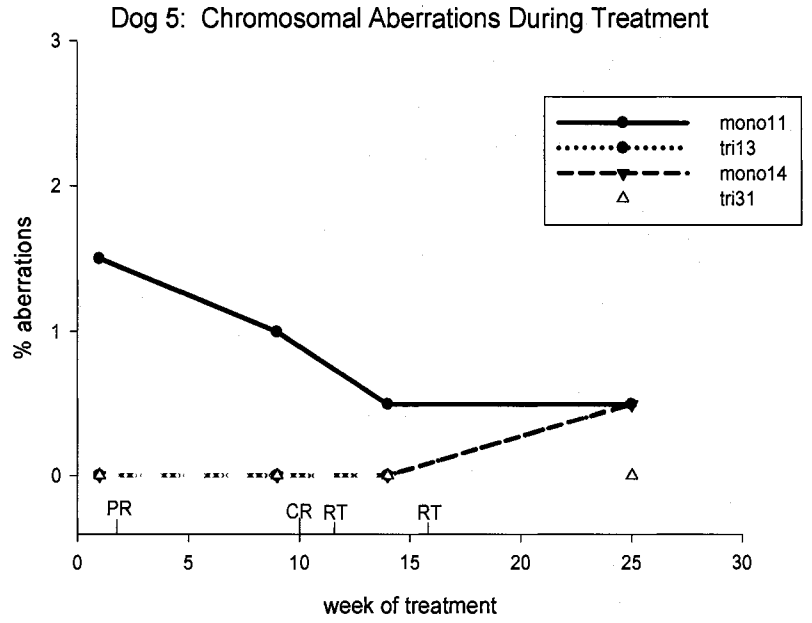


Figure 3.17: Comparison of percentage of numerical aberrations and mean chromosome counts during treatment for Dog 4. Mono=monosomy, tri=trisomy, CR=clinical remission, PR=partial remission, RT=radiation therapy.



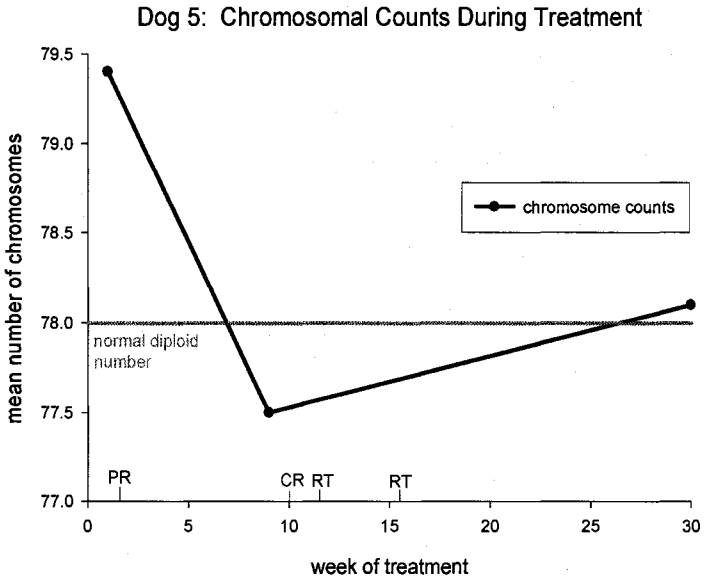
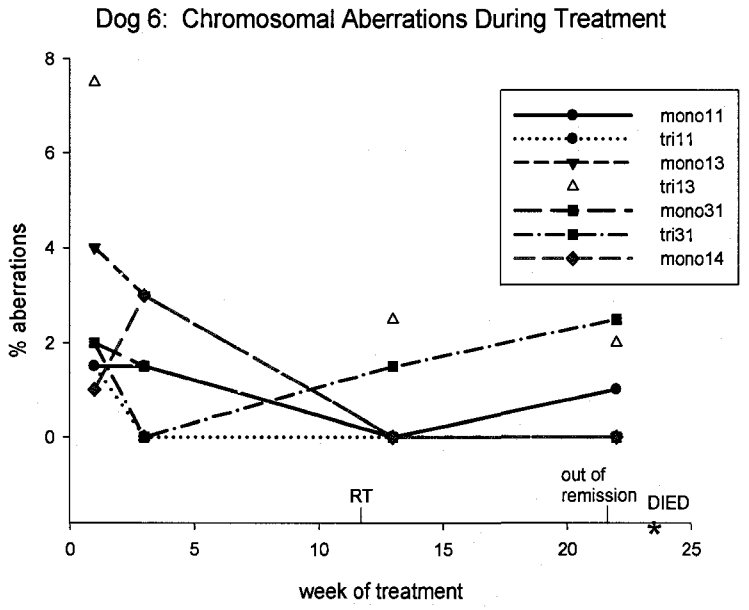


Figure 3.18: Comparison of percentage of numerical aberrations and mean chromosome counts during treatment for Dog 5. Mono=monosomy, tri=trisomy, CR=clinical remission, PR=partial remission, RT=radiation therapy.



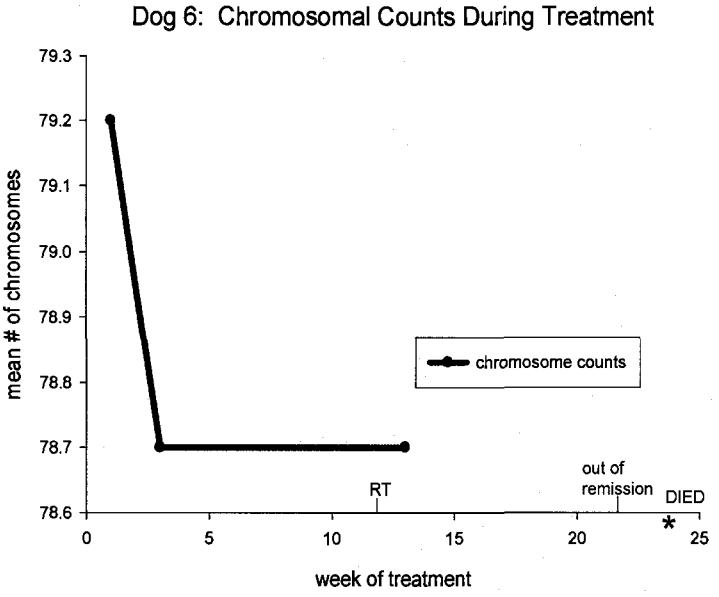


Figure 3.19: Comparison of percentage of numerical aberrations and mean chromosome counts during treatment for Dog 6. Mono=monosomy, tri=trisomy, CR=clinical remission, PR=partial remission, RT=radiation therapy.

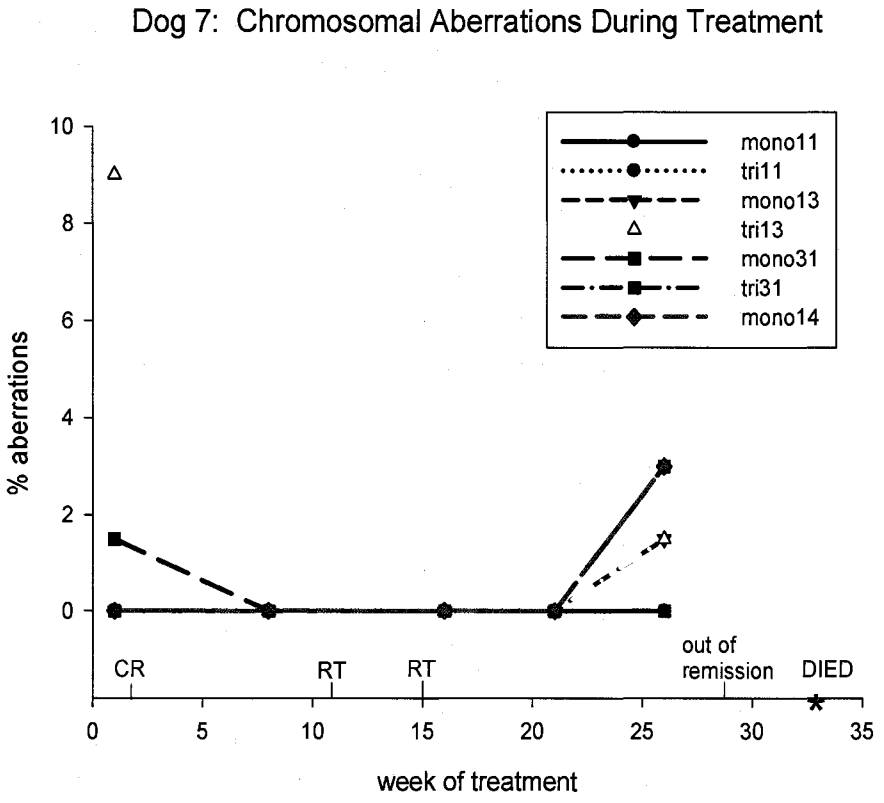


Figure 3.20: Percentage of numerical aberrations during treatment for Dog 7. Mono=monosomy, tri=trisomy, CR=clinical remission, PR=partial remission, RT=radiation therapy.

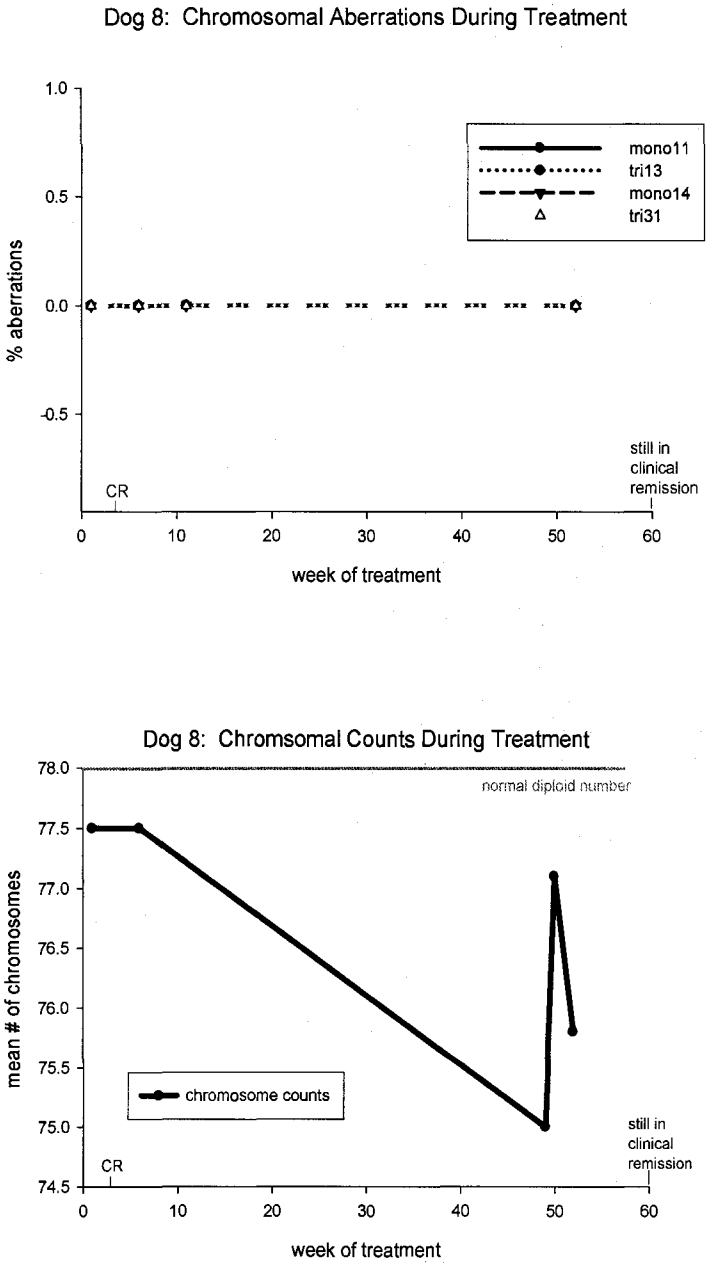
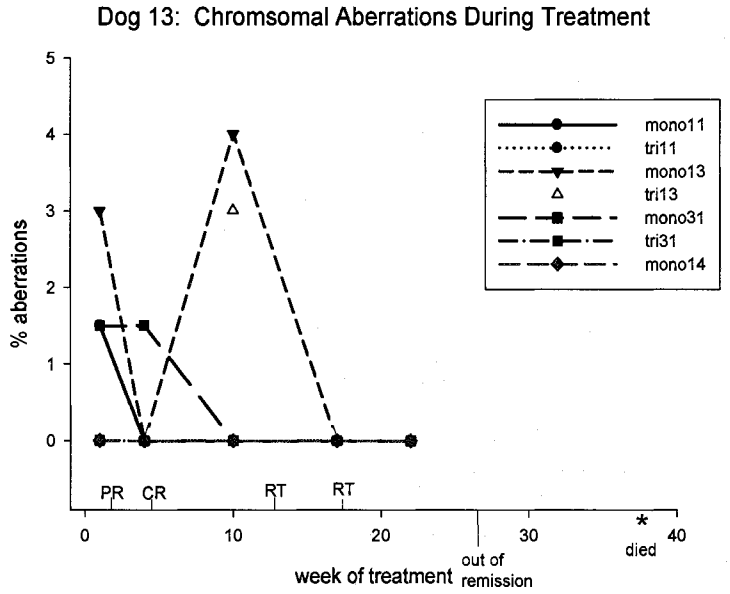


Figure 3.21: Comparison of percentage of numerical aberrations and mean chromosome counts during treatment for Dog 8. Mono=monosomy, tri=trisomy, CR=clinical remission, PR=partial remission, RT=radiation therapy.



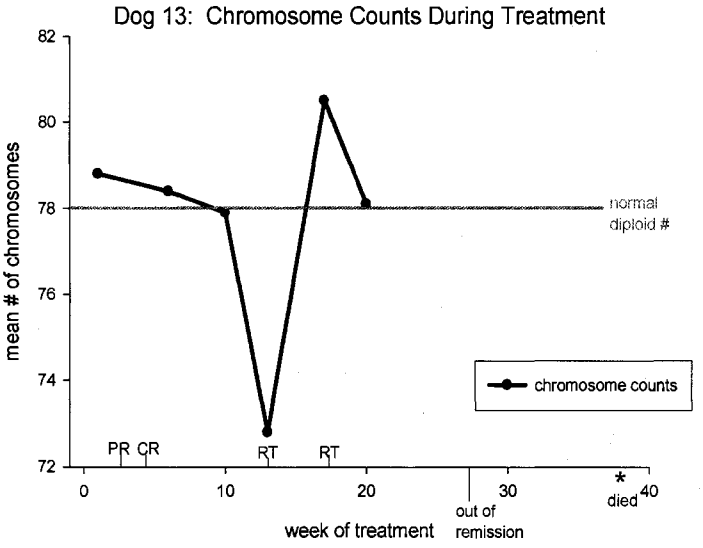


Figure 3.22: Comparison of percentage of numerical aberrations and mean chromosome counts during treatment for Dog 13. Mono=monosomy, tri=trisomy, CR=clinical remission, PR=partial remission, RT=radiation therapy.

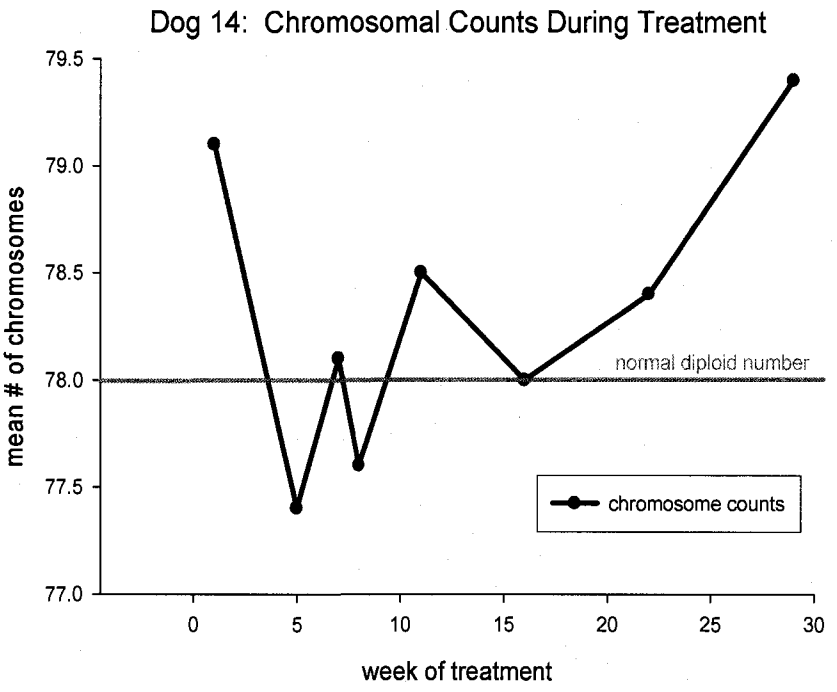


Figure 3.23: Mean chromosome counts during treatment for Dog 14.

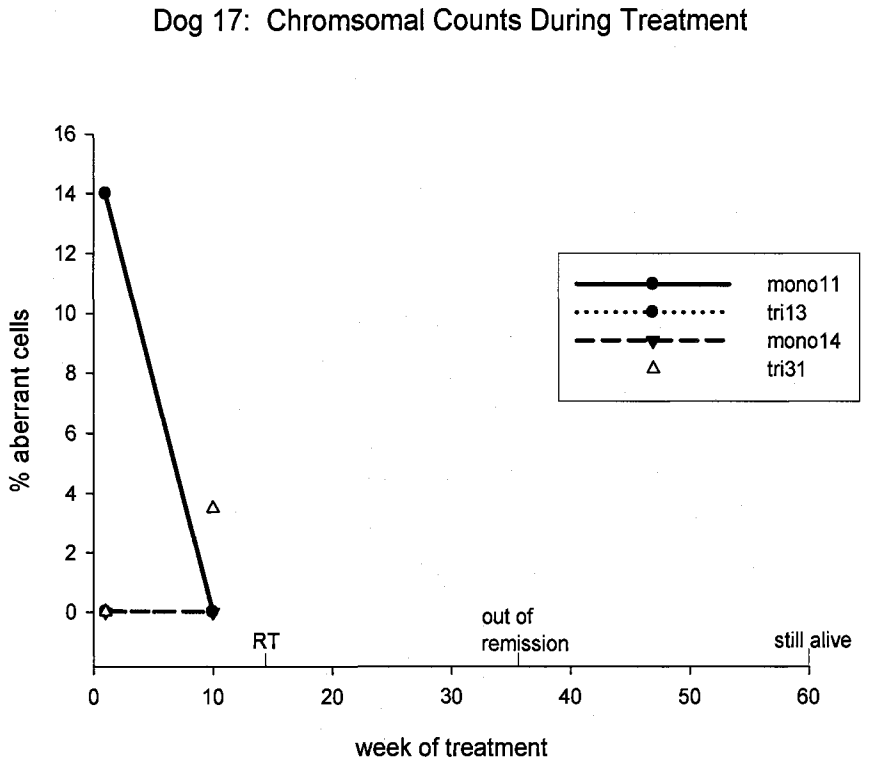


Figure 3.24: Percentage of numerical aberrations during treatment for Dog 17. Mono=monosomy, tri=trisomy, CR=clinical remission, PR=partial remission, RT=radiation therapy.

DISCUSSION

Chromosome counts

A study on human Hodgkin's Lymphoma (HL) followed chromosomal aberrations through the course of the patient's treatment using whole chromosome paints on peripheral blood samples. Since canine chromosome probes are not commercially available, chromosome counts were first performed on dogs with lymphoma to determine whether numerical aberrations could be detected in the peripheral blood. A total of 14 lymphoma patients had chromosome counts conducted at various time points. In addition, ten normal dogs and five dogs with solid tumors had chromosome counts performed at various time points to serve as controls. The aim was to count 20 metaphase cells from each sample. However, some samples fell short of this target due to a low mitotic index or metaphase cells that had overlapping chromosomes making it impossible to definitively quantify the number of chromosomes.

The control dogs each had chromosomes counted from at least one time point and most of them had counts performed at sequential time points at least one month apart. The average chromosome counts for the normal control dogs varied from 77.56 to 78 and the ranges were 75 to 78 meaning that an occasional cell had fewer than the normal diploid number of 78. None of the cells from the normal control dogs were hyperploid. For the solid tumor control dogs, the average chromosome counts varied from 77.67 to 78 and the ranges were 75 to 79. All of the abnormal counts in the solid tumor dogs, as from one cell with 79 chromosomes, were hypoploid. However, it is possible that a chromosome could be lost during slide preparation, which could account for some of the abnormal counts whereas the gain of a chromosome is more likely due to a genetic

mutation. Despite the slight variation in chromosome numbers, the modal numbers for all control dogs was 78, demonstrating that dogs without cancer and those with non-hematopoietic tumors contain the correct diploid number of chromosomes that did not vary over time.

In contrast to the control dogs, the dogs with lymphoma had a much wider range in chromosome count variation. The presence of tumor cells in the peripheral blood is likely due to tumor extravasation which is well documented in a variety of human tumors(7). The extravasated lymphoma cells would be mixed in with the normal lymphocyte count so a high fraction of lymphoma cells would not be expected. The percent of abnormal counted cells in the lymphoma patients varied from 10 to 100% although the abnormal counts were generally closer to 50%. The chromosomal modal number ranged from 76 to 79 chromosomes. In a study of 61 dogs with lymphoma, Hahn et al. found a modal chromosomal range of 71 to 85, however his counts were performed on the tumor, not peripheral blood(6).

In this study, routine bloodwork revealed that one of the dogs with lymphoma was leukemic at one timepoint (week 22 of treatment). The normal range for lymphocytes is 1.0 to 4.8 x 10^3 lymphocytes/μl whereas this dog was measured at 10.1 x 10^3 lymphocytes/ μl. The pathologist's record confirmed the presence of numerous blasts with prominent nucleolar irregularity. However this diagnosis did not appear to affect the chromosome counts. The percentage of abnormal cells was 50%, which was the same percentage as week 13 when the dog was not leukemic. Additionally, the modal number of chromosomes and the range of chromosome numbers were identical in weeks 13 and 22 (Table 3.3).

One noticeable trend was the greater disparity between chromosome counts during the course of chemotherapy and/or radiation therapy. The majority of the dogs were on the CHOP chemotherapy regimen which lasts approximately 19 weeks. Cyclophosphamide is a well-known DNA-damaging agent. In human oncologic studies, it has been shown to cause DNA breaks, sister chromatid exchanges, and anueploidy(8;9). Vincristine has been determined to cause aneuploidy, as well as other anomalies such as chromatid breaks and acentric fragments in human lymphocytes(10;11). Research in human cells has demonstrated that Adriamycin causes a wide variety of aberrations including inter- and intra-chromatid and inter- and intra-chromosomal aberrations(12;13). M'Kacher's study which followed human HL patients during their treatment found that the frequency of chromosomal aberrations were significantly increased with both chemotherapy and radiation therapy. His study determined differences between various chemotherapy protocols, however all drug regimens produced statistically significant increases in aberrations. An even greater increase in aberrations was seen following radiation therapy with the size of the radiation field having a considerable effect(2). Ionizing radiation is a well-documented inducer of chromosome aberrations. Lymphocytes and rapidly dividing cells such as tumoral cells are particularly sensitive to the damaging effects of radiation(14). The increase in chromosomal aberrations noted during the course of lymphoma treatment for the dogs is likely induced primarily by the treatment modalities, although some may be due to disease progression.

Upon the completion of chemotherapy and radiation therapy, the frequency of numerical aberrations decreased. This is consistent with M'Kacher's findings with the

aberrations in human Hodgkin's lymphoma patients. The HL patients experienced the greatest decline in the frequency of aberrations in the first six months post-treatment although they continued to decline at subsequent time points(2).

FISH data

The dogs in the control populations were also analyzed cytogenetically with the four single locus probes. Ten normal dogs and five dogs with solid tumors had at least one time point evaluated, with the majority of dogs having two or more time points scored. An occasional single monosomy was detected at some of the time points. Three of the five monosomies were identified in metaphase cells which could have missing chromosomes lost during the slide preparation technique. Another explanation could be a problem in hybridization or merely a random aberrant cell. Since 200 cells were analyzed and only one aberration was found at any one time in the control dog samples, the percentage is lower than the 1% cutoff chosen for statistical study.

The numerical aberrations were followed during the course of treatment for a select group of the dogs with lymphoma. Two dogs had two time points analyzed, two dogs had three time points, four dogs had four time points, and five dogs had five time points. All of the time points were at least one month apart. A general pattern emerged with a greater percentage of aberrations at the first time point and then diminishing during the course of treatment. Often the percentage of aberrations would increase dramatically before the time of disease recurrence, similar to the results of the chromosome counts. During weeks 1 through 20, the dogs underwent chemotherapy and radiation therapy. This time period did not appear to affect the frequency of numerical

aberrations. This differed from the chromosomal counts that appeared heavily influenced by the treatment modalities. However, this may only reflect the four chromosomes that were examined and conceivably other numerical, or even structural, aberrations could be present. The percentage of numerical aberrations and average chromosomal counts were graphed above one another in order to compare them at identical time points. In some regards, this is ineffective in that equal numbers of monosomies and trisomies would negate each other in the counts and the cell would appear normal despite having anomalies. For example, Dog 8 did not have any numerical aberrations in CFAs 11, 13, 14, or 31; however, his chromosome counts reflected hypoploid counts at all five time points, signifying that there were numerical abnormalities occurring that were not evident in this study (Figure 3.21).

This is the first study to evaluate the correspondence of chromosomal aberrations in the tumor and peripheral blood in canine lymphoma. The positive results garnered indicate promise for use as a diagnostic tool. Further research needs to be conducted to determine the prognostic significance of the additional aberrations found in canine lymphoma. It is highly likely that an abundance of lymphoma subsets exist in canine lymphoma, just as in human NHL. Once that is established, a peripheral blood sample from a dog diagnosed with lymphoma could be cytogenetically analyzed to better aid the clinician and owner toward an appropriate treatment protocol that is ideal for the particular subset of lymphoma. This method utilizing peripheral blood is superior to relying on tumor tissues given that blood samples could be transported from a veterinary clinic to a laboratory with cytogenetic capabilities, unlike tumor samples. Additional research needs to be undertaken to explore the role of chromosomal changes during the

course of treatment which could be performed using peripheral blood. This could lead to a predictive assay to evaluate response to treatment and possibly predict the time of recurrence.

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CHAPTER IV

GENERAL DISCUSSION

The first step in this research endeavor was to use chromosome counting techniques to ensure that aneuploid cells could be detected in the peripheral blood of canine lymphoma patients. Not only were abnormal counts found at the time of diagnosis, but the modal number of chromosomes often changed dramatically during the course of treatment. Once the presence of aneuploid cells was established, a more thorough investigation of the specific chromosomes was undertaken using single locus probes for canine chromosomes 11, 13, 14, and 31. The most important objective was to confirm that the aberrations identified in the peripheral blood corresponded to those found in the tumor. The results overwhelmingly indicated a direct association between the aberrations in the tumor and blood.

The numerical aberrations were followed over time using both chromosome counting and FISH techniques. The two methods had different approaches toward analyzing ploidy. The chromosome counting method was able to account for all of the chromosomes in a mitotic cell. In cells where the count number did not equal the normal diploid number of 78, it was evident that a numerical aberration was present. However, even in cells with the correct diploid number, there could be numerical chromosomal aberrations. For example, if there is a trisomy of one chromosome and a monosomy of a different chromosome, the two aberrations would cancel out when counted and the cell

would appear numerically normal. The FISH technique is able to look at the specific chromosomes that are involved in the numerical aberrations but due to the time constraints in developing canine single locus probes only four chromosomes were analyzed. In looking at the data of dogs with both chromosome counting and FISH analyses, there were instances when the four chromosome probes did not detect any aberrations during the entire treatment period yet the chromosome counts fluctuated. This could indicate that chromosomes other than CFAs 11, 13, 14, or 31 were implicated in those specific cases. Both Hahn and Thomas have reported numerous aberrant chromosomes in canine lymphoma in addition to the four analyzed in this project(1;2). Another possibility is that the chromosomes that are commonly aberrant in canine lymphoma were resolved by a successfully targeted chemotherapy yet other chromosomes were adversely affected due to the DNA damaging affects of the treatment.

This research provides a new and novel method of following disease through the cytogenetic changes evidenced in the peripheral blood. The data suggests that the specific aberrant chromosomes change during the course of treatment, which could be due to disease progression or response to chemotherapy and/or radiation therapy. A trend developed in the FISH data over time suggesting that aberrations tend to resolve during the course of treatment and then increase prior to the resurgence of clinical signs. This is in agreement with findings in human Hodgkin's lymphoma(3). Future research is needed to determine a prognostic value for specific aberrations but the potential exists for early intervention during the course of treatment. The current protocol is to wait until clinical signs are evident before implementing a rescue chemotherapy regimen. If the end of

remission could be detected prior to the onset of clinical signs, perhaps a rescue protocol could be implicated earlier and give rise to longer survival times.

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APPENDICES

LIST OF ABBREVIATIONS

°C	degrees Celsius
µl	microliter
ACUC	Animal Care and Use Committee
AF	antifade
AgNOR	argyrophilic nucleolar organizer region
CD	cluster of differentiation
CFA	canis familiaris (canine chromosome)
CHOP	Cyclophosphamide, Adriamycin, Vincristine, Prednisone (chemotherapy drug protocol)
cm	centimeter
CO_2	carbon dioxide
COT	product of DNA concentration (Co) and time of incubation (T)
CR	complete remission
CSU-VMC	Colorado State University Veterinary Medical Center
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dTTP	deoxythymidine-5'-triphosphate
dUTP	2'-deoxyuridine-5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
FISH	fluorescence *in situ* hybridization
G2	Gap 2 (cell cycle)
Gb	gigabase
H_2O	water
HL	Hodgkin's Lymphoma
HAS	homo sapiens (human chromosome)
Kb	kilobase
KCl	potassium chloride
L	liter
LB	Luria-Bertani
Mb	megabase
mg	milligrams
ml	milliliter
mM	millimolar
mono	monosomy
ng	nanogram
NCI	National Cancer Institute
NHL	Non-Hodgkin's Lymphoma
PCR	polymerase chain reaction
PR	partial remission
PSA	prostate specific antigen

rpm	revolutions per minute
RT	radiation therapy
STS	sequence-tagged site
TE	tris-EDTA
tri	trisomy
U	unit
WHO	World Health Organization

RPMI Supplemented Media

for culture of blood and tumor samples

Makes 1 Liter

RPMI 1640 supplemented with L-glutamine & hepes.....795 ml

Fetal calf serum.....200 ml

Penicillin/streptomycin (50X).....5 ml

1. using sterile technique in a tissue culture hood, add fetal calf serum and pen/strep to the medium
2. filter sterilize through 0.2 μm Nalgene filter unit
3. refrigerate at 4°C

Heat Inactivation of Fetal Bovine Serum

Makes 0.5 Liter

1. pre-heat a water bath to 60°C

2. thaw bottle of fetal bovine serum at room temp for several hours or overnight at 4°C

3. swirl the thawed serum to mix and place in the 60°C water bath for 40-45 minutes

4. filter sterilize with a 500 ml Nalgene 0.22 um filter

5. using sterile technique, aliquot serum into sterile 50 ml conical tubes labeled with the lot number and date

6. store aliquots at –20°C

Peripheral Blood Culture

Day 1:

1. using sterile technique, combine 10 ml $RPMI^{+}$ media, 1 ml canine blood collected in a sodium heparin tube, and 10 µg/ml pokeweed mitogen in a TOT75 flask
2. incubate at 37°C for 3 days

Day 4:

1. add 100 µl colcemid (100 ng/ml) to flask, incubate at 37°C for 60 minutes
2. transfer flask contents to 15 ml conical tube
3. centrifuge for 8 minutes at 1000 RPM, aspirate supernatant
4. resuspend the pellet gently, add 10 ml of 37°C 75mM KCl
5. incubate at 37°C for 30 minutes
6. add 500 µl of fresh 3:1 methanol:acetic acid fixative to tube, gently invert to mix
7. centrifuge for 8 minutes, aspirate supernatant
8. resuspend pellet gently, add 8 ml fixative, invert tube gently to mix
9. centrifuge for 8 minutes, discard supernatant
10. repeat steps 10-12 three more times

Lymph Node Culture

Day 1:

1. collect lymph node sample in a culture dish with 4 ml 37°C RPMI medium

2. gently agitate node to free cells with sterile surgical blade

3. set up culture with a final cell concentration of 0.75 X 10^6 cells/ml in 10 ml of media in a TOT75 flask

4. incubate at 37°C for 15 hrs

Day 2:

11. add 100µl colcemid (100 ng/ml) to flask, incubate for 60 minutes at 37°C

12. transfer flask contents to 15 ml conical tube

13. centrifuge for 8 minutes at 1000 RPM

14. aspirate supernatant

15. resuspend the pellet gently, add 10ml of 37°C 75mM KCl

16. incubate at 37°C for 30 minutes

17. add 500 µl of fresh 3:1 methanol:acetic acid fixative to tube, gently invert to mix

18. centrifuge for 8 minutes, aspirate supernatant

19. resuspend pellet gently, add 8 ml methanol:acetic acid fixative

20. invert tube gently to mix

21. centrifuge for 8 minutes, discard supernatant

22. repeat steps 10-12 three more times

Preparing BAC DNA for extraction

LB broth recipe

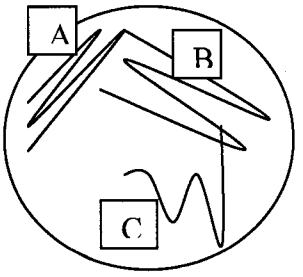
1. dissolve 10g tryptone, 5g yeast extract, and 10g NaCl in 800 ml distilled water
2. adjust the pH to 7.0 with 1 N NaOH
3. adjust the volume to 1 liter with distilled water, sterilize by autoclaving

Growing of BAC Clones:

1. add 2.5 ml bacterial medium + 30 μg/ml chloramphenical in a 15 ml conical tube
2. sterilize inoculating loop under a flame, cool loop on side of BAC tube, dip loop into BAC tube, swish loop in the conical tube containing the medium and broth
3. put conical tube at an angle on shaker set on 225 RPM overnight at 37°C

Making single colony preps:

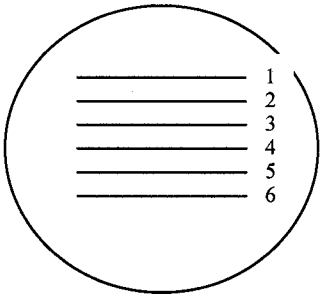
1. make LB agar plate with 20 ml agar + 20 μg/ml chloramphenicol
2. sterilize inoculating loop under flame, dip loop into liquid culture and make isoloation colony lines on the plate as follows:



Part C will have isolated colonies

3. place the plate upside down at 37°C, without CO_2 or water, overnight to grow
4. choose an individual colony for isolation of BAC using a sterile toothpick

5. using a single colony, plate onto another agar plate as follows:



6. allow colonies to grow overnight in a 37°C incubator
7. for each line, put 15 µl of PCR water and one colony in a PCR tube
8. vortex for five minutes
9. run PCR and gel
10. take ¼ of the chosen line and drop into 5 ml of LB broth
11. allow colony to grow overnight on a shaker at 225 RPM at 37°C
12. centrifuge the culture at 2.500 RPM for 5 minutes
13. resuspend bacteria in 1 ml of a 9:1 LB broth:glycerol solution and store at -20°C
14. grow 3 µl of bacterial solution in 250 ml LB broth, then extract BAC DNA

PCR Amplification Program for BACs

dH_2O.....17.25 µl
buffer.....2.5 µl
$ClMg_2$.....0.75 µl
dNTPs.....2.0 µl
primers.....1.0 µl
taq polymerase.....0.25 µl
bacteria.....1.25 µl
25 µl

step #	temperature (°C)	time
1	94	3 minutes
2	94	45 seconds
3	55	30 seconds
4	72	1 minutes 30 seconds
5	go to step 2 (30 times)	
6	72	10 minutes
7	4	30 minutes

Isolation of BAC DNA using Qiagen Plasmid Maxi kit

P1 Buffer (resuspension buffer)

50 mM Tris-Cl, pH 8.0
10 mM EDTA
100 μg/ml RNase A

1. dissolve 6.06g Tris base + 3.72g $Na_2EDTA\text{-}2H_2$) in 800 ml distilled water
2. adjust the pH to 8.0 with HCl. Adjust the volume to 1 liter with distilled water
3. add 100 mg RNase A per liter of P1

P2 Buffer (lysis buffer)

200 mM NaOH, 1% SDS (w/v)

1. dissolve 8.0g NaOH pellets in 950 ml distilled water, 50 ml 20% SDS (w/v) solution
2. final volume should be 1 liter

P3 Buffer (neutralization buffer)

3 M potassium acetate, pH 5.5

1. dissolve 294.5g potassium acetate in 500 ml distilled water
2. adjust the pH to 5.5 with glacial acetic acid
3. adjust the volume to 1 liter with distilled water

QBT Buffer (equilibration buffer)

750 mM NaCl
50 mM MOPS, pH 7.0
15% isopropanol (v/v)
0.15% Triton X-100 (v/v)

1. dissolve 43.83 NaCl, 10.46g MOPS (free acid) in 800 ml distilled water
2. adjust the pH to 7.0 with NaOH
3. add 150 ml pure isopropanol and 15 ml 10% Triton X-100 solution (v/v)
4. adjust the volume to 1 liter with distilled water

QC Buffer (wash buffer)

1 M NaCl
50 mM MOPS, pH 7.0
15% isopropanol (v/v)

1. dissolve 58.44g NaCl and 10.46g MOPS (free acid) in 800 ml distilled water
2. adjust the pH to 7.0 with NaOH
3. add 150 ml pure isopropanol
4. adjust the volume to 1 liter with distilled water

QF Buffer (elution buffer)

1.25 M NaCl
50 mM Tris-Cl, pH 8.5
15% isopropanol (v/v)

1. dissolve 73.05g NaCl and 6.06g Tris base in 800 ml distilled water
2. adjust the pH to 8.5 with HCl
3. add 150 ml pure isopropanol
4. adjust the volume to 1 liter with distilled water

Alkaline lysis:

1. combine 250 ml LB broth and 100 µl chloramphenicol (30µg/ml) in 500 ml Erlenmeyer flask

2. sterilize inoculating loop under a flame, cool loop in agar, scrape off 1/3 of a bacterial line, drop into the flask containing LB broth

3. allow bacteria to grow overnight in a shaker at 225 PRM at 37°C

4. transfer culture into a nalgene high speed centrifuge bottle

5. centrifuge sample at 6000 RPM for 20 minutes, discard the supernatant

6. resuspend the bacterial pellet in 25 ml Buffer P1

7. add 25 ml Buffer P2, mix gently and thoroughly by inverting 4-6 times, incubate at room temperature for 5 minutes

8. add 25 ml chilled Buffer P3, mix immediately and gently by inverting 4-6 times and incubate on ice for 30 minutes

9. transfer to a Sepcor widemouth flat bottom bottle, centrifuge at 11.500 RPM for 30 minutes at 4°C

10. retain supernatant containing plasmid DNA by pouring it into new Sepcor bottles
11. centrifuge at 11.500 RPM for 15 minutes at 4°C

12. retain the supernatant containing the plasmid DNA, avoid the chunks when pouring

13. precipitate the DNA by adding 0.7 volumes of room temperature isopropanol to the lysate

14. centrifuge at 10000 RPM for 30 minutes at 4°C

15. carefully decant the supernatant to retain the DNA pellet

16. redissolve the DNA pellet in 500 ml TE buffer, pH 8.0

DNA purification:

17. prewarm QF Buffer to 70°C

18. add 12 ml Buffer QBT to the 500 µl DNA in TE in a 50 ml conical tube with collar between column and tube

19. equilibrate the column tip by adding 10 ml Buffer QBT and allow it to flow via gravity

20. apply the DNA solution to the column and allow it to flow via gravity

21. wash the column tip with 15 ml Buffer QC two times

22. elute the DNA with 8 ml Buffer QF using a fresh 50 ml tube in order to retain the DNA-containing supernatant

23. precipitate the DNA by adding 0.7 volumes of room temperature isopropanol to the elute DNA

24. mix, transfer to round bottom centrifuge tubes and centrifuge at 15.00 x g for 30 minutes at 4°C

25. carefully decant the supernatant and wash DNA pellet with 1.5 ml room temperature 70% ethanol, transfer to a microcentrifuge tube

26. centrifuge at 15.000 x g for 15 minutes at 4°C

27. air dry the DNA pellet for 10 minutes, redissolve the DNA in 200 µl TE buffer, pH 8.0

Probe Labeling

Roche Nick Translation Kit

reagent	volume (µl)
dH2O	5.0
Buffer (10X)	2.0
dNTPs (1A;1G:1C)	6.0
dTTP	1.3
labeled d-UTP	0.75
BAC DNA	3.0
DNase/polymerase	2.0

PCR: 15°C for 3 hours, 65 °C for 10 minutes

Fluorescence *in situ* Hybridization

Day 1:

1. in an eppendorf tube, combine:

hybridization mix	20 µl
canine COT-1 DNA	4 µl
CFA11 probe (spectrum red)	2.5 µl
CFA13 probe (spectrum red)	1.25 µl
CFA14 probe (FITC)	3 µl
CFA31 probe (FITC)	3 µl
sterile water	up to total volume of 30 µl

2. apply 30 µl of probe mixture to slide
3. cover with 22 X 40 coverslip
4. rubbercement edges to seal
5. codentaure the probe and slide using PCR machine
6. incubate overnight in a humid chamber at 37°C

Day 2:

1. pre-warm coplin wash jars to 45.5°C
2. wash slide in jars 1 through 6 for 2.5 minutes each
3. apply 60 µl DAPI/AF to slide
4. cover with 22 X 55 mm coverslip
5. visualize with fluorescent microscope

Coplin jar wash recipes:

jars 1 and 2.....50% formamide in 2XSSC pH 5.8
jars 3 and 4.....2XSSC pH 7.4
jars 5 and 6.....2XSSC pH 7.4 + 0.1% igepal CA-630

Hybridization Mix

for fluorescence *in situ* hybridization

makes 10 ml

formamide.....6 ml
dextran sulfate solution*.....2.5 ml
blocking reagent.....0.5 ml
water.....1 ml

aliquot into eppendorf tubes; store at -20°C

*dextran sulfate solution:

80% dextran sulfate
8XSSC
4X phospate buffer
4X blocking reagent

Canine COT-1 DNA

preparation from whole blood

Reagents:

Ammonium chloride (NH4Cl)
Chloroform
Ethanol, absolute
HCl
Isoamyl alcohol
Isopropanol (2-propanol)
Phenol
Potassium carbonate (KHCO3)
Proteinase K
Sodium acetate
Sodium chloride
Sodium EDTA (Na2EDTA)
Sodium dodecyl sulfate (SDS 10%)
Tris EDTA buffer, pH 8.0

Preparation:

Lysis buffer-

NH4Cl.....8.29g f.c. [155mM]
KHCO3.....1g f.c. [10mM]
Na2EDTA.....0.034g or 200µl EDTA 0.5M f.c. [0.1mM]

1. Fill to 1000ml with distilled water

2. Adjust to pH 7.4 with 1M HCl or NaOH for each use

SE buffer-

NaCl.....4.39g f.c [75mM]
Na2EDTA.....8.41g or 50ml EDTA 0.5M f.c. [25mM]

1. Fill to 1000ml with distilled water

2. Adjust to ph 8.0 with 1M NaOH for each use

Sodium acetate-

3M sodium acetate.....246g/L

1. Adjust to pH 5.2 with CH3COOH

Procedure:

1. collect 10 ml canine blood in sodium heparinized tube
2. add 30 ml lysis buffer to 10 ml blood, shake gently, incubate for 30 min on ice
3. centrifuge at 1200 rpm for 10 min at 4°C
4. remove supernatant (blood waste), add 10ml lysis buffer, resuspend the pellet
5. centrifuge at 1200 rpm for 10 min at 4°C
6. remove supernatant, add 5ml SE buffer, resuspend the pellet
7. centrifuge at 1200 rpm for 10 min at 4°C
8. remove supernatant, add 5ml SE buffer, resuspend the pellet, add 40µl proteinase K (10mg/ml), and 250µl 20% SDS, shake gently
9. incubate overnight in a 37°C water bath
10. add 5ml SE buffer and 10ml phenol, shake by hand for 10 min
11. centrifuge at 3000 rpm for 5 min at 10°C
12. transfer the supernatant into a new tube (note: retain the supernatant), add 10ml phenol/chloroform/isoamyl alcohol (25:24:1), shake by hand for 10 min
13. centrifuge at 3000 rpm for 5 min at 10°C
14. transfer supernatant to a new tube (note: retain the supernatant), add 10ml chloroform/isoamylalcohol (24:1), shake by hand for 10 min
15. centrifuge at 3000 rpm for 5 min at 10°C

16. transfer the supernatant into a new tube (note: retain supernatant), add 300µl 3M sodium acetate (pH 5.2) and 10ml isopropanol, shake gently until DNA is precipitated, use a glass pipette to capture DNA

17. wash DNA in 70% ethanol and dissolve the DNA in 0.5-1.0ml TE buffer overnight at 4°C

18. sonicate for a total of 2.5 min in 30 sec bursts at 50% duty cycle, 5 output control

19. precipitate DNA: split sample into tubes of 0.5 ml each. Add 50 µl sodium acetate (3M) and 1.65ml 100% ETOH, put in -80 freezer overnight

20. centrifuge tubes at maximum speed in microcentrifuge for 30 min

21. discard supernatant, put tubes upside down in warm room for 15min

22. resuspend DNA in each tube with 100µl PCR water

23. measure concentration of DNA

24. store COT-1 DNA in freezer

List of reagents and suppliers

item	quantity	supplier	catalog #
cell culture			
RPMI medium with 20mM HEPES and L-glutamine	500 ml bottle	Sigma-Aldrich	R7388
Penicillin-streptomycin	100 ml bottle	Invitrogen	15140-122
Demecolcine solution 10 μg/ml	10 ml bottle	Sigma-Aldrich	D1925
Pokeweed mitogen	5 mg powder	Sigma-Aldrich	L8777
Fetal bovine serum	500 ml bottle	Sigma-Aldrich	F4135
Culture flasks, 25 cm^2, canted neck	100 flasks	Corning	CLS430372
Culture dish	20 dishes	Sigma-Aldrich	Z358762
Sodium heparin blood collection tubes	100 tubes	Kendall	321254
Jamshidi needle	10	Kendall	8881247111
cell fixation			
Acetic acid, ACS reagent	500 ml bottle	Sigma-Aldrich	320099
Methanol	4 L bottle	Sigma-Aldrich	179337
Microscope slides	72 slides	Corning	CLS29483X1
15 ml conical tubes	800 tubes	Sigma-Aldrich	Z707724
DAPI	10 mg vial	Roche	10236276001
ProLong Gold anti-fade reagent	10 ml bottle	Invitrogen	P36930
FISH			
Formamide, ACS certified	500 ml bottle	Fisher	F84-1
Igepal CA-630	50 ml	Sigma-Aldrich	18896
Blocking reagent	50 g	Roche	11096176001
Chloramphenicol	200 mg vial	Sigma-Aldrich	C6455
Plasmid Maxi Kit	25 reactions	Qiagen	12163
Nick Translation Kit	50 reactions	Roche	10976776001
BACs			
CFA 11	255bp	CHORI	RP81-376H9
CFA 13	199bp	CHORI	RP81-265L22
CFA 14	197bp	CHORI	RP81-376O15
CFA 31	125bp	CHORI	RP81-376G11

CHROMOSOMAL ABERRATIONS IN CANINE LYMPHOMA

Client information sheet

We appreciate your interest in our clinical studies investigating chromosomal changes in canine lymphoma patients receiving a multi-drug chemotherapy protocol. Your clinician will review the disease characteristics as well as explain the treatment options. This handout will provide information about the goals of this study as well as patient responsibilities.

Presently, our canine patients that receive chemotherapy for lymphoma are monitored for response status based on palpation of the size of their peripheral lymph nodes. Recent studies in human lymphoma cases have shown that response status may be predicted based on changes in the chromosomes within the blood and lymph node. The goal of this study is to evaluate changes in the chromosomes in the lymph node and blood before and during treatment for lymphoma. We will correlate chromosomal abnormalities with treatment response. These results will provide oncology clinicians with valuable information regarding your pet's response to treatment using a relatively noninvasive method. In the future, this may lead to earlier detection of treatment failure. As a result, treatment protocols could be adjusted such that a better outcome is achieved.

Patients- Who is eligible?

This study is limited to canine patients who have been diagnosed with lymphoma and have not received any chemotherapy treatment for their disease. Additionally, the owners have elected to treat their pet with a CHOP-based multi-drug protocol with half body radiation treatment "sandwiched" into the protocol.

The study- What is involved?

Your pet will undergo several diagnostic procedures which are included in the staging of clinical patients. This will include a physical examination, blood work, bone marrow aspirate and lymph node biopsy. (Please understand that all patients are sedated and provided with pain medication prior to undergoing a bone marrow aspirate and lymph node biopsy.) Several of these samples will be evaluated for chromosome changes. Patients will receive chemotherapy according to the schedule for the CHOP based multi drug protocol. At monthly intervals, blood samples will be evaluated for chromosomal abnormalities. Additionally, a blood sample at the time of recurrence will be drawn.

Financial incentive-What will CSU provide?
Once eligibility for the study has been determined, the study will pay for the bone marrow aspirate, lymph node biopsy, histopathologic grading, blood draws, and additionally, the radiation therapy costs will be $500 instead of the normal price of $1100, a savings of $600.

Owner commitment- What will I do?
Once your pet has been diagnosed with lymphoma, the owner is responsible for all charges with the exception of the bone marrow aspirate, lymph node biopsy, blood draws, histopathology charges, and five hundred dollars of the radiation therapy charges. Owners are responsible for making all appointments with the receptionist and keeping all appointments as required by the treatment protocol.

Any questions?
Please don't hesitate to request additional information from your oncology clinician or study protocol advisors.

Dr. Susan Plaza-Clinical trial coordinator
(970) 297-4001

Dr. Sue LaRue- Principal Investigator
Radiation Oncologist
(970) 297-0334

Jennifer Devitt-graduate student- Cell and Molecular Biology
(970) 297-4082

Krina Galvin- appointment receptionist
(970) 297-4478

OWNER CONSENT AGREEMENT

I agree to enter my dog into this study protocol. I understand that the purpose of this research is to investigate chromosomal changes in canine patents with lymphoma being treated with a multi drug protocol with radiation therapy. The goals of this study are to predict treatment failures before the appearance of clinical signs so that treatment protocols may be started sooner and lead to an improved outcome.

I have been informed of the procedures that will be performed. I understand the treatment options available to me. I have had the opportunity to ask questions about the study and understand that I may ask questions at any time. I agree to have chemotherapy treatments administered on time as recommended and return for the recheck examinations. The initial bone marrow aspirate, lymph node biopsy, and histopathology charges will be covered by the study as well as five hundred dollars applied to the radiation therapy charges. I understand that I will be financially responsible for all additional charges.

I understand that the study records may contain personal identifying information that we agree to keep confidential. I agree to donate blood samples from my dog during the normal course of diagnostics and treatment of this disease. I understand that these samples will be used in studies of canine cancer. I understand that by signing this Consent Form, I give up all future claims to these samples and any experimental results that may be derived from their investigational use. If I have any questions or concerns, I may contact my oncology clinician or the protocol coordinators.

Signature of the owner/agent **Date**

Witness **Date**

Colorado State University

Animal Care and Use Research/Teaching Protocol Review Form
Form A-100 (Rev 8/02/04)

ACUC approval of this protocol review form is necessary prior to animals being obtained, housed or manipulated for research or teaching purposes.

This form should be used for new protocols, and for renewing protocols at the end of every third approval period. Submit one signed original and 12 copies (double-sided preferred) to the Regulatory Compliance Office, 321 General Services Building. **With your ACUC application, provide ONE copy of the complete funding proposal.** Please make sure that all required signatures are obtained on the final sheet of the form before submission.

Please answer each question. If the question does not apply to your research, respond with an "N/A."
Do not answer a question by referring to your response to another question; this form is designed to collect necessary information in a grouped format.

PART I—Basic Protocol Information

1. Investigator Information

a) Principal Investigator: Susan LaRue, DVM, PhD
(PI must be faculty member, administrative professional, or permanent research associate)
b) Department: Rad. & Env. Health Sci 4 -Digit Campus Zip Code: 1620
c) Campus Phone: 297-0334
d) E-mail: slarue@colostate.edu
e) Secondary Contact name/phone/email: Jennifer Devitt/297-4082/jdevitt@colostate.edu
f) List researchers/staff qualified to carry out this protocol: Jennifer Devitt
g) If surgery is involved, list the name of each investigator who will perform surgery and type of ACUC authorization (a, b, or c; if c, provide description):
Each investigator performing surgery must be authorized to do so by the ACUC by fulfilling one of the following requirements:
a. Completed AN550, the surgery training course
b. Obtained a DVM degree
c. Has previous experience with this surgical procedure (provide description)
Clinical Oncology Staff, all have obtained DVM degree

2. Project Information

a) Project/Course Title: Chromosomal Abnormalities in Peripheral Lymphocytes as a Prognostic Indicator of Canine Lymphoma
b) Funding Agency:
c) Funding Agency Deadline:

d) Which is this application:

☒ For a new project

☐ A major amendment to an existing project. List ACUC number: - A_ -0

☐ A competing continuation. List ACUC number: - A_ -0

☐ A renewal/*non*-competing continuation List previous ACUC number: - A_ -0

3. Animal Information

Enter one **SPECIES/STRAIN** in each box and report vertically → (if more than 4, list on separate attachment)	canine			
Sex(s):	either			
Age/weight range:	any			
NUMBER to be used in Year 1:				
NUMBER to be used in Year 2:				
NUMBER to be used in Year 3:				
TOTAL NUMBER for the lesser of 3 years or duration of project				
SOURCE of animals:	client owned animals with lymphoma			

USDA PAIN CATEGORIES: A painful procedure is defined as any procedure that would reasonably be expected to cause more than slight or momentary pain and/or distress in an animal to which that procedure is applied. Animals exhibiting signs of pain, discomfort, or distress such as decreased appetite/activity level, decreased mobility, adverse reactions to physical contact, open sores/necrotic skin lesions, abscesses, lameness, conjunctivitis, corneal edema, and photophobia are expected to receive appropriate relief unless written scientific justification is provided in the A-100 protocol and approved by the ACUC. **Indicate which level(s) apply for each species. If listing more than one, indicate how many animals at each pain level. Example: B (20 mice) and C (15 mice). If an animal is used for multiple procedures, count it in the most painful category.** **Category B:** breeding, conditioning only, or holding colony. **Category C:** No more than momentary or slight pain or distress and no use of pain-relieving drugs; or no pain or distress. Examples: euthanized for tissues; observation under normal conditions; positive rewards; routine injections (not Freund's Adjuvant); tattooing, blood sampling. **Category D:** Pain or distress appropriately relieved with anesthetics, analgesics and/or tranquilizer drugs or other methods for relieving pain or distress. Examples: Needle biopsy, non-survival or survival surgeries, terminal cardiac blood collection; exposure of blood vessels for catheter implantation; induced infections or antibody production. **Category E:** Unrelieved pain or distress. Examples: toxicological or microbial testing or infectious disease research that requires continuation until clinical symptoms are evident or death occurs; application of noxious stimuli; prolonged restraint; use of paralyzing drugs for restraint; infliction of burns or trauma.	D			

If using animals from other protocols, identify the PI and protocol number, and briefly list the procedures the animals have undergone on the protocols; identify the individual animals by identification number if applicable:

4. Project Activity Duration

a) Start Date *(date you first expect to order/obtain animals)*: Ocotober 20, 2005
(*Unless continuation,* ***start date should not be prior to ACUC review date***)
b) Stop Date *(date you expect to be finished using animals)*: October 20, 2007

5. Project Summary *(Provide a summary of the project in <200 words, using lay language suitable, for example, for release to a newspaper. Include rationale and goals of the research project)*: Lymphoma is an extremely common canine tumor that can be located in any lymph node, organ, and/or bone marrow. Untreated, survival times are around one month. Chemotherapy can initiate and temporarily maintain remission, with average remission times of one year. Chromosomal abnormalities in lymphoma are predictive of outcome. We will compare chromosomes found on the initial lymph node excision with those in blood samples. We will also use immunophenotyping to classify the tumor using a bone

marrow aspirate in addition to histology performed on the excised lymph node.This is unique because chromosomes cannot be evaluated from lymph nodes after a patient has gone into remission. Blood will be drawn from dogs throughout chemotherapy and at failure to look for trends in chromosomal abnormalities that may be indicative of treatment response. Cytogenetic analysis will be performed using classic cytogenetic techniques and fluorescent in situ hybridization (FISH). FISH uses a molecular probe to paint each chromosome a different color to facilitate identification. We hypothesize that the frequency of abnormalities will correlate with treatment response, and that abnormalities will increase prior to treatment failure. If this proves true, simple blood samples could be obtained on lymphoma patients at predetermined time points to predict recurrence before evidence of clinical signs. Treatment protocols could be initiated sooner, leading to better outcome.

6. To help ACUC streamline the review process, please answer the following about procedures involved in your protocol:

a) Deep anesthesia followed by euthanasia of animals and tissue harvest. No other procedures are conducted on animals ..... ☐
Yes ☒No

b) Deep anesthesia followed by terminal surgery for research or teaching purposes. No other procedures are conducted on animals ..... ☐
Yes ☒No

c) Only minor procedures with minimal pain or discomfort of the animals (such as blood sampling) ☒ Yes ☐No

d) Only observation of field animals only ..... ☐
Yes ☒No

e) Only clinical evaluation of animals during routine reevaluations in hospital ..... ☐ Yes ☒ No

f) Involves the use of food animals on studies designed to improve production efficiency and do not involve surgery or other invasive procedures .... ☐
Yes ☒No

g) Is this ACUC application <u>solely</u> for a breeding colony at CSU .... ☐
Yes ☒No

7.Animal care:

a) Location of housing: CSU-VTH Ward 3

b) Location of procedures: B-109

c) Will Lab Animal Resources provide the care ..... ☐
Yes ☒No

c) Attending veterinarian (LAR or specify other): Clinical Oncology Staff (William Dernell, Chief)

d) Location of medical records: with patient

8. Living animals are required for this project because:

a) Complexity of the processes studied cannot be duplicated/modeled using in vitro models.....☒ Yes ☐ No

b) Not enough information known about processes being studied to design non-living models.....☐ Yes ☒ No

c) Pre-clinical studies in living animals are necessary prior to human testing.... .....☐ Yes ☒ No

d) This study requires tissue harvested from animals prior to in vitro testing..... .....☐ Yes ☒ No

e) Currently this is the best method to accomplish the required teaching objectives .....☐ Yes ☒ No

f) Populations are being studied in natural or semi-natural environments ....☐ Yes ☒ No

g) Animal behavior is being studied .....☐ Yes ☒No

h) Other (please specify):

9. To comply with USDA Policy 12, provide documentation of a literature search to certify that 1) alternatives to each potentially painful/distressful procedure contained in this protocol have been sought, 2) the work is not duplicative of previous studies and 3) the fewest number of animals will be used to obtain valid results.

a) For automated literature searches, provide answers to <u>each</u> question below:

Date search performed: June 15, 2005

Keywords used: tumor cytogenetics, canine, human, peripheral blood, lymphoma cytogenetics

Period covered by search: 1966-present

Names of databases searched: Medline

Did the search reveal applicable alternatives.....☐ Yes ☒No

If yes, please explain:

b) The Animal Welfare Act allows other means of conducting a search to certify the above. If you used an alternative search strategy, provide information on the strategy, methods and sources:

c) If this is a teaching protocol, please specify why there are no alternatives to using live animals:

10. This species has been selected because:

a) Anatomy, physiology, behavior or agent susceptibility of species uniquely suited to the study☐ Yes ☐No

b) Lowest phylogenetic species providing adequate size, tissue, or anatomy for proposed study ..... ☐ Yes ☐ No

c) This species provides a particularly good model for the human or other animal disease or process ..... ☐ Yes ☐ No

d) Previous studies which form the background for this project used this species ..... ☐ Yes ☐ No

e) The objective of this study is to provide information about the target species ..... ☒ Yes ☐ No

f) Other (please specify):

11. The ACUC requires a power calculation be provided or an explanation why a power calculation is not feasible for this project. Complete one or more of the following to justify the number of animals you will use.

a) This is a pilot study ***(a total of 12 or more animals typically indicates to the ACUC that the project is not a pilot)***; describe how numbers were estimated:

b) The group size was determined using a statistical package ***(specify the package and power level expected, based on the calculation):***

c) This is a teaching protocol (specify species and number of animals and number of students):

d) This study involves tissue harvested from animals for in vitro studies (explain number of animals requested for amount of tissue needed):

e) This study involves breeding animals (list number of breeding adults used/number of offspring produced each year):

f) This is a capture/release population study attempting to maximize sample size within logistical constraints ..... ☐ Yes ☐ No

g) This is an observational, non-manipulative study; animal numbers cannot be predicted: ..... ☒ Yes ☐ No

h) Other (please describe):

12. Is this a field study? ..... ☐ Yes ☒ No

If no, move to question 13. If yes, please provide the following information:

a) Briefly describe the capture device (e.g. trap, net, electroshock, etc).

b) What is the maximum amount of time animals will spend in trap or net?

c) Is there a possibility lactating females will be captured?

d) How will animals spending time in capture device be shielded from harsh environmental conditions (e.g. heat, cold, rain, etc.)?

e) During what season and at what time of day will capture take place?

f) What method of marking animals will be used? In general, toe clipping is not acceptable.

g) What insulative bedding will be used?

PART II—Surgical and Other Manipulations

13. Will surgical procedures be involved (Y/N)?

If no, move to question #19. If yes, complete question 13-18, below.

Will any individual animal undergo more than one operative procedure..... ☐

Yes ☒No

If yes, please justify:

14. Surgery will be:

a) Survival or Terminal: survival

b) Major or Minor: minor

Major surgery penetrates or exposes a body cavity or produces substantial impairment of physiological or psychological function (e.g. laparatomy, thoractomy, joint replacement, limb amputation).

15. Briefly describe operative procedure or provide ACUC approval number for SOP.

A palpable popliteal lymph node will be excised through a skin incision approximately 4-6 cm in length (depends on LN size), after blunt dissection to free it from surrounding tissue. Any vessels will be ligated and subcutaneous tissue and skin will be closed in a routine manner.

16. Pre-anesthetics, anesthetics, and/or sedatives

(Please provide the following information for each drug used. Complete question 22 below to provide information on analgesia.)

a) Drug: pre-anesthetic regimens are tailored to each patient's unique needs. Common drugs used alone or in combination are atropine (0.4mg/kg), acepromazine (0.01-0.2mg/kg), fetanyl (0.01mg/kg), Valium (0.2mg/kg), oxymorphone (0.05-0.1mg/kg), generally given SQ 30-40 minutes prior to induction. Anesthesia is induced with propofol (6-8mg/kg) IV and maintained with isoflorane in oxygen after endotracheal intubation. Sedation (if needed) is also tailored to each patient's unique needs using the same drugs listed as pre-anesthetics.

b) Initial Dose (mg/kg):

c) Route:

d) Supplemental Dose (mg/kg):

Route:

Frequency:

17. Sterile Technique

a) Will sterile instruments be used..... ☒

Yes ☐No

If yes, explain method of sterilization: Steam or Sterade gas- dependant on VTH central supply policies

b) If multiple surgeries will be performed on the same day, how are instruments sterilized between uses?

c) Sterile gloves worn ..... ☒
Yes ☐No

d) Sterile drapes used ..... ☒
Yes ☐No

e) Animal hair/fur/wool clipped ..... ☒
Yes ☐No

f) Explain skin preparation (agent and prep): Hibiclens or Betadine followed by alcohol

g) Sterile gown worn ..... ☐
Yes ☒No

h) Sterile mask worn ..... ☒
Yes ☐No

i) Head cover and foot cover worn ..... ☒
Yes ☐No

18. Describe anesthetic monitoring and post-operative recovery/care, including frequency and location of post-op monitoring: patients will have ECG (continuous) and Doppler blood pressure (every 5 minutes) monitoring done throughout the procedure. Post procedure patients are monitored until extubated and considered "cage safe" and ambulatory according to the guidelines of the clincal anesthesia department at the VTH.

19. Non-surgical manipulations (list the following information for each):

a) Agent: bone marrow aspirate as a standard procedure
b) Vehicle: 16 gauge needle
c) Route: proximal humerus
d) Volume:
e) Frequency: once, at time of tumor biopsy
f) Duration:

Experimental Diet ..... ☐
Yes ☒No describe):

Fluid collection ..... ☒
Yes ☐No
If yes, list following information for each:
Fluid: blood
Collection Site/Method: intravenous
Volume Collected: 1 ml
Frequency: every 2 weeks for 4 months and at tumor recurrence
Percent of total blood volume withdrawn: neglible (<0.01% each time for 18 kg animal)

20. Describe any adverse effects that may occur *secondary* to experimental agents, procedures or field manipulations:

21. Methods to be used for monitoring animal well-being will include:
(Answer Y for all that apply)

a) Use of clinical scoring system ..... ☐
Yes ☒No
Attach or provide ACUC SOP number:
Frequency and Duration:

b) Observation for changes in behavior, posture and activity ..... ☒
Yes ☐No
Frequency and Duration: at time of procedure and prior to release to owner

c) Observation for pain and discomfort ..... ☒
Yes ☐No
Frequency and Duration: at time of procedure and prior to release to owner

d) Observation of procedural area for local irritation/infection..... ☒
Yes ☐No
Frequency and Duration: at time of procedure and prior to release to owner

e) Observation for decreased activity/inability to move ..... ☐
Yes ☒No
Frequency and Duration:

f) Assessment of daily food/water consumption..... ☐
Yes ☒No
Frequency and Duration:

g) Other (describe):

22. Analgesia

Will animals experience more than momentary pain/distress ..... ☐
Yes ☒No

(If yes, complete below. If no, move to question #23)

a) Analgesic Drug:
b) Dose (mg/kg):
c) Route:
d) Frequency:
e) Duration:

The ACUC requires animals receive analgesia for 72 hours post surgery. **If no analgesic will be used to eliminate a potentially painful or distressful condition, provide justification**:

23. Overview of procedures to be conducted with animals.

a) Will any of the following occur?
If any yes answer is given, describe under item f below

a) Physical restraint greater than holding or transporting animals ..... ☐
Yes ☒No

b) Use of paralytic drugs (must be scientifically justified) ..... ☐
Yes ☒No

c) Unusual housing conditions..... ☐
Yes ☒No

d) Food or water deprivation other than pre-surgery ..... ☐
Yes ☒No
e) Extreme environmental conditions ..... ☐
Yes ☒No
f) Describe and justify any "yes" answer above:

b) Provide a brief description of experimental groups, key procedures, frequency and type of sampling, and endpoints. You can summarize if specific information is provided elsewhere, but *a response here is required.*

We are comparing cytogenetic changes in canine lymphoma biopsy samples to those found in peripheral lymphocytes. At initial diagnosis we will evaluate the tumor, bone marrow aspirate, and the peripheral blood. At alternating weeks during treatment, we will look only at peripheral blood (as most of these patients are in remission and DON'T have tumor present to sample. We will also look at cytogenetic changes at recurrence, which will be from 6 to 18 months following diagnosis, in most patients. Preliminary work by our lab has shown that the number of chromosomes seen in peripheral blood is not normal in dogs with lymphoma. This study will establish whether or not these changes correspond to the cytogenetic changes seen in in tumor. Changes in peripheral lymphoctye cytogenetics will also be evaluated during the course of treatment, when there is not lymphnode available. This provides a unique way of studying response to treatment. Finally, peripheral blood cytogenetics will be evaluated at the time of recurrance. This could lead to a method of screening patients to determine if treatment failure is imminent.

PART III—Training and approvals

24. Will animals or their wastes or experimental agents be, or possibly be:

a) Biohazardous (infectious agents or rDNA/transgenics) ..... ☐
Yes ☒No
If yes, identify agents or rDNA use, describe potential risk to personnel/environment and risk management steps you've taken. Cite IBC approval number.
See website http://www.research.colostate.edu/rcoweb/ib.htm for information on required approvals.

b) Radioactive ..... ☐
Yes ☒No
See website http://www.ehs.colostate.edu/radiation/ or contact Jim Abraham, 491-3736 for information on required approvals.

c) Use of controlled drugs (including HCG and Ketamine) (Y/N) ..... ☐
Yes ☐No
If yes, list whose drug cabinet will be accessed.
See website http://www.research.colostate.edu/rcoweb/dr.htm for information.

d) Carcinogenic to humans or other animals (Y/N) ..... ☐
Yes ☒No
Contact Environmental Health Services at 491-6745 for information on required approvals.

25. Documentation of Training

a) CSU Animal Care Handbook read and provided to staff ..... ☒
Yes ☐No
Details:

b) Specific or targeted training performed on site ..... ☒
Yes ☐No
Describe (who, by whom, topics, etc.):

c) PI has a written description of SOPs available ..... ☐
Yes ☒No
Specify location of SOPs related to the species used in this project:

d) Pertinent training/education of people handling animals ..... ☒
Yes ☐No

e) Other (describe):

PART IV—Euthanasia

26. Will euthanasia be performed? ..... ☐
Yes ☒No

If yes, move to question 27. If no, complete following information to specify what will happen to animals at study end.

a) Adoption ..... ☐
Yes ☐No

b) Transfer to other studies ..... ☐
Yes ☐No

c) Sold at auction (hoof stock only) ..... ☐
Yes ☐No

d) Released into home territory..... ☒
Yes ☐No

27. Describe experimental endpoints or clinical signs that will determine when euthanasia will be performed. (Death is not an acceptable endpoint unless extensively justified). Describe euthanasia method to be used should unanticipated complications arise and euthanasia becomes necessary.

28. Euthanasia method/agent:

Should be consistent with guidelines published by the AVMA Panel of Euthanasia. See http://www.avma.org/resources/euthanasia.pdf.

a) Species:
b) Agent/Method:
c) Dose (mg/kg):
d) Route:

29. I understand that changes in the approved protocol must be submitted in writing to the ACUC as a protocol amendment and approved by the ACUC prior to implementation. Such changes include, but are not limited to: species, animal numbers, animal-related procedures, animal restraint, food/water deprivation, euthanasia, PI, research staff, and the like. Minor changes can be emailed to Michael.Suniga@Colostate.edu for review by one or more ACUC members; significant changes (e.g. a large increase in animal numbers, adding an invasive procedure) usually require a new A-100 be submitted for review by the ACUC at its next regularly scheduled meeting.

Please read the following before you sign this form:

As Principal Investigator, I:

Assure that these studies do not unnecessarily duplicate previous experiments.

Will abide by all relevant portions of the Public Health Service Policy and the USDA Animal Welfare regulations and guidelines concerning activities involving animals. For full text, see http://www.research.colostate.edu/rcoweb .

Agree to furnish ACUC with any relevant information on animal use it requests.

Assume responsibility for the ethical conduct of this project to protect the welfare of the animals.

Agree to attend and have my key personnel attend appropriate ACUC training opportunities.

Assure that personnel conducting animal procedures will be appropriately qualified and trained in these procedures. Assure that all individuals performing surgery under this protocol have been authorized by the ACUC to do so, as required by ACUC.

Understand that my signature acknowledges that I have reviewed this form and am responsible for this project.

Principal Investigator signature _____ **Date** _____

As Department Head, I understand that my signature on this form acknowledges that I have read this application and approve of this research.

Department Head signature _____ **Date** _____

Note: Alternate faculty signature for Department Head must be specifically delegated to another faculty member by the Department Head in advance.