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DISSERTATION

VACCINE-ASSOCIATED CARCINOGENESIS IN CATS

Submitted by

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Department of Radiological Health Sciences

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2000

COLORADO STATE UNIVERSITY

COLORADO STATE UNIVERSITY

NOVEMBER 8, 2000

WE HERBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY ELIZABETH A. MCNIEL ENTITLED VACCINE-ASSOCIATED CARCINOGENESIS IN CATS BE ACCEPTED AS FULFILLING IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

VACCINE-ASSOCIATED CARCINOGENESIS IN CATS

Animal models have been extremely valuable to cancer research for the basic study of carcinogenesis, evaluation of therapeutic approaches, and understanding the function of cancer related genes. There are scientific and ethical problems with traditional rodent models. As an alternative, spontaneously occurring tumors in pets have great potential to be informative regarding human cancer. The cell and molecular biology of pet animal tumors is relatively poorly defined, however. My commitment to the development of this research niche led me to pursue a PhD and is the basis for my thesis work. As a paradigm for the study of the carcinogenesis of companion animal tumors and the development of new cancer models, I investigated feline vaccine-associated sarcomas.

Epidemiologic evidence strongly associates vaccination of cats for rabies and feline leukemia virus with the development of soft tissue sarcomas at the administration site. I investigated two etiologic theories for vaccine-associated sarcoma. In part I of my dissertation, I address the first, which is the induction of cancer by the vaccine itself. I found that feline vaccines containing aluminum adjuvant and aluminum adjuvant itself are cytotoxic and mutagenic in cultured mammalian cells, whereas vaccines that do not contain adjuvant do not induce killing or mutation. I further demonstrated that cytotoxicity and mutagenicity of adjuvanted vaccines depends upon induction by aluminum hydroxide of reactive oxygen species.

Part II concerns my work to improve the existing A_L mutagenicity assay that I used in evaluating feline vaccines. I created high throughput system that that saves a great deal of time and expense. The assay that I developed utilizes flow cytometry to phenotypically characterize and enumerate mutant cells and to rapidly provide mutational spectra. Finally in this part, I describe a method for comparing mutational spectra using the Euclidean distance formula and cluster analysis.

In Part III, I have addressed a second etiological theory for vaccine-associated carcinogenesis, namely genetic susceptibility. Evidence supports the idea that certain cats are predisposed to develop this cancer. To investigate genetic susceptibility and determine genetic loci important in vaccine-associated carcinogenesis, I developed cytogenetic techniques for use in cats. The first step in cytogenetic studies is the preparation of metaphase nuclei for evaluation. Peripheral blood lymphocytes are frequently used for karyotyping because they are easy to collect and culture. I found, however, that feline lymphocytes were hypersensitive to apoptosis in vivo, making collection of mitotic cells more difficult than with other species. I was able to modulate apoptosis and obtain satisfactory metaphase yields by adding IL-2 to pokeweed-stimulated lymphocyte cultures. In part III, I also present the use of a novel form of fluorescent R-banding for karyotyping cats, by which I was able to identify a variable anomaly in the feline karyotype: the deletion of a band from one of the F2 homologues. I found this abnormality in 2 unrelated, clinically normal cats and in a normal feline cell line. Whether this lesion has biologic significance remains to be determined. I also

karyotyped 3 cats with vaccine-associated sarcoma and failed to identify chromosomal aberrations that might be linked to cancer susceptibility. Additional work is required to reach any conclusions in this regard. Finally, I developed comparative genomic hybridization (CGH) for use in cats. CGH is a fluorescent in situ hybridization technique which can be used to rapidly screen the entire genome for numerical chromosome aberrations, for use in cats. I demonstrated the use of CGH to evaluate a tumor using a feline bronchoalveolar cell line.

In summary, I have studied feline vaccine-associated sarcoma as a paradigm for studies of spontaneous cancer in companion animals and as a model for human disease. This work allowed me to develop a variety of techniques that I can apply to the study of this particular cancer as well as other animal tumors. In addition, I found that feline vaccines induce mutation through an ROS-mediated mechanism. Based upon these findings and the tentative suggestion that genetic susceptibility plays a role in vaccine-associated carcinogenesis, I propose, and am investigating, a new hypothesis: cats, some more than others, are sensitive to ROS-induced cancer. This system could provide a model to shed light on the relationship between ROS and carcinogenesis in humans.

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ACKNOWLEDGEMENTS

My experience as a graduate student in the Department of Radiological Health Sciences at Colorado State University has been an incredibly positive educational experience. The entire faculty, students, and staff have contributed to creating this positive learning environment and I feel indebted to everyone for these years of help and support. There are certain individuals who were particularly instrumental in my successful completion of my doctorate. I would, first like to acknowledge my advisor, Dr. Susan LaRue. If not for Sue, I would have been unaware of the opportunities that existed in this department. She is a truly unique person, combining a tremendous enthusiasm for teaching and research, a highly developed sense of loyalty and ethics, boundless energy, and a warm and caring personality. Second, I must acknowledge my co-advisor, Dr. Charles Waldren, who opened his lab to me and invested a great deal of time and effort into my training. He has always been available to offer advice concerning my research and career decisions, and to discuss science and ideas. Both Charles and Sue have always shown faith in my abilities and have listened openly and encouragingly to my ideas and opinions. I consider them the ultimate mentoring team. I hope that I can create a laboratory based upon the exemplary model that they have provided for running a laboratory, conducting research, and teaching graduate students. I must also acknowledge my other committee members, Dr. Joel Bedford for allowing me free access to his laboratory and for his support

throughout my tenure in this department, and Dr. Barbara Powers for all of her help with tumor pathology.

Diane Vannais, with her incredibly extensive and well-developed grasp of science, is in a class by herself and I am particularly grateful for all of her help. I do not think I could have achieved what I have without her help in the laboratory. Many others, including Allen Christian and Russ Drabek were indispensable in my lab training and owe them a great deal for their support and friendship. I must also acknowledge the students that helped me with this work including Emily Hall and Eric Hoots. I feel incredibly lucky to have been able to work with them and I am sure they will both be successful scientists. Finally, I am grateful for the support of the other graduate students in this department including Christine Anderson, Adam Holmes, Nadira Trncic, Larry Dugan, Yualin Peng, Larry Dugan, And Sandra Gunselman. I could not have earned this degree without all of the individuals I have mentioned and many more that have offered their help to me these last few years.

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PART I

ADJUVANTED FELINE VACCINES ARE MUTAGENIC IN CULTURED MAMMALIAN CELLS

CHAPTER 1. INTRODUCTION:

Pet animal models for human cancer.

Simple models can shed light on complex biologic systems. However, the loss of potentially important modifying and interacting variables is an inherent flaw in simplified systems. As a result, no single model can explain all aspects of a biologic process. All in vivo and in vitro systems have advantages and drawbacks in scientific investigation. The development and application of several different models is required to understand complex biological phenomena including disease such as cancer.

The value of animal models in disease research is well documented. Animal models are indispensable in cancer research for studies of carcinogenesis, evaluation of therapeutic approaches, and understanding the function of cancer related genes. Rodents, have been widely used because they are small, easy to house and care for, can be manipulated genetically, have multiparous births and short life spans so that endpoints can be reached more rapidly. There are, however, scientific and ethical problems with traditional rodent models. First, inbred genetically identical animals have a limited genetic background, significantly influencing their physiology and causing strain specific effects. On the other hand, inbreeding advantages, in that genes accounting for strain differences, such as altered susceptibility to cancer, can be investigated. It is very difficult to extrapolate results obtained in a specific rodent strain to a genetically heterogeneous population such as humans, however. Second, there are numerous physiologic and

metabolic differences between rodents and humans, further complicating extrapolation among species. Third, many animal studies are performed on tumors induced by high doses of known carcinogens or by transplantation of tumor cells. These types of induced tumors are fundamentally different from tumors that developing spontaneously as in humans. Fourth, rodents are short-lived making it difficult to assess long term effects of clinical treatments. Finally, there is increasing ethical concerns about using animals in research and that gaining approval to carry out animal studies is more difficult than ever before.

The pet animal (dog and cat) cancer model has several advantages. 1) Most tumors in pets occur *naturally* in contrast to other animal models in which tumors are induced via carcinogens, genetic manipulation or implantation. The spontaneous nature of tumors in pets facilitates epidemiologic and etiologic study. This type of study is particularly informative because pets share the environment and lifestyles with their owners. 2) Cancer is common in pets, particularly dogs. Certain cancers occur *even more frequently* than in the human population. Therefore, pets are a readily accessible source of patients and tumor material for study. 3) Cancers in animals are like those occurring in humans both morphologically and clinically which facilitates extrapolation between species. 4) Dogs and cats are as genetically diverse as humans, relieving the problem of genetic background. Yet, some inbreeding occurs producing large families of related animals with complete pedigrees for investigating genetic influences. 5) Tumors in pets can be studied in clinical, therapeutic situations, where animal welfare is the single most important issue, as is the case for human patients in clinical studies, yet it is much easier to conduct these studies in animals than in humans. For example, tissue and blood

samples, even multiple samples at different stages of the disease or therapy, can be easily obtained during routine therapeutic and diagnostic procedures, particularly because of a frequent requirement for sedative, anesthetic, and analgesic drugs in normal procedures. Clinical studies are much less expensive at veterinary institutions and owner compliance is quite good.

Studies of spontaneous tumors in dogs and cats can overcome many of the limitations of traditional animal models. They provide great potential to contribute to understanding carcinogenesis and tumor biology and to the development of therapeutics. Veterinary cancer centers such as the Comparative Oncology Unit at Colorado State University have long recognized the value of pet animal cancer models and are using the large number of clinically well characterized animal tumors to evaluate experimental therapeutics. However, the cell and molecular biology of pet animal tumors is relatively poorly defined. My interest in exploiting this valuable resource for the study of cancer was largely responsible for my undertaking of a PhD. I elected for several practical and theoretical reasons to begin my efforts to develop cancer models with the study of an interesting cancer in cats, the vaccine-associated sarcoma. The investigation of the etiology of this cancer is the basis for my PhD work and this dissertation.

Feline vaccine associated sarcoma: an important veterinary problem.

Significance. Vaccination has been used to prevent human disease for at least 300 years and, although the procedure has been improved, the principles behind vaccination are unchanged[1-3]. Exposing an individual to an inactive or less virulent form of a microbe, virus or bacterium, can "prime" the immune system to overcome a subsequent challenge with the disease-causing organism[1]. Immunization programs are critical to infectious

disease control, as underscored by the eradication of smallpox and the prevention of such diseases as poilio and measles in the last century. Similarly, vaccination of pets and other domestic animals is a fundamental procedure in general veterinary practice and plays a significant role in disease prevention and, also, in the maintenance of human and animal health by vaccination against rabies.

Most microbial organisms have a limited host range so that common infectious organisms infecting one species rarely infect animals of another species[1]. For example, influenza viruses are not passed to cats and dogs from humans, and canine parvovirus or feline leukemia virus do not infect humans[1]. As a consequence, humans and pets do not share similar vaccination schedules and products, but all vaccines are produced by similar techniques. Live, attenuated vaccines such as measles-mumps-rubella used in people, contain a nonvirulent form of the virus achieved by viral tissue passage and usually result in long-lasting immunity. Other vaccines consist of killed organisms or subunits of organisms, such as influenza vaccine. These killed vaccines may not induce a strong immune response unless the antigen is coupled with a vaccine adjuvant. Various adjuvants have been used, but aluminum gels are most common. Adjuvants are thought to function by creating a persistent depot of antigen for presentation to immunologic cells[4]. For humans, biologic products are considered drugs and are regulated by the Food and Drug Administration which requires proof of efficacy and extensive safety evaluation to be approved for use in humans. The United States Department of Agriculture regulates animal biologics, including vaccines. The United States Department of Agriculture requires demonstration of vaccine efficacy, but safety requirements are limited to acute,

severe toxicity. The actual ingredients in animal vaccines are unknown as this because this is considered proprietary knowledge.

Despite the more lenient vaccine safety requirements in animals, adverse reactions have been uncommon, but there is good evidence that vaccination site sarcomas are associated with certain vaccines administered to cats[5-10]. It is tremendously unsettling to veterinarians that a beneficial and seemingly innocuous procedure such as vaccination could induce an aggressive malignancy. Fraught with ethical, clinical and legal issues for veterinarians, feline vaccine associated sarcoma has become an important and controversial problem in veterinary medicine. Concerns about this disease have prompted veterinary organizations to form a task force to address such issues as dissemination of information about the disease to veterinarians and the public, procedural recommendations for vaccination, and distribution of funds for researching the etiology and treatment of this cancer. Research is underway at numerous veterinary institutions to control this problem[8,11-16]. On the other hand, feline vaccine-associated sarcoma appears to provide an animal cancer model for cancer susceptibility and carcinogenesis.

Epidemiology. Vaccination of cats became prevalent nationwide in the middle to late 1980s. The reasons for this change were, first, a rabies epidemic in raccoons in the eastern United States revived public health concerns and second, the first vaccines against the retrovirus, feline leukemia virus were released for veterinary use [8,11,17-21]. Hendrick and Goldschmidt postulated a relationship between vaccination and the development of soft-tissue sarcomas in cats[5]. These pathologists observed a great increase in the number feline biopsy submissions of subcutaneous granulomas and soft-tissue sarcomas at anatomic locations used for vaccination of cats. These observations

were noticed shortly after a 1987 change in the Pennsylvania State rabies regulations[22] requiring rabies vaccination for cats[5].

Initial reaction was mixed to the association between vaccination and the development of tumors in cats, with some skeptical editorials appearing in veterinary journals[23,24]. But anecdotal reports and epidemiologic studies from all over the United States began to provide support for the relationship between vaccination and the development of tumors[6,7,25-28]. The largest of these studies statistically compared the occurrence of soft-tissue sarcomas at vaccine injection sites with soft-tissue sarcomas at other non-injection sties. A close temporal relationship between vaccination of a cat and the development of a tumor at vaccination sites was reported[10]. Further, the risk of soft-tissue sarcoma was shown to increase with the number of vaccines administered, evidence for a dose-response relationship. The risk of soft-tissue sarcoma at the vaccination site was reported to be 50%, 127%, and 175% higher after 1, 2, and 3 vaccines, respectively, than in cats that were not vaccinated at that site[10]. Both feline leukemia virus vaccines and rabies vaccines are associated with an increased risk of soft-tissue sarcoma[10]. A multivalent product protecting cats against panleukopenia, calici, and viral rhinotracheitis viruses, has not been epidemiologically associated with tumor development. In the spring of 1999, based on the accumulated data, the World Health Organization declared adjuvanted feline vaccines carcinogenic[29].

Histology. Histologically, the tumors at vaccination sites are classified as soft-tissue sarcomas of various types[6,7,12,25]. Most are fibrosarcomas, but others are classified as malignant fibrous histiocytoma, leiomyosarcoma, and soft-tissue osteosarcoma[7,12,25,25]. The tumors have anaplastic and aggressive histologic features such as poorly differentiated cellular morphology, high mitotic index, necrosis, and invasiveness[7,12,25]. Nodules of lymphoid and granulomatous inflammation frequently occur at the periphery and the center of the tumor often is necrotic[7,12,25]. In many tumors, a grayish granular to crystalline foreign material can be observed[7,12,25](Figure 1.1). Based on X-ray spectra obtained through electron probe microanalysis, this material has been shown to be composed of aluminum and oxygen [6].

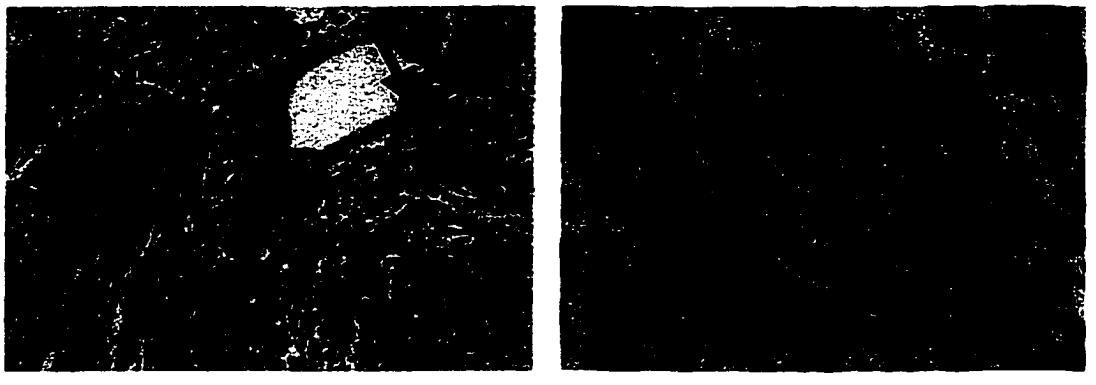

Figure 1.1. Hemotoxylin and eosin stained histologic section from a feline vaccine-associated sarcoma. A) Low power photomicrograph (400X) showing the tumor with a nodule of lymphocytic and granulomatous inflammation (arrows). B) High power photomicrograph (1000X) demonstrating anaplastic cellular morphology.

Clinical Behavior of Tumors. Clinically, these tumors are characterized by their invasive local behavior (Figure 1.2). They are not encapsulated within the subcutaneous space (Figure 1.3). Radical resection may result in extended local control in some cases[30]. Recurrence rate after conservative surgery alone is high, as with invasive soft tissue

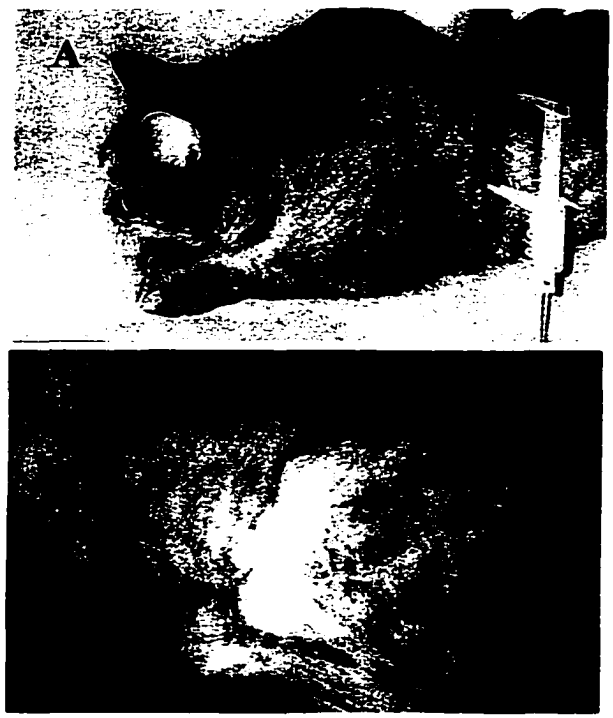

Figure 1.2. These pet cats developed fibrosarcomas following routine vaccination. The subcutaneous intrascapular space is commonly affected. The cat in panel A has a bulky, poorly defined dorsal, intrascapular mass characteristic of this cancer. Panel B is a dorsal view of a cat with a multinodular tumor in the intrascapular space. There are multiple subcutaneous nodules. A surgical scar traverses the body indicating that this tumor has recurred following surgical

sarcoma in humans[31,32]. Adjunctive radiation therapy seems to improve local control and is commonly used[32]. Despite aggressive histologic features, the metastatic rate of feline vaccine associated sarcomas is relatively low, with evidence of distant spread found in less than 20% cases[30,33,34]. Metastasis is recognized primarily in the lung. At this time, local disease is the life-limiting factor. The low metastatic rate may be inherent to the biology of the tumor, in that mechanisms for distant invasion are slow to develop. Many and possibly all cats with vaccine-associated sarcoma are euthanized rather than allowed to die "naturally," which can make estimation of metastatic rate somewhat subjective and dependent on factors like a cat owner's perception of quality of life or the animal's overall health status.

Etiology. Mechanisms by which vaccination leads to tumor development are not understood. At least four theories have been proposed. First, carcinogenicity of the

vaccines themselves must be considered. Aluminum has been identified in vaccine associated tumors and aluminum gels are the adjuvant most commonly used in vaccines [4,6]. Aluminum adjuvant has been shown to persist at the site of injection in experimental animals for at least 1 year following administration[4]. Aluminum preparations and other vaccine components have not been definitively linked to cancer, however[35].

A second theory is that the critical factor in vaccine-associated carcinogenesis stems from aluminum induced inflammation[36]. It is known that a prolonged local immune response which sometimes results in granuloma development, is required for effective vaccination[36]. Further, the role of inflammation in tumorigenesis is well recognized[37,38]. Reactive oxygen species, including the oxygen radicals such as O_2^{-} ', $OH^{\cdot}$, and $ROO^{\cdot}$, as well as H_2O_2, are known to be generated during the inflammatory response [37,38], which can lead to DNA damage and mutation[39-43]. In addition, chemical mediators such as cytokines and growth factors, released by infiltrating inflammatory cells and stromal cells, are mitogenic, enhancing proliferation of damaged cells[37,38]. Other sarcomas occurring in cats associated with physical trauma and inflammation are well-documented such as posttraumatic ocular sarcomas and fracture site sarcomas [44-46].

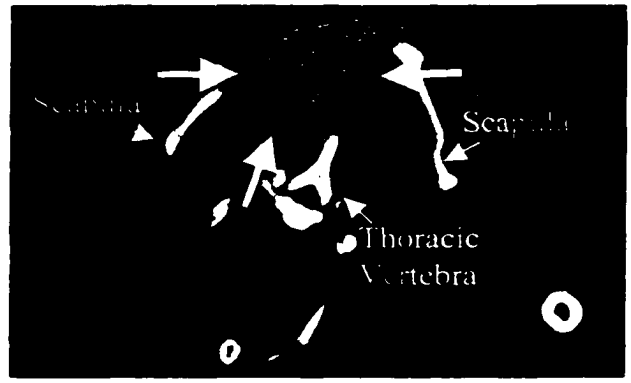

Figure 1.3. Computerized Tomagraphy (CT) (transverse plane) of a cat with vaccine-associated sarcoma in the intrascapular space. The tumor is a poorly-defined, unencapsulated, invasive mass characteristic of this cancer.

Third, feline retroviruses, both exogenous and endogenous within the genome, are ubiquitous in the feline population so that a viral etiology for vaccine associated sarcoma must be considered[47,48]. Evidence to date does not, however support this theory. For example, one group failed to identify feline leukemia virus (FeLV) or feline sarcoma virus (FeSV) by immunohistochemistry or polymerase chain reaction (PCR) in tumors[48]. In fact, cats with vaccine associated tumors rarely test positive by ELISA for FeLV or feline immunodeficiency virus (FIV).

Finally, regardless of the carcinogenic effector of vaccination, be it inflammation or some vaccine component such as aluminum, there is evidence that individual susceptibility is important in the development of vaccine associated sarcoma in cats.

Cancer susceptibility. Although the pattern of inheritance is not established and the evidence is somewhat anecdotal, a predisposition to vaccine-associated carcinogenesis in certain cats is suspected. First, a relatively small number of vaccinated cats develop tumors at the site of injection. The actual incidence of feline vaccine-associated sarcoma has been difficult to determine due to incomplete vaccination histories and limited follow-up, but is estimated to be between 1 in 1000 and 1 in 10,000 vaccinated cats[26,36]. Most cats regularly receive multiple vaccines and never have tumors develop. Second, cats that have vaccine-associated tumors are younger than cats with soft-tissue sarcomas that are not associated with vaccination[7]. As in humans, developing cancer as a child or young adult strongly suggests a genetic predisposition.[49,50] Third, the latency period between vaccination and tumor development is short, ranging from a few weeks to 2 years[7,12,25,36], suggesting that tumorigenesis is accelerated in these individuals. Fourth, there are reports of vaccine-associated tumors in siblings which suggests an shared

genetic component to tumor susceptibility[51]. Finally, some cats have more than one primary vaccination site sarcoma, suggesting that tumor development in these individuals is a common event compared to other cats[25].

Feline vaccine associated sarcoma: a valuable and unexplored model for carcinogenesis.

The study of feline vaccine associated sarcomas has the potential to be an informative model for the study of carcinogenesis and tumor biology. First, the unique carcinogenic association of vaccination to tumor development and the well-recognized stepwise progression of lesions from inflammatory to tumor provide a fascinating model for the study of tumorigenesis. This cancer is one of few spontaneously occurring tumors in any species that has a recognizable cause. Second, genetic susceptibility may play a role in the development of this cancer.

There is no evidence that vaccination of species other than cats results in tumorigenesis, but genetic predisposition to cancer is known to be important in humans. For example, there are a plethora of familial cancer syndromes including retinoblastoma (associated with germline mutation in *Rb)*, Li-Fraumeni (associated with germline mutation in *p53)*, Wilm's tumor (associated with mutation at a number of *WT* loci), colon cancer (associated with inherited mutation in *MSH2*, *APC*, *MLH1* and others), and breast cancer (associated with mutation in BRCA1 and BRCA2 genes at least) to name just a few. Individuals are also predisposed to cancer by less penetrant genetic mutations, such as polymorphisms in detoxifying enzymes.[52-57] The impact of the low penetrance genes on cancer risk is modified by lifestyle factors, exposures, and other genetic traits. These traits may be very important in the occurrence of 'sporadic ' as well as inherited cancer in

humans[55]. Predisposition syndromes can be very difficult to study in human populations, because the highly penetrant genes are rare and the less penetrant genes are difficult to detect. Therefore animal models for genetic susceptibility are particularly important and may be highly informative. For a further discussion of cancer predisposition, refer to chapter 9.

Statement of the problem. I set out, therefore, to investigate two of the etiologic theories for vaccine-associated sarcoma; first, the role of vaccines and aluminum in carcinogenesis and second, the role of genetic susceptibility. My dissertation is composed of three parts, each addressing a separate component of my experimental studies. In the first part, I present studies investigating the mutagenicity of feline adjuvanted vaccines using the A_L system, an in vitro mammalian cell assay. Through the application of the A_L mutation assay to the study of feline vaccines, I became interested in the measurement of DNA damage and mutation. Consequently, I worked to improve the efficiency and informative value of the A_L mutation assay, which comprises the second part of my dissertation, including: 1) the development of an improved, high-throughput version of the A_L mutation assay using flow cytometry and 2) the mathematical comparison of mutational spectra. Finally, in the third part, I present the development of cytogenetic techniques to study feline tumors as an approach to investigating genetic contributions to vaccine induced carcinogenesis including inherited susceptibility.

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CHAPTER 2: ADJUVANTED FELINE VACCINES ARE MUTAGENIC.

Introduction.

Epidemiologic evidence supports a relationship between vaccination of cats for rabies or feline leukemia virus with the development of soft-tissue sarcomas at the site of administration[1-8]. These tumors are locally invasive and histologically aggressive[9]. The mechanism for carcinogenesis is not known[10,11]. As discussed in chapter 1, persistence of aluminum hydroxide from vaccine adjuvant at the vaccine site and in tumors has been demonstrated and may be involved in the pathogenesis[8,12].

Mutations that inactivate tumor suppressor genes and the activate proto-oncogenes, are critical to carcinogenesis[13-15]. Therefore, it is not surprising that induction of mutation is a common characteristic and a likely underlying mechanism involved in carcinogenesis[16]. The hypothesis driving the present study was that the adjuvanted feline vaccines that are associated with tumor development in cats, as well as aluminum adjuvant preparations, would be mutagenic thus providing a mechanism for vaccine-associated carcinogenesis. As a corollary, I hypothesized that feline vaccines administered without adjuvants would be nonmutagenic or less mutagenic. I tested these hypotheses using a sensitive, in vitro, mammalian, somatic cell assay, the A_L assay, to quantify mutagenicity of vaccines.

The A_L mutation assay, developed by Waldren and his co-workers, has been used for 30 years to evaluate the mutagenicity of a variety of agents including radiations,

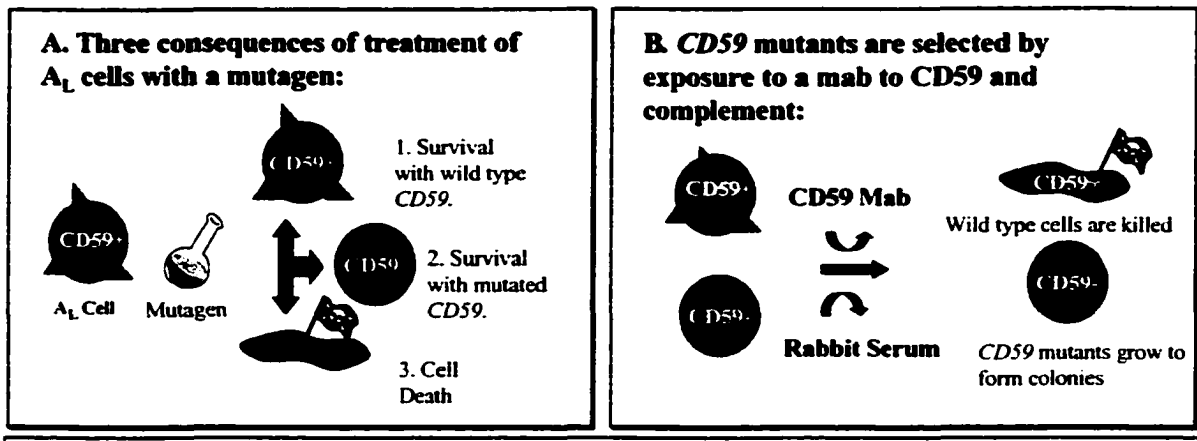

Figure 2.1. Diagram depicting the principle behind the A_L mutation assay. Panel A: When A_L cells are exposed to a mutagen, a cell may survive and be 1) wild-type at the *CD59* locus, or 2) mutated at the *CD59* locus, or 3) the cell may be killed. Thus surviving cells are either wild-type or mutant. Panel B: The mutagen treated surviving cells are exposed to a monoclonal antibody to CD59 with serum as a source of complement which kills wild-type cells, thus selecting for *CD59* mutants.

drugs, asbestos, and arsenic[17-24]. Human-hamster hybrid A_L cells contain Chinese hamster ovary (CHO) chromosomes plus a single human chromosome 11. A_L cells express human cell surface antigens including CD59 encoded by the *CD59* gene at 11p13.5. After exposure to a mutagen, the surviving A_L cells are either wild-type for CD59 or are mutated to $CD59^-$. Anti-CD59 antibody, E7.1, plus rabbit serum as a source of complement is used to kill wild-type cells leaving $CD59^-$ mutants to form colonies as illustrated in Figure 2.1. The mechanics and rationale for the use of this and other mutation assay systems is discussed in detail in chapter 4.

Materials and Methods.

Vaccines and Aluminum Adjuvant. The vaccines evaluated are commercially available and include rabies vaccines (Imrab®1 and Imrab®3 from Rhone Merieux, Inc, Athens Georgia; Durarab®1 and Durarab®3 from Immunomed Corporation, Wheatridge, Colorado), feline leukemia virus vaccine (Fel-O-Vax Lu-K® from Fort Dodge

Laboratories, Fort Dodge, Iowa) and feline panleukopenia/calici/rhinotracheitis vaccine (Protex®-3, Intervet, Millsboro, Delaware). An aluminum hydroxide adjuvant preparation was also evaluated (Alhydrogel® Superfos Biosector available in the US from Accurate Chemical & Scientific Corporation, Westbury, New York.

Cells. Human-hamster hybrid A_L cells[25](AND-6[26]) were cultured in F12 medium supplemented with 4% heated (56° C for 30 minutes) newborn calf serum, 3% heated (56° C for 30 minutes) fetal calf serum, 1 mM l-glutamine, 50 Units/ml penicillin, 50 ug/ml streptomycin, and 20 mM hepes. Cultures were kept at 37°C in an incubator containing a humidified atmosphere of 5% CO_2 in air. A_L cells are available from American Type Culture Collection, Manassas, Virginia.

Clonogenic survival assay[27]. To evaluate vaccines for cytotoxicity, a known number of A_L cells were plated, incubated for 3 hours to allow cells to attach, exposed to various dilutions of vaccine for 24 hours or 4 days and then rinsed. Fresh medium was added and plates were incubated for 7-10 days at which time they were fixed, stained, and the colonies were counted. Results were expressed as surviving fraction (SF) and graphed against dose to obtain survival curves.

SF = (number of colonies/number of cells plated) / (CE)

CE = (number of colonies on untreated control plates/number of cells plated on control plates)

Mutation Assay. (Figure 2.1) Concentrations of the vaccines resulting in 20% - 80% killing were selected for evaluation in the mutation assay. Two to three doses and untreated samples were tested simultaneously in duplicate. Sufficient numbers of A_L cells

were plated to result in at least 10^5 surviving cells following exposure to vaccine at a given concentration (based upon cytotoxicity data). Cells were plated, incubated for three hours, exposed to vaccine for 4 days, rinsed, and growth medium was re-added. Cells were subcultured every 2-3 days to maintain log phase growth for an expression period after which time the progeny of mutant surviving cells no longer have CD59 surface antigen present. On days 10 and 12, cells were harvested, counted, resuspended in a serum-free medium containing 1% fetal calf serum with 1 mM l-glutamine, 50 Units/ml penicillin, 50 ug/ml streptomycin, and 20 mM HEPES. 2 x 10^5 cells were plated in 100-mm tissue culture plates, and incubated for three hours. Rabbit serum (2% v/v; Covance, Denver, Pennsylvania) and human serum (0.1% v/v; Sigma-Aldrich) as a source of complement and anti-CD59 monoclonal antibody (0.5% v/v; E7.1 clone from mouse hybridoma[28])were added. The plates were incubated for 10 days, fixed, stained, and the colonies were counted. Cloning efficiency (CE) was determined on plates treated with rabbit serum in the absence of monoclonal antibody (three plates for each treatment). Results were expressed as induced mutants/10^5 surviving cells (induced M_f) and plotted against dose, where:

CE = (average colony counts on plates treated with rabbit serum/number of cells plated);

M_f = Mutant frequency = (10^5)*(number of colonies on treated plates /number of cells plated)/(CE for treated cells);

Spontaneous M_f = (10^5)*(number of colonies on untreated plates /number of cells plated)/(CE for untreated cells);

And Induced M_f = Mutant frequency = M_f - Spontaneous M_f.

The numbers of mutants (M_f) and the number of wild-type cells (Number of cells plated – M_f) was compared for treated and untreated samples by Chi square.

Results.

Four commercially available adjuvanted feline vaccines and an aluminum hydroxide gel preparation commonly used as a vaccine adjuvant were evaluated (Imrab®1, Imrab®3, Fel-O-Vax Lu-K®, Alhydrogel®). Initially, I tested the vaccines and aluminum hydroxide gel for cytotoxicity in A_L cells following 24 hour and 4 day exposures. Exposure to these resulted in dose dependent killing of A_L cells after both 24-hour and 4-day exposures. (Figure 2.2 A and B) Survival curves for rabies and feline leukemia virus vaccines are similar following 24-hour and 4-day exposures. There was some variation in the degree of cytotoxicity from experiment to experiment. Twenty-four-hour and four-day survival curves for aluminum hydroxide gel were considerably different, with the short exposure resulting in more cytotoxicity at each dose. Because adjuvanted vaccines and Alhydrogel are formulated as suspensions, the contents settle out (visibly) when the vials are left sitting. Although the vials were agitated prior to use, there is a possibility that a slightly different dose of adjuvant/vaccine is delivered in each experiment. Because 24-hour and 4-day aluminum gel cytotoxicity experiments were performed at different times, the results were particularly likely to differ. To test this hypothesis, 24-hour and 4-day cytotoxicity experiments were performed side by side. There was no difference in 24-hour and 4-day survival curves in side-by-side experiments(Figure 2.2 C).

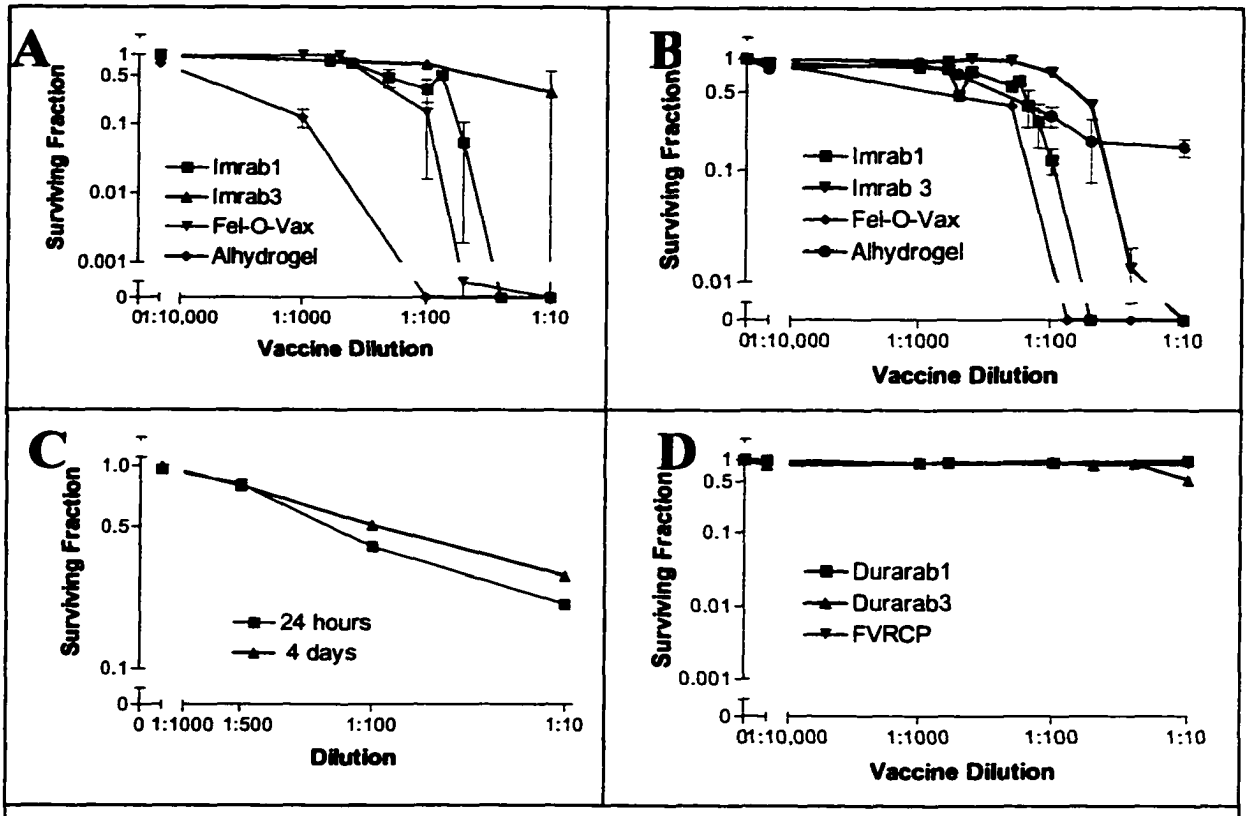

Figure 2.2. Survival of A_L cells following vaccine and aluminum gel exposure. The data points represent the mean and standard error of multiple experiments. A) 24-hour exposure to Imrab1(10 experiments), Imrab3 (2 experiments), and Fel-O-Vax (3 experiments) and Alhydrogel (3 experiments) B) 4-day exposure to Imrab1(14 experiments), Imrab3 (8 experiments), and Fel-O-Vax (1 experiment) and Alhydrogel (4 experiments) C) simultaneous 24-hour and 4-day exposure to Alhydrogel (2 experiments) D) 4-day exposure to vaccines not containing adjuvant including Durarab1(2 experiments), Durarab3(1 experiment), and Protex(1 experiment).

To evaluate the role of vaccine adjuvant in cytotoxicity, I tested non-adjuvanted rabies vaccines (Durarab®1 and Durarab®3) and a trivalent feline modified live virus vaccine (does not contain adjuvant) for panleukopenia, calici, and rhinotracheitis (Protex®-3). These non-adjuvanted products resulted in minimal toxicity.(Figure 2.2 D)

I used the A_L assay to determine whether exposure to vaccine and aluminum hydroxide induces mutation in vitro. The compounds were evaluated at concentrations

roughly equivalent to the LD_{20}, LD_{50}, and LD_{80} (doses that kill 20, 50, and 80% of cells respectively). The two rabies vaccines and aluminum hydroxide gel induce mutation in a dose-dependent manner. The mutation induction curves for both rabies vaccines are similar and appear to reach a maximum at a dilution of 1:100. For doses causing similar cytotoxicity, the aluminum hydroxide preparation appears to be less mutagenic than rabies vaccines. The non-adjuvanted, trivalent, panleukopenia, calici, and rhinotracheitis vaccine did not induce mutation above spontaneous levels when evaluated at concentrations similar to those evaluated for rabies.

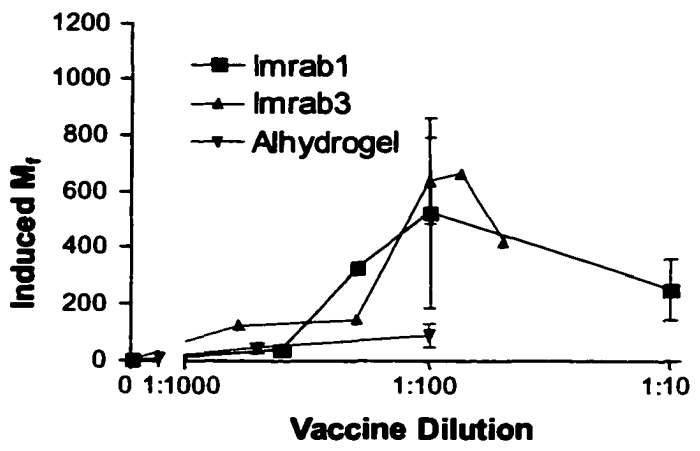

Figure 2.4. Induction of CD59- A_L cell mutants following 4-day exposure to rabies vaccines (Imrab1 and Imrab3) and aluminum hydroxide adjuvant preparation (Alhydrogel). Data points reflect the mean and standard error of 2 experiments using Imrab1 and Alhydrogel) and a single experiment using Imrab3.

Agent	Dose	SF	M_f
Imrab1	**0**	1.0	0
	1:400	0.77	39*
	1:200	0.57	328*
	1:100	0.12	1194*
Imrab3	**0**	1.0	0
	1:600	0.95	127*
	1:200	0.95	146*
	1:100	0.75	638*
	1:75	0.38	666*
	1:50	0.01	423*
Alhydrogel	**0**	1.0	0
	1:1000	0.92	6
	1:500	0.72	43*
	1:100	0.31	88*

Table 2.1. Mutation Induction and cytotoxicity by rabies vaccines and aluminum. SF = Surviving Fraction, M_f = Induced mutant frequency. * significantly greater than spontaneous level by chi-square.

Conclusions.

These data demonstrate that certain feline vaccines, containing adjuvants, and an aluminum hydroxide adjuvant preparation are mutagenic in vitro. Mutagenicity of adjuvanted feline vaccines and aluminum hydroxide gel suggest a mechanism for

carcinogenesis in vaccinated cats. Further, the lack of toxicity of products without adjuvant implicates the vaccine adjuvant as the component responsible for DNA damage and mutation. The rabies vaccines evaluated demonstrate a plateau to mutant induction curves. The reason for this is not clear. It is possible that protective cellular mechanisms are induced by high doses of vaccine or aluminum which could also account for induction of fewer mutants by aluminum hydroxide gel than by rabies vaccines. Another possibility is that at high doses, there is a very high frequency of genomic rearrangements resulting in translocation rather than deletion of the *CD59* locus without loss of gene function. Cytogenetic evaluation of treated cells would be helpful in determining the frequency of translocations.

If aluminum in the vaccine adjuvant is primarily responsible for the mutagenicity and carcinogenicity of vaccines, one would expect that aluminum gel alone to exhibit similar mutant yields as the adjuvanted vaccines which I did not observe. I found decreased mutant yield by Alhydrogel. I am suspicious that the suspension nature of this product may have altered the results. For example, as demonstrated in figure 2.2, 24-hour exposure to Alhydrogel resulted in significantly more killing in Panel A than for 4-day exposure in panels B and C and for 24-hour exposure in panel C. This suggests a difference in the exposure dose in these experiments and also in cats receiving vaccines rather than the length of exposure. The fact that the killing data is based upon multiple experiments suggests that the exposure differences were not due to incorrect dilutions, but some alteration in the product. It is possible, therefore, that the doses used in mutation experiments were inadvertently lower than intended. Further evaluation of aluminum hydroxide products should be performed.

These findings generate doubts about the safety of adjuvanted vaccines for both humans and animals. Neurologic toxicity due to aluminum has been demonstrated in hemodialysis patients and experimentally[29,30]. Occupational aluminum exposure is associated with pulmonary fibrosis and granulomatosis, alveolar proteinosis and encephalapthy[31]. More recently, aluminum has been implicated in developmental and degenerative neurologic disease such as Alzheimer's[29,32]. Recently, aluminum adjuvant in human vaccines has been associated with the development of a characteristic myositis[33].

There is very little previous evidence that aluminum is carcinogenic and mutagenic, however. There is some epidemiologic support for aluminum-associated cancer in humans in occupational settings. Working in aluminum reduction plants or aluminum smelters and the development of hematopoietic tumors, lung tumors, or bladder tumorsis reported[31]. Small numbers and confounding factors muddy these results, however[31]. Aluminum preparations have been associated with the development of tumors in lab animals. Intrapleural aluminum oxide inoculation produced 1 tumor in 35 Wistar rats[31]. Aluminum foil introduced to the subcutaneous space resulted in sarcomas in 8 of 18 rats[31]. In one study, but not in another, subcutaneous injection of aluminum-dextran caused sarcoma development in mice[31]. Other animal studies of aluminum preparations have not demonstrated carcinogenicity[31]. In fact, an anti-tumor effect by aluminum has been seen in lab animals with induced tumors[31].

Results of previous mutagenicity testing of aluminum are mixed. Aluminum salts, including Al_2O_3 that I evaluated here with the Alhydrogel preparation, were not mutagenic in bacterial assays including the Ames' test, but have been shown to inhibit cell division and cause chromosome aberration in plants[31]. Aluminum oxides have been

shown to increase HPRT mutation dimethylnitrosamine-treated CHO cells, but $AlCl_3$ did not induce mutation in the TK locus of L5178Y cells[31]. Finally, chromosome aberrations in the bone marrow of aluminum chloride injected mice have been observed. It is possible that mutagenicity is associated with only certain forms of aluminum or with some other component of the aluminum hydroxide preparation. Certain formulations of adjuvants and vaccines may be more mutagenic than others. The mutagenicity of these vaccines does not directly imply carcinogenicity of vaccines in humans. however.

It is likely that vaccine induced mutation is not the only factor involved in the development of vaccine-associated tumors. In fact. cats and dogs receive the same rabies vaccines, yet vaccine-associated tumors have not been recognized in dogs. This implies that cats have some inherent susceptibility to vaccine-associated carcinogenesis. Furthermore, there is some evidence suggesting that there are individual differences among cats in their susceptibility to vaccine-associated tumors. Complete characterization of the etiology of this cancer will require identification of susceptibility factors. Determining the mechanism for vaccine and aluminum hydroxide induced mutagenesis may be critical to understanding feline susceptibility to tumorigenesis and ultimately, to preventing it.

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CHAPTER 3: FELINE VACCINES INDUCE DNA DAMAGE AND MUTATION THROUGH THE GENERATION OF REACTIVE OXYGEN SPECIES.

Introduction.

Adjuvanted feline vaccines are linked to the development of sarcomas in cats at the site of vaccination. In the last chapter, I demonstrated that adjuvanted feline vaccines and aluminum adjuvant are mutagenic and that vaccine-induced mutation depends upon the presence of vaccine adjuvant. Mutagenicity suggests a mechanism for vaccine-associated carcinogenesis.

Direct tumor induction by vaccines is unlikely since dogs and cats receive the same rabies vaccine yet dogs do not appear to develop vaccine-associated tumors. In addition, certain cats may be more susceptible to tumorigenesis than others suggesting that some factor governs predisposition to vaccine-associated tumorigenesis. Understanding the mechanism of vaccine and aluminum induced mutagenesis could be critical to determining this susceptibility factor and thus to preventing this tumor in cats and to assessing the risk of vaccines and aluminum adjuvants to humans and other animals.

Aluminum (atomic number = 13; MW = 27.0; density = 2.7 g/cm^3) is a light, silvery metal that is solid at room temperature and belongs to Group 13 of the periodic table of elements[1]. Aluminum is neurotoxic, but data concerning mutagenicity and carinogenicity are sparse and results are unclear [3-5]. There is evidence that aluminum toxicity may be mediated by oxidative stress. Although Aluminum lacks redox potential,

aluminum induced lipid peroxidation has been observed[6]. Further, aluminum induces stress responses in plants including the up-regulation of anti-oxidant enzymes such as superoxide dismutase, glutathione-*S*-transferase, and peroxidase, suggesting oxidative stress is a component of aluminum exposure[7]. In addition, aluminum inactivates glucose-6-phosphate dehydrogenase, an enzyme involved in protection from oxidative stress via the pentose phosphate shunt[8-11].

Many "heavy" metals are mutagenic and carcinogenic including arsenite, chromate, nickel, and lead. One mechanism by which metals induce DNA damage and mutation is through induction of reactive oxygen species (ROS) and development of oxidative DNA lesions[12]. Oxidative DNA damage may be an important carcinogenic mechanism. There is evidence that most cancer results from oxidative damage[13]. This is disputed by many, however[14].

I hypothesized that adjuvanted feline vaccines and aluminum hydroxide adjuvant induce mutations through an ROS-mediated mechanism. I tested this hypothesis in vaccine and aluminum exposed A_L cells by first, evaluating the effects of free radical modulators on in vitro cytotoxicity and mutagenicity and second, by demonstrating the induction of ROS in these cells[14-16].

Materials and Methods.

Vaccines and Aluminum Adjuvant. The vaccines evaluated are commercially available and include rabies vaccines (Imrab®1 and Imrab®3 from Rhone Merieux, Inc, Athens Georgia), feline leukemia virus vaccine (Fel-O-Vax Lu-K® from Fort Dodge Laboratories, Fort Dodge, Iowa) and feline panleukopenia/calici/rhinotracheitis vaccine (Protex®-3, Intervet, Millsboro, Delaware). An aluminum hydroxide adjuvant preparation

was also evaluated (Alhydrogel® Superfos Biosector available in the US from Accurate Chemical & Scientific Corporation, Westbury, New York).

Cells. Human-hamster hybrid A_L cells[17](AND-6[18]) were cultured in F12 medium supplemented with 4% heated (56° C for 30 minutes) newborn calf serum, 3% heated (56° C for 30 minutes) fetal calf serum, 1 mM l-glutamine, 50 Units/ml penicillin, 50 ug/ml streptomycin, and 20 mM HEPES. Cultures were kept at 37°C in an incubator providing a humidified atmosphere of 5% CO_2 in air. A_L cells are available from American Type Culture Collection, Manassas. Virginia.

Clonogenic survival assay[19]. A known number of A_L cells were plated, incubated for 3 hours, exposed to various dilutions of vaccine either alone or with N-acetylcysteine (NAC) or t-buthionine-(S,R)-sulfoximine (BSO) for 24 hours or 4 days and then rinsed. Fresh medium was added and plates were incubated for 7-10 days at which time they were fixed, stained, and the colonies were counted. Results were expressed as surviving fraction (SF) and graphed against dose to obtain survival curves.

SF = (number of colonies/number of cells plated) / (CE)

CE = (number of colonies on untreated control plates/number of cells plated on control plates)

Mutation Assay. (Figure 2.1) Vaccines or aluminum hydroxide gel and untreated samples were tested simultaneously and in duplicate. Sufficient numbers of A_L cells were plated to result in at least 10^5 surviving cells following exposure to vaccine at a given concentration (based upon cytotoxicity data). Cells were plated, incubated for three hours, exposed to vaccine and NAC for 24 hours or 4 days. The plates were then rinsed and growth medium was re-added. Cells were subcultured every 2-3 days to maintain log

phase growth for an expression period after which time the progeny of mutant, surviving cells no longer have CD59 surface antigen present. On days 10 and 12, cells were harvested, counted, resuspended in serum-free medium containing 1% fetal calf serum with 1 mM l-glutamine, 50 Units/ml penicillin, 50 ug/ml streptomycin, and 20 mM HEPES. 2 x 10^5 cells were plated in 100-mm tissue culture plates, and incubated for three hours. Rabbit serum (2% v/v; Covance, Denver, Pennsylvania) and human serum (0.1% v/v; Sigma-Aldrich) as a source of complement and anti-CD59 monoclonal antibody (0.5% v/v; E7.1 clone from mouse hybridoma[20]) were added. The plates were incubated for 10 days, fixed, stained, and the colonies were counted. Cloning efficiency (CE) was determined on plates treated with rabbit serum in the absence of monoclonal antibody (three plates for each dose). Results were expressed as induced mutants/10^5 surviving cells (induced M_f) and plotted against dose, where:

CE = (average colony counts on plates treated with rabbit serum/number of cells plated);

M_f = Mutant frequency = (10^5)*(number of colonies on treated plates /number of cells plated)/(CE for treated cells);

Spontaneous M_f = (10^5)*(number of colonies on untreated plates /number of cells plated)/(CE for untreated cells);

And Induced M_f = Mutant frequency = M_f - Spontaneous M_f.

The numbers of mutants (M_f) and the number of wild-type cells (Number of cells plated – M_f) was compared for treated and untereated samples by Chi square.

ROS Production. To measure the production of reactive oxygen species (ROS), a fluorescent indicator dye, 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate,

di(acetoxymethyl ester) (CDCFDA; Molecular Probes, Eugene, Oregon) was used. CDCFDA enters cells passively and is trapped intracellularly by the action of cellular esterases. In its reduced form, CDCFDA does not fluoresce. When CDCFDA becomes oxidized by intracellular ROS and is excited by a 490-nm laser, the dye emits light of approximately 520-nm wavelength (green fluorescence). Log phase A_L cells were trypsinized, counted, and incubated with CDCFDA (10 uM) for 30 minutes at 37° C in the dark. Cells were then treated with vaccines (1:10 dilution) for 30 minutes at 37° C in the dark. Samples were then rinsed, resuspended in phosphate buffered saline, and transferred to ice for 10 minutes. Flow cytometric detection of relative fluorescence per cell was performed immediately. H_2O_2 was added to one sample as a positive control and one tube was left untreated as the baseline control.

Flow cytometry was performed on a Coulter® Epics® XL-MCL cytometer. A flow rate between 300 and 600 cells per second was used to detect green fluorescence in individual cells. Samples were "gated" based on 90° vs forward light scatter histogram to eliminate debris and cell clumps from the analysis. At least 10,000 cells per sample were evaluated and the data was saved as a list mode file and analyzed with FCS Express© software (Version 1.0; DeNovo Software, Thornhill, Ontario, Canada). The position of the fluorescent peak was determined for each sample. Results are expressed as percent of baseline fluorescence defined by:

% baseline fluorescence = (relative fluorescent peak position for treated sample/relative fluorescent peak position of untreated sample)x 100.

Results.

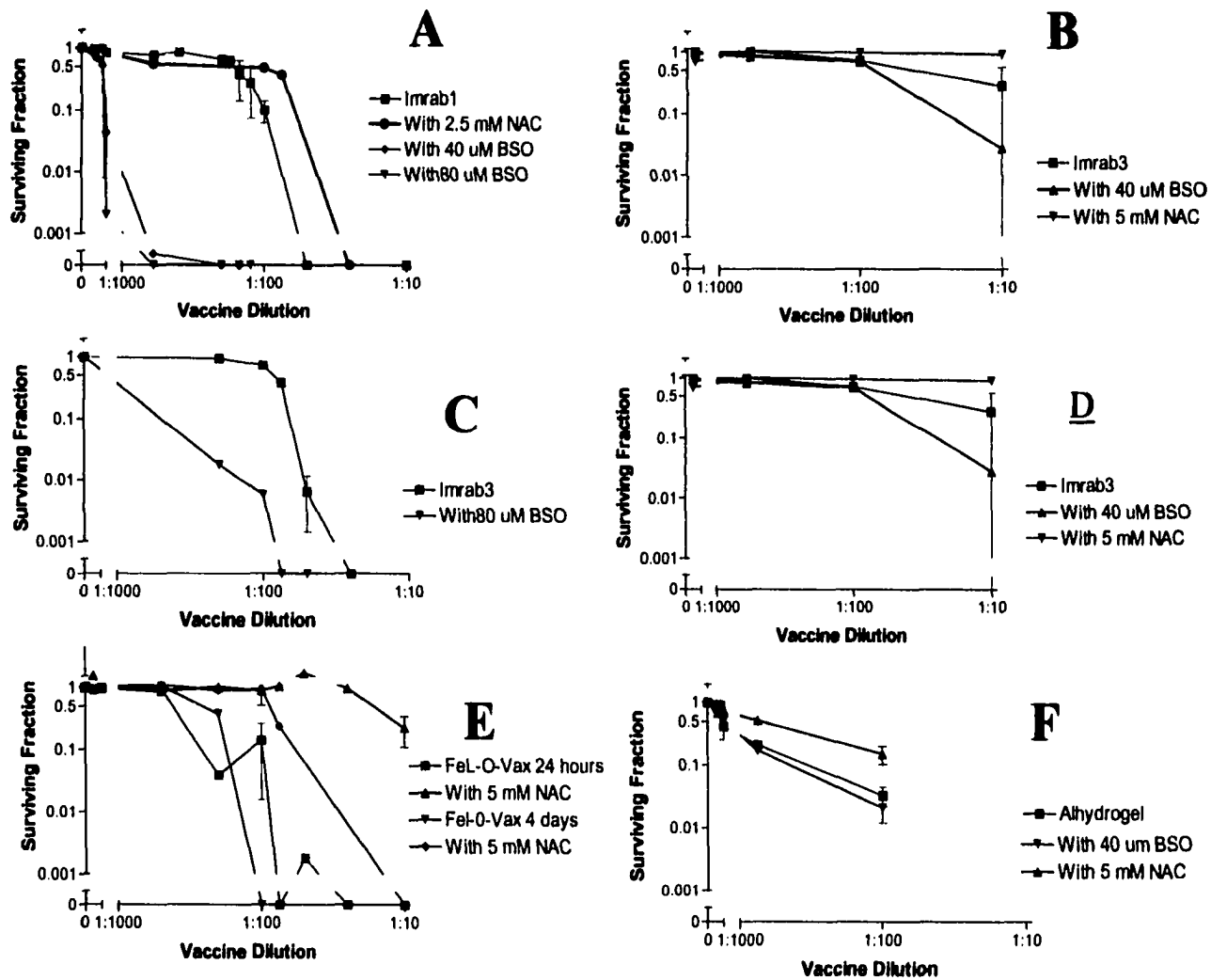

Figure 3.1. The effect of the cellular antioxidant capacity on killing of A_L cells by adjuvanted vaccines and aluminum hydroxide gel. N-acetylcysteine (NAC) enhances cellular antioxidant capacity whereas t-buthioninie (S,R) sulfoximine (BSO) depletes antioxidant capacity. Survival curves are shown for A_L cells with and without NAC or BSO exposed to: Imrab1 A) for 4 days and B) for 24 hours, Imrab3 C) for 4 days and C) for 24 hours, Fel-O-Vax E) for 24 hours or 4 days, and Alhydrogel F) for 24 hours.

Effect of Cellular Antioxidants on Vaccine-Induced Cytotoxicity and Mutagenicity. Two commercially available rabies vaccines (Imrab1 and Imrab2), a feline leukemia virus vaccine (Fel-O-Vax) and aluminum hydroxide gel (Alhydrogel) were evaluated to determine the effect of cellular antioxidants on cytotoxicity in A_L cells. NAC, which enhances cellular glutathione levels and directly scavenges free radicals, consistently decreased cell killing by these compounds[21-26]. BSO, which acts to deplete cellular glutathione, enhanced cytotoxicity of all three vaccines, but had minimal impact upon the toxicity of aluminum hydroxide gel [27-30]. (Figure 3.1) The effect of NAC on mutagenicity of the Imrab3 and aluminum hydroxide gel was also evaluated. (Figure 3.2) Mutagenicity of both the vaccine and the aluminum gel were significantly reduced by NAC. These results suggest that oxidative mechanisms are involved in vaccine and aluminum induced cytotoxocity and mutagenicity. The impact of BSO on mutagenicity has not yet been evaluated.

Vaccine induced cellular ROS production. Flow cytometric experiments with the dye, CDCFDA, were performed to measure the putative increase in cellular ROS in A_L cells following exposure to vaccines.(Figure 3.3) Induction of ROS was demonstrated by

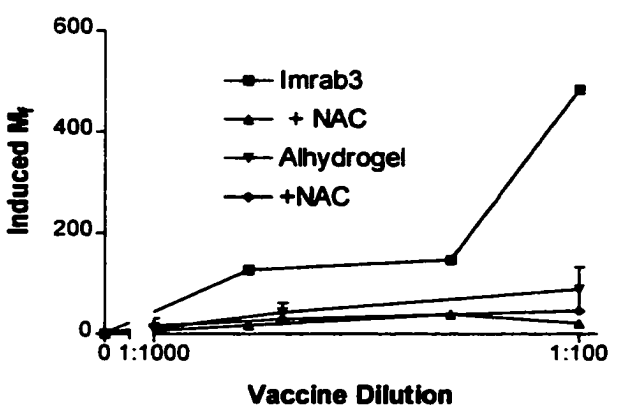

Figure 3.2. Mutation induction at the CD59 locus of A_L cells by 24-hour exposure to rabies vaccine (Imrab 3) and aluminum hydroxide gel (Alhydrogel) with and without NAC (5 mM).

intracellular oxidation of the indicator dye to a fluorescent product. When A_L cells were exposed to adjuvanted vaccines, but not when exposed to the non-adjuvanted Protex vaccine. This suggests that the adjuvant is responsible for ROS induction.

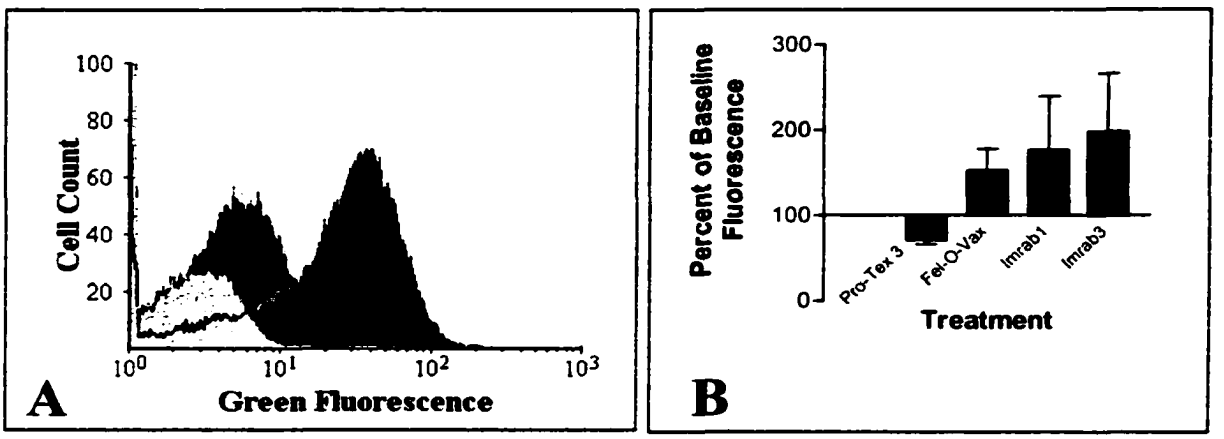

Figure 3.3. CDCFDA staining of A_L cells. A) Flow cytometric histogram showing controls which included unstained (light gray), CDCFDA stained, untreated (dark gray) and H_2O_2 (250 uM) treated (black) A_L cells. H_2O_2 induces a 600% increase in fluorescence over untreated cells. B) Induction of CDCFDA staining by exposure to vaccines (1:10 dilutions) for 30 minutes expressed as a percent of baseline (untreated cells) fluorescence.

Conclusions.

ROS are induced by vaccines and aluminum and appear to be involved in *in vitro* cytotoxicity and mutagenicity in A_L cells. This has never been demonstrated previously. Oxidative DNA damage has been implicated in tumorigenesis. Evidence for this includes: 1) Oxidative DNA damage due to endogenous production of mitochondrial ROS is a frequent event[13]. 2) With increasing age, animals accumulate oxidative lesions in DNA[31,32]. 3) Oxidative base lesions can cause mispairing of DNA bases and mutation[33,34]. 4) Differences in oxidative lesions are observed in DNA spectra between normal and tumor tissue[35-38]. 5) Diets high in anti-oxidants reduce the risk of cancer[39-42].

6) Oxidants and oxidative stress can induce transformation in vitro[43-47]. To my knowledge, however, no tumor has been demonstrated to result directly from oxidative damage.

It is conceivable that oxidative damage is primarily responsible for the development of feline vaccine-associated sarcoma. Inflammation at the vaccination site, which also induces local production of ROS, may enhance the oxidative stress induced by these vaccines. Feline vaccine-associated sarcoma may provide the only animal model of the role of oxidative DNA damage in carcinogenesis. Further investigation of oxidative lesions in vaccine-associated tumors is indicated.

Genetic susceptibility appears to be important in vaccine-associated carcinogenesis. If oxidative mechanisms are suspected in the development of these tumors, sensitivity to oxidative damage may account for the predisposition in cats. Cats, in general, are sensitive to oxidants. Heinz bodies, red blood cell lesions resulting from the oxidation of hemoglobin, are considered a normal finding only in cats and may be found in seemingly excessive numbers in healthy cats[48,49]. Cats can become severely intoxicated from minimal exposures to oxidant toxins such as onions or onion powder in baby food, propylene glycol present in some pet foods, and acetominophen[50-54]. Oxidant toxicity in the cat is characterized by excessive Heinz body formation and hemolytic anemia. Cats may also have low levels of tissue antioxidants compared to other species[55]. In humans, we know that factors like anti-oxidant capacity, enzyme expression, and DNA repair capacity are variable in individuals and may contribute to susceptibility to cancer[46,56,57]. The same is probably true of cats.

Cats can provide a model for cancer susceptibility due to genetic polymorphism and chemical exposure, a process that may be a critical to human spontaneous tumor development[56]. I am initiating studies to evaluate antioxidant capacity, sensitivity to oxidative lesions, and repair capabilities of cats with and without feline vaccine associated sarcoma.

The role of oxidative DNA damage in vaccine-associated sarcoma, also suggests a mechanism for prevention of this tumor. Vaccines that induce little oxidative stress or vaccines containing antioxidants might greatly decrease the risk of tumors. The pharmaceuticals industry should consider ROS induction when developing drugs and vaccines, particularly for cats.

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   Ref Type: Electronic Citation

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PART II: BUILDING A BETTER MUTATION ASSAY

CHAPTER 4: DETECTION OF MUTATION BY FLOW CYTOMETRY IN HUMAN-HAMSTER HYBRID A_L CELLS: A HIGH THROUGHPUT MAMMALIAN CELL MUTATION ASSAY.

Introduction.

Measuring carcinogenic risk. Determining the carcinogenic potential of chemicals and treatments in the human population is difficult, time consuming, and expensive. Human epidemiologic studies have been helpful in identifying carcinogens and quantifying the risk to humans based on actual exposures of the population[1]. Reports of increased occupational cancer risk, breast cancer in nuns and scrotal cancer in chimney sweeps, appeared as early as the 18th century[1]. Unfortunately, epidemiology has limitations including the observational, as opposed to experimental, nature of studies, the difficulty in identifying *specific* causes for these observations, the existence of confounding variables including choice of control population, and a requirement for high dose exposures for an observable effect[1]. Although human data is most relevant, it is rarely available.

Animal carcinogenesis studies, based upon the assumption that agents that cause cancer in animals are likely to cause cancer in humans and vice versa, have been used as an alternative to human epidemiologic data. These studies are not always applicable to humans, however. Animal carcinogenesis studies evaluate exposures to the maximum tolerated dose (MTD) of a chemical[2]. These high-dose, long-term exposures, particularly coupled with physiologic and genetic species differences, may have little

relevance to the human population[2]. Additionally, the time and expense involved in long term animal studies, running millions of dollars and requiring 3-4 years to perform[3]. Only about 1% of all chemicals have been evaluated in animal studies[3].

Mutation assays. Short-term bioassays based upon the measurement of mutation were designed to provide an experimental method to rapidly and inexpensively predict carcinogenicity. The fundamental premise of in vitro assays is that the mutagenic potential of an agent correlates with its carcinogenic potential in humans. In support of this idea is the abundant experimental evidence demonstrating that mutations, defined as a heritable alteration in DNA, drive carcinogenesis from the initiating event to the development of an increasingly malignant phenotype[4-6]. In fact, induction of mutation is a common characteristic and almost certainly the underlying mechanism of most carcinogenic agents regardless of whether DNA damage results directly or indirectly from the exposure[7,8]. As a result, mutation assays are important in the evaluation of drug and chemical safety and in investigations of carcinogenic mechanisms[9].

Mutation assays have been developed in a number of species including bacteria, plants, yeast, and mammalian cells[10-30]. Although most assays are entirely in vitro, transgenic mouse, fish, and rat assays are available[10,31].

Mechanics of mutagenicity testing. In all cases, mutagenicity testing requires two steps: 1) exposure to an agent and 2) identification of mutation. Mutations can be identified in various ways. Commonly, detection of mutation is based upon selection of an altered cell phenotype. These types of assays evaluate the integrity of a specific genetic locus or loci for mutation. For instance, cells that are mutated in a particular gene may become resistant to antibiotic (reversion mutation) or have altered nutritional requirements, such

that mutant cells can be selected and differentiated from wild-type cells[30,32]. Reversion assays detect a very limited kind of mutations[10,33]. Other assays evaluate for a specific type of mutation such as chromosome aberrations and micronuclei[13,34]. In general, a lag or expression period is required between exposure to an agent and detection of to allow for processing of DNA damage and for expression of an altered phenotype.

The predictive value of mutation assays is assessed by correlating mutagenicity data with rodent and human carcinogenicity data. Existing assays are limited by: 1) the accuracy of the assay in predicting carcinogenic potential, and/or 2) the considerable time, labor, and expense involved, particularly in characterizing *both* the number and types of mutations.

The accuracy of a mutation assay is related to its ability: 1) to detect various types of mutations including small point mutations and large chromosomal deletions and re-arrangements, and 2) to mimic expected human exposure with respect to dose, cellular uptake, and metabolism[17,21,35,36 26,33,37,38]. Bacterial assays have relatively low sensitivity and specificity compared to mammalian assays because 1) only small point mutations can be detected with these systems and 2) bacteria are fundamentally different from eukaryotes in many aspects of genome organization, cell structure, and metabolism[33]. Bacterial assays are relatively easy and inexpensive to perform however and have become the most widely used tests for mutagenic/carcinogenic agents[39]. Mammalian mutation assays are more predictive of carcinogenicity, but tend to be relatively time consuming, laborious, and expensive[39]. (Table 4.1) Ideal mutation assays would combine the high throughput capacity of bacterial systems with the greater predictivity provided in mammalian somatic cell systems.

Table 4.1. Predictivity of common mutagenicity assays.

Mutagenicity Assay	Sensitivity	Specificity	Concordance
Ames Test	53%	53%	53%
Mouse Lymphoma	77%	30%	59%
Chromosome Aberrations	62%	60%	61%
Sister Chromatid Exchange	87%	30%	64%

Accuracy of bacterial (Ames) and mammalian cell (Mouse lymphoma, chromosome aberrations, and sister chromatid exchange) in predicting chemical carcinogenicity. Sensitivity is the % of carcinogens that test positive. Specificity is the % of noncarcinogens that test negative. Concordance is the % agreement between carcinogenicity and assay data.

The A_L mutation assay. The A_L assay, developed by Waldren and his co-workers, has been used for 30 years to evaluate a variety of agents for mutagenicity[40-55]. Human-hamster hybrid A_L cells contain CHO chromosomes plus a single human chromosome 11[56].(Figure 4.1) Human cellular antigens coded for by chromosome 11 including the cell surface antigen, CD59 encoded by the *CD59* gene at 11p13.5, are expressed on A_L cells. After exposure to a mutagen, the surviving A_L cells are either wild-type for *CD59* or are mutated to $CD59^-$.(Figure 2.1) (Note: CD59 refers to the protein whereas *CD59* refers to the gene coding for the protein.) In the traditional assay, anti CD59 antibody (E7.1) plus rabbit serum, as a source of complement, is used to kill wild-type cells, leaving $CD59^-$ ($E7.1^-$) mutants to form colonies. Mutational spectra are determined by using polymerase chain reaction (PCR) or Southern analysis to detect the presence or absence of genes that map along chromosome 11.

With data collected over 30 years from a number of labs, we have demonstrated that the A_L mutation assay in its present form which relies on complement-mediated

eradication of CD59 expressing cells, can be a very accurate predictor of carcinogenicity. I here describe the results of my work in which I used flow cytometry to significantly enhance throughput and facilitate generation of mutational spectra which promises to improve predictivity. Mutants, which were detecting by complement-mediated selection requiring 10-15 days for colony formation, can now be measured by loss of surface antigens in a few minutes.

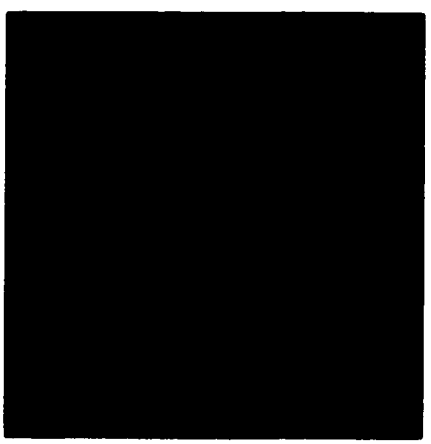


Figure 4.1. Fluorescence in situ hybridization of human DNA labeled with Lissamine (red) onto nuclei of A_L cells which are counterstained with the DNA intercalating dye, DAPI (blue). A metaphase nucleus is seen shown. Humans chromosome 11 fluoresces in red among the blue fluorescing CHO chromosomes.

Materials and Methods.

Reagents. Rabbit serum (Covance, Denver, Pennsylvania); Human Serum (Sigma-Aldrich); FACS buffer (1% Bovine Serum Albumin, 10 mM Na Azide in PBS); 0.5% paraformaldehyde (in PBS); 0.5 mM EDTA (in HBSS);

Antibodies. Unconjugated: Anti-CD59 (E7.1 clone from mouse hybridoma). [57] **Alexa Fluor 488™- conjugated:** goat anti-mouse IgG (Molecular Probes, Eugene, Oregon). **Phycoerythrin conjugated:** Anti-CD59 (Mem43 clone), Anti-CD3, and Anti-CD56 (Caltag Laboratories; Burlingame, CA). Anti-CD59 (H19 clone), Anti-CD5, Anti-CD6, Anti-CD20, Anti-CD44, Anti-CD56, Anti-CD81, Anti-CD82, Anti-CD90, Anti-CD98,

and Anti-CD151 (Pharmingen; San Diego CA). **Cy-Chrome™-conjugated:** Anti-CD56 (Pharmingen; San Diego, CA).

Cells. Human-hamster hybrid A_L cells (AND-6[58]) were cultured in F12 medium supplemented with 4% heated (56° C for 30 minutes) newborn calf serum, 3% heated (56° C for 30 minutes) fetal calf serum, 1 mM l-glutamine, 50 Units/ml penicillin, 50 ug/ml streptomycin, and 20 mM hepes. Cultures were kept at 37°C in an incubator containing a humidified atmosphere of 5% CO_2 in air. A_L cells are available from American Type Culture Collection, Manassas, Virginia.

Clonogenic survival assay[59]. To evaluate chemicals for cytotoxicity, a known number of A_L cells were plated, incubated for 3 hours to allow for cell attachment, exposed to the chemical at various concentrations for 3 to 24 hours, rinsed and fresh medium was added. For evaluation of radiation, cells were irradiated in suspension and then plated. Plates were incubated for 7-10 days, fixed, stained, and the colonies were counted. Colony counts were adjusted for cloning efficiency determined on untreated control plates. Results were expressed as surviving fraction (SF) and graphed against dose to obtain survival curves.

SF = (number of colonies/number of cells plated) / (CE)

Standard (complement-mediated killing) Assay. (Figure 2.1) (I will refer to the standard assay as the CMC (Complement-mediated cytotoxicity) assay). Sufficient numbers of A_L cells were plated to result in at least 10^5 surviving cells following

exposure to a given dose of an agent. For chemicals, cells were plated, incubated for three hours, exposed for 3-24 hours, rinsed, and then fresh media was added. For irradiations, cells were irradiated in suspension and then plated. Cells were subcultured every 2-3 days to maintain log phase growth for an expression period after which time the progeny of mutant surviving cells no longer have CD59 surface antigen present. On days 10 and 12, cells were harvested, counted, resuspended in serum-free media containing 1% fetal calf serum with 1 mM l-glutamine, 50 units/ml penicillin, 50 ug/ml streptomycin, and 20 mM HEPES. 2 x 10^5 cells were plated in 100-mm tissue culture plates, and incubated for three hours. Rabbit serum (2% v/v; Covance, Denver, Pennsylvania) and human serum (0.1% v/v; Sigma-Aldrich) as a source of complement and anti-CD59 monoclonal antibody (0.5% v/v; E7.1 clone from mouse hybridoma[57]) were added. The plates were incubated for 10 days, fixed, stained, and the colonies were counted. Cloning efficiency (CE) was determined on plates treated with rabbit serum in the absence of monoclonal antibody (three plates for each treatment). Results were expressed as induced mutants/10^5 surviving cells (induced M_f) and plotted against dose, where:

CE = (average colony counts on plates treated with rabbit serum/number of cells plated);

M_f = Mutant frequency = (10^5)*(number of colonies on treated plates /number of cells plated)/(CE for treated cells);

Spontaneous M_f = (10^5)*(number of colonies on untreated plates /number of cells plated)/(CE for untreated cells);

And Induced M_f = Induced Mutant frequency = M_f - Spontaneous M_f.

The numbers of mutants (M_f) and the number of wild-type cells (Number of cells plated – M_f) was compared for treated and untreated samples by Chi square.

Flow Cytometry. (Figure 4.2) A_L cells were treated with a mutagen and subcultured for 10-12 days as described in the previous section. The cells were then harvested using 0.5 mM EDTA, to prevent trypsin degradation of surface antigens, and counted. The cells were resuspended in cold FACS buffer, pelleted by centrifugation, and incubated on ice for 30-40 minutes with phycoerythrin-conjugated monoclonal antibody to human CD59

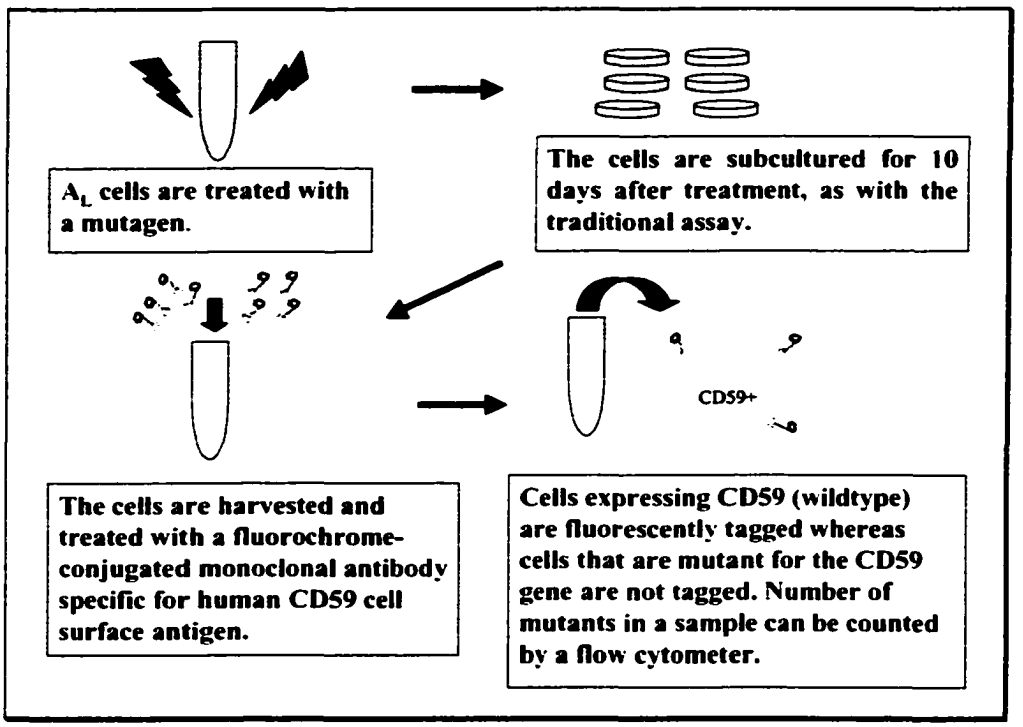


Figure 4.2. Flow cytometric A_L assay.

(Mem43 clone). The cells were rinsed twice in cold FACS Buffer and resuspended in 0.5% cold paraformaldehyde. After a fifteen-minute incubation on ice, the samples were centrifuged and resuspended in FACS Buffer (10^6 cells/ml). Untreated A_L served as the positive control and mutant A_L cells (128 cells) that lack all but the distal p-arm of chromosome 11 served as a negative control[60,61]. CHO-K1 cells were stained with these antibodies to demonstrate lack of cross-reaction with hamster antigens.

Flow cytometry was performed on a Coulter® Epics® XL-MCL cytometer (Beckman Coulter Inc, Fullerton, California). Samples were gated, based upon 90 degree vs forward light scatter characteristics, to eliminate cell clumps and debris from the analysis. Voltage across the photomultiplier tubes was adjusted to obtain analyzable histograms with good separation of fluorescent and non-fluorescent cells. Flow rates between 300 and 600 cells per second were used to collect data from about 10^5 cells per sample. List mode files were collected for each experiment. Data analysis was performed with FCS Express© software (Version 1.0; DeNovo Software, Thornhill, Ontario, Canada). Histogram Regions were set to enumerate non-fluorescent and fluorescent cells. The percent of cells/events in the non-fluorescent region of untreated wild-type cells was taken as background and was subtracted from the percent of cells in the non-fluorescent region of treated samples. Results were expressed as induced M_f = number mutants/ 10^5 surviving cells.

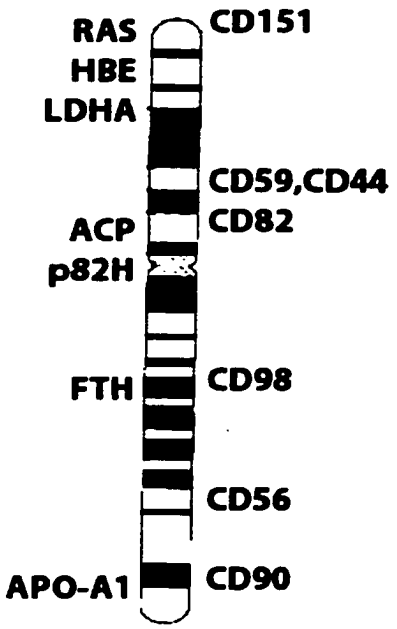


Figure 4.3. Ideogram of human chromosome 11 showing positions of gene markers used in the generation of mutational spectra using the traditional (left) and flow cytometric (right) A_L assay.

Generation of mutational spectra with molecular biologic techniques[62,63]. Mutational spectra were determined by evaluating mutant clones: 1) for the absence or presence of the 4 exons of the *CD59* gene using PCR and 2) for the absence or presence of other human genes mapped to chromosome 11 using PCR or

Southern analysis. (Figure 4.3) (Data provided by D.Vannais)

Generation of mutational spectra by flow cytometry. Chromosome 11 codes for a number of cell-surface antigens that are expressed on A_L cells.(Figure 4.3) The expression of several of these antigens was evaluated by staining and fixing with wild-type A_L, mutant A_L (128), and CHO-K1 cells with phycoerythrin-conjugated monoclonal antibodies, as described above.

Results.

Sensitivity and specificity of traditional A_L assay. The A_L assay has been used for thirty years in various laboratories to investigate mutagenicity. The results of the A_L assay correlate strongly with carcinogenicity data, more so than other widely used assays.

Flow cytometric detection of CD59 surface antigen on A_L hybrid cells. Monoclonal antibodies to different epitopes of CD59 are available.(Figure 4.4) A_L cells can be labeled with fluorochrome-conjugated monoclonal antibodies for rapid differentiation of wild-type (*CD59+*) from mutant (CD59-) cells. (Figure 4.5)

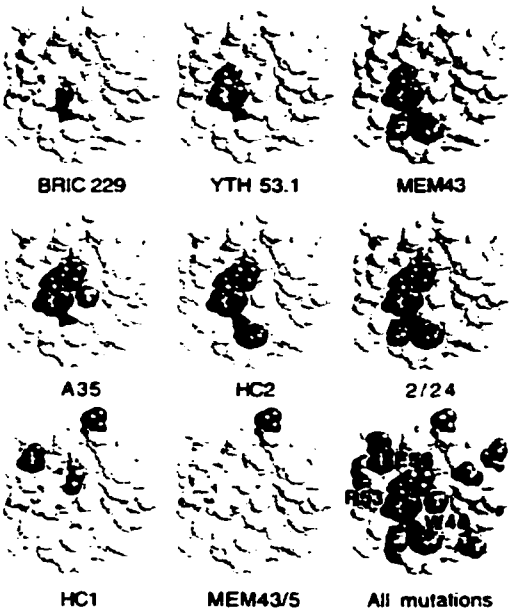


Figure 4.4. Diagramatic representation of the CD59 molecule based upon mutational and structural data. The binding epitopes for various antibodies to CD59 are depicted. (Reproduced from The Journal of Experimental Medicine,1997,185(3), p.513. by copyright permission of Rockefeller University Press.)

Accuracy of CD59 detection by flow cytometry (reconstruction experiments). To determine the accuracy of CD59 detection, Mutant A_L cells were mixed with wild-type cells in known proportions (0.1% to 1% mutant cells) and these samples were stained and fixed for flow cytometry. Flow cytometric detection of CD59 antigen was compared to the actual proportion of mutant cells. Identification of CD59 surface antigen with phycoerythrin-conjugated Mem43 antibody is highly accurate. I can identify as few as1 mutant cell (CD59-) in a population of 1000 A_L cells with calculated sensitivity and specificity exceeding 99%. (Figure 4.5; Table 4.2)

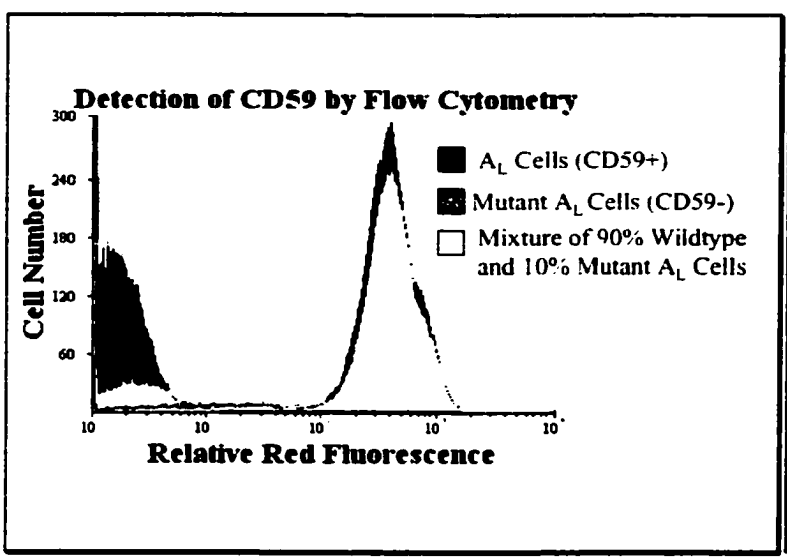


Figure 4.5. Histogram demonstrating flow cytometric detection of CD59 with a phycoerythrin labeled monoclonal antibody (Mem43) against a particular epitope of this surface antigen. Results from three populations of A_L cells are shown, including wild-type (*CD59+*), mutant (CD59-), and a population produced by mixing wild-type with known mutant cells.

Table 4.2. Accuracy of CD59 detection by flow cytometry of mixed populations of A_L cells:

Actual Proportion of mutant cells:	Number of CD59- cells identified:	Number of *CD59+* cells identified:	% CD59- cells: (corrected for background)
0	544	142,218	0
.1	758	140,441	0.16
.2	261	44,909	0.2
.5	1494	140,649	0.68
1	1642	94,948	1.34

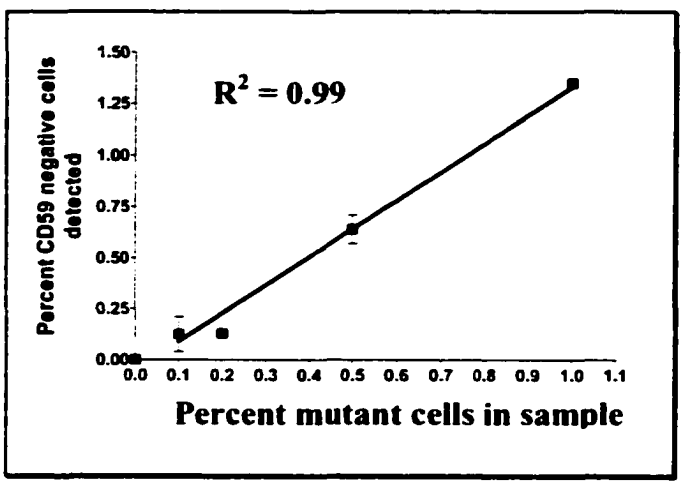


Figure 4.5. Graph demonstrating the accuracy of CD59 detection with flow cytometry. Mixed populations of A_L cells were created by adding known proportions of mutant and wild-type cells. The cell mixtures were stained for CD59 and run through the flow cytometer. The percent CD59- cells detected by flow cytometry and corrected for background is plotted against the actual proportion of mutants in the mixture. A regression line is shown

Comparison of mutagenicity data generated by the flow cytometric assay with the CMC A_L assay. To determine how well flow cytometric assay compares to the traditional complement-mediated assay, and therefore carcinogenicity data, I evaluated the same A_L cell population using both the traditional and the flow cytometric assay. Mutation induction curves were generated for populations exposed to EMS, ^{137}Cs gamma irradiation, and carbon particle irradiation (LET = 100 keV/uM). (Figure 4.7) No significant difference was seen between the mutation dose-response curves generated with the complement-mediated and flow cytometric assays.

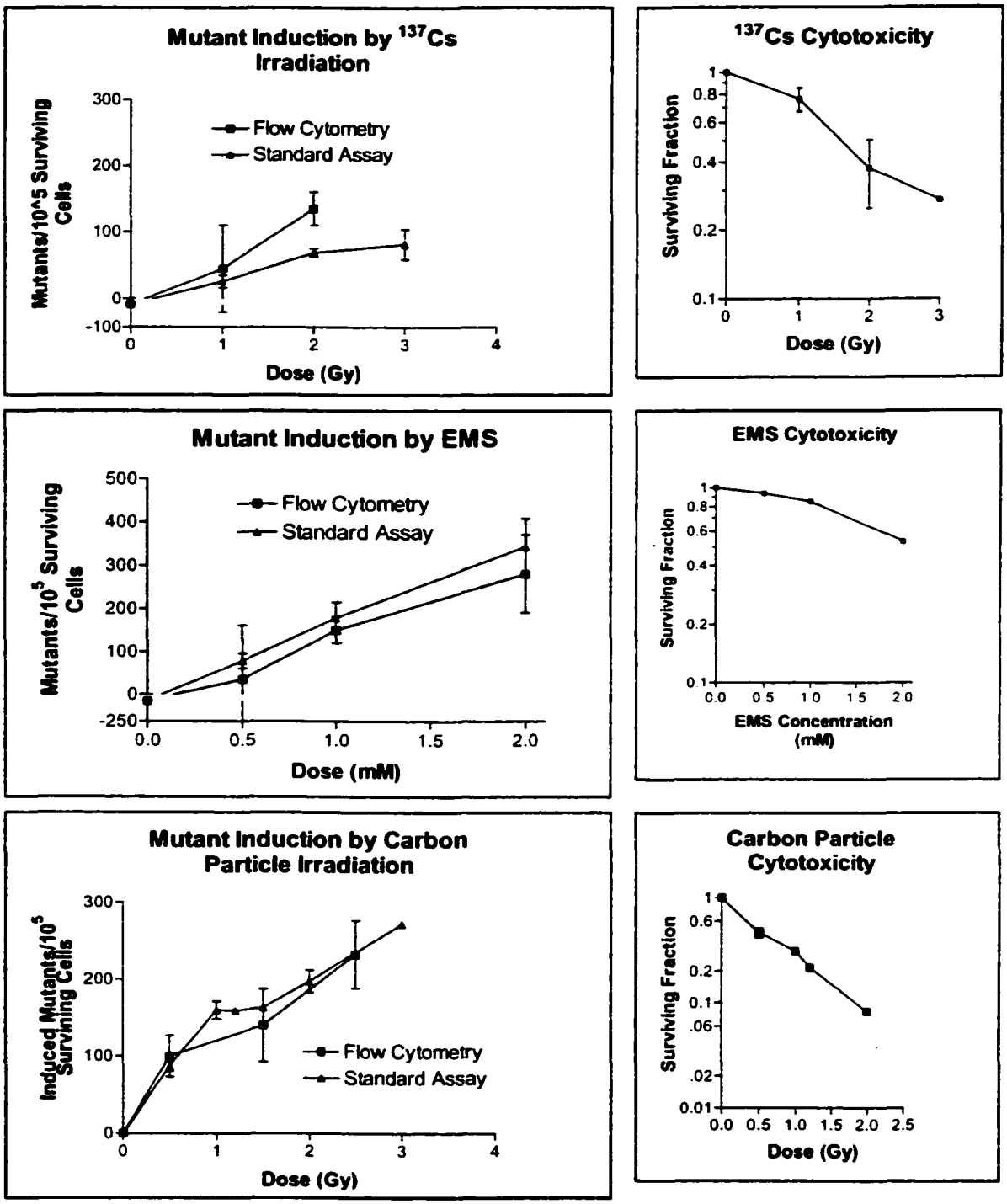


Figure 4.7. Mutation induction (left) and survival (right) curves for ^{137}Cs (Dose rate = 2 Gy/min; Do = 1.5) and carbon particle (290 MeV/nucleon; Do = 0.8; LET = 100keV/micron) irradiation and EMS exposure. Mutation data was obtained using both the standard and the flow cytometric A_L assays.

Generation of mutational spectra with flow cytometry. .Human chromosome 11 is known to code for a number of cell-surface antigens, in addition to CD59. I used flow cytometry to determine which antigens are expressed on A_L cells. (Table 4.3) I found seven human cell surface antigens that are expressed. The monoclonal antibody against human CD81, however, cross reacted with parental CHO cells, and was therefore excluded from further studies.

Table 4.3. Expression of human cell surface antigens on A_L CHO x human hybrid cells:

Surface Antigen:	Stains A_L Cells?	Stains CHO-K1 Cells?
CD3	No	No
CD5	No	No
CD6	No	No
CD20	No	No
CD44	Yes	No
CD56	Yes	No
CD81	Yes	Yes
CD82	Yes	No
CD90	Yes	No
CD98	Yes	No
CD151	Yes	No

Mutational spectra, defined as a collection of mutations resulting from a specific treatment or exposure, for A_L CD59- mutants have been generated using molecular biologic techniques to determine the absence or presence of chromosome 11 genes[62.63]. The presence or absence of these genes, which are mapped to defined regions of the chromosome, provides information regarding the size of the mutation resulting in the CD59- phenotype. The genes for the human cell surface antigens that are expressed in A_L cells map to similar positions on chromosome 11.(Figure 4.3) It is possible, therefore, to obtain the same sort of data for mutational spectra by evaluating either the expression of human cell surface antigens with flow cytometry or the presence of chromosome 11

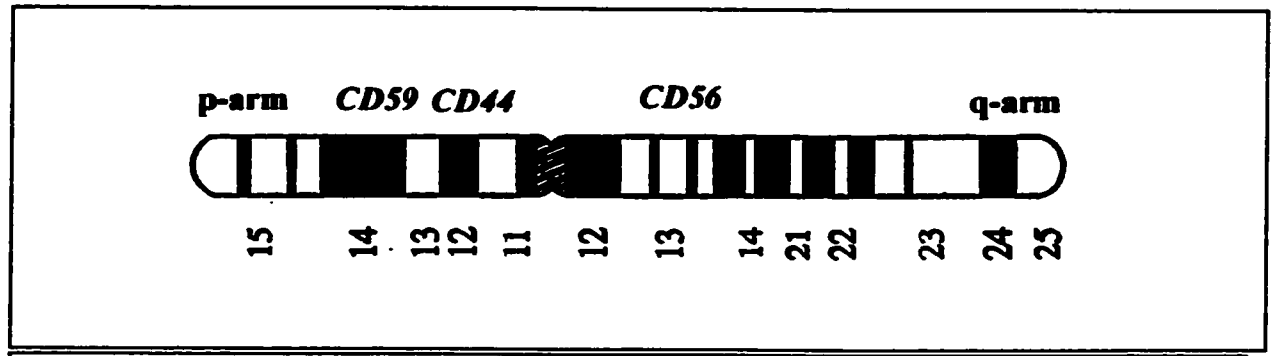
genes with molecular methods, but flow cytometry is relatively simple and quick compared to PCR and southern analysis, however. PCR and southern analysis might require several days to complete, whereas, flow cytometry can be performed in a few hours.

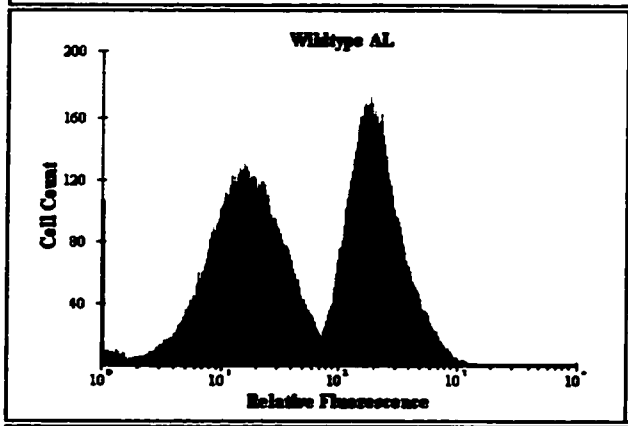
To demonstrate the generation of mutational spectra with flow cytometry, I selected five CD59- mutants, previously evaluated by molecular DNA techniques for the presence and absence of chromosome 11 genes, and stained these with monoclonal antibodies to five human cell surface antigens.(Table 4.4) Only one of the mutants (Mutant 128) evaluated revealed an inconsistency between the flow cytometric and molecular data. This particular clone had been subcultured numerous times for over five years since the last molecular analysis, arousing suspicion that genetic changes might have occurred, accounting for the discrepancy. We re-evaluated the 128 mutant clone by PCR and determined that the clone had lost the genetic markers, FTH and APO, since the earlier evaluation. This further demonstrated the accuracy of the flow analysis.

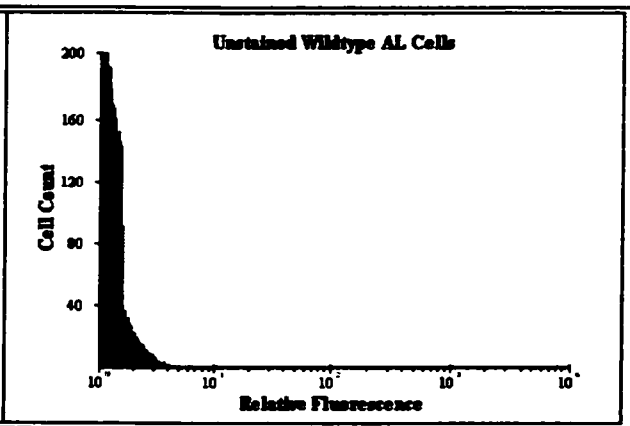
Table 4.4. Comparison of methods for obtaining mutational spectra:

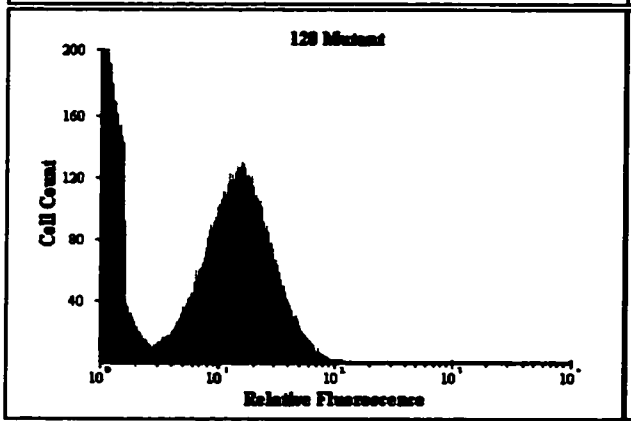
	Presence or Absence of Chromsome 11 Genetic Markers:						
	RAS	*CD59* gene	CAT	ACP	FTH	APO	
Wild-type A_L	+	+	+	+	+	+	
Mutant Lines:							
1 (128)	+	-	-	-	- (+)	- (+)	
2 (901)	+	+	+	+	-	-	
3 (1039-6)	+	-	-	+	+	+	
4 (1039-2)	+	-	-	+	+	+	
	Presence of Human Surface Antigens:						
	CD151	CD59	CD44	CD82	CD98	CD56	CD90
Wild-type A_L	+	+	+	+	+	+	+
Mutant Lines:							
1 (128)	+	-	-	-	-	-	-
2 (901)	+	+	+	+	-	-	-
3 (1039-6)	+	-	-	+	+	+	+
4 (1039-2)	+	-	-	+	+	+	+

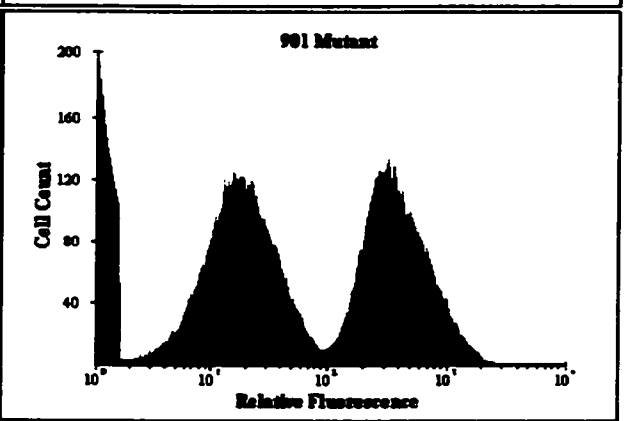
Flow cytometry is, therefore, an accurate, simple, and inexpensive method for determining mutational spectra relative to Southern and PCR analysis. The molecular techniques may cost $2000 whereas the flow cytometric data could be obtained for a few hundred dollars. Simultaneous detection of cell surface antigens with multicolor flow cytometry would increase the speed and simplicity of this assay. I have demonstrated proof of principle by detecting expression of three cell surface antigens simultaneously in three different A_L mutants. (Figure 4.8) I can readily determine the presence or absence of three differentially stained cell surface antigens in mutant and wild-type cells.











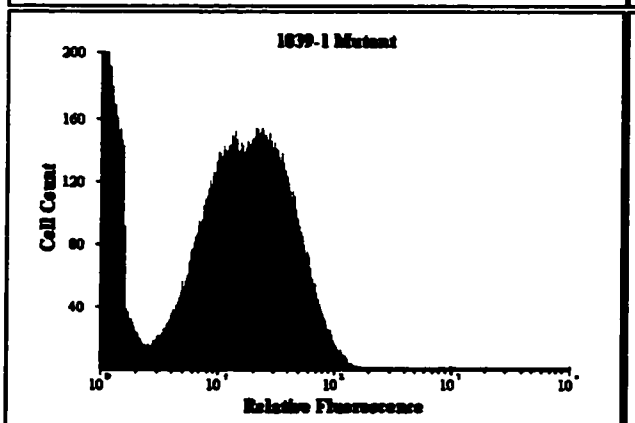


Figure 4.8. Simultaneous, flow cytometric, detection of three cell surface antigens on A_L cells stained with FITC-conjugated CD59(Green), PE-conjugated CD44(red), and Cy-Chrome-conjugated CD56 antibodies. A: Idiogram of chromosome 11 indicating the positions of the genes coding for the three antigens. B-F: Histograms for wild-type A_L (B) stained and (C) unstained are shown along with mutants: (D) p-arm deleted, (E) q-arm deletion, and (F) the *CD59* gene deleted.

Discussion.

Determining the carcinogenic risk of drugs and chemicals to humans is a complicated, yet important process, both scientifically and monetarily. Unfortunately, epidemiologic and animal carcinogenicity data are unavailable for most individual chemicals not to mention mixtures. Mutagenicity testing can help in predicting carcinogenicity, but many short-term assays are inaccurate and others are laborious and expensive. The flow cytometric A_L assay offers a potentially accurate and relatively inexpensive, high throughput, mammalian, mutagenicity test. I have demonstrated that the traditional A_L assay is an accurate predictor of carcinogenicity and that the flow cytometric assay correlates well with the traditional assay. Evaluation of a variety of chemicals in different laboratories is required to further validate the flow cytometric A_L assay.

Our data indicates less precision with flow cytometry compared to the CMC assay based on larger standard error in the mutant induction experiments using irradiation and EMS. This difference may be explained by 1) error in the flow cytometric technique or 2) inherent fluctuations in the flow cytometric detection of mutants. Technical limitations may be overcome by collecting data from more cells in a given sample and by optimizing instrumentation and sample preparation. I collected data for 10^5 cells per sample, but with automation, large numbers of cells ($>10^6$) could be analyzed with minimal effort. The apparent lack of precision may reflect heterogeneity in the type of mutants present in the sample. In the flow cytometric assay, various mutants may reveal various patterns of fluorescence, whereas in the *CMC* version of the A_L assay, mutations that eliminate binding of the CD59 antibody are phenotypically the same, i.e. these cells survive and

form colonies after treatment with monoclonal antibody and complement. Point mutations may decrease the binding affinity of the CD59 antibody, resulting in a relative decrease, but not absence, of fluorescence as detected by flow cytometry. Large deletions would be expected to completely eliminate antibody binding and hence, fluorescence. A third class of mutants with variable levels of fluorescence occurs secondary to mutations in the CHO genes encoding the glycosylphoshotidylinositol (GPI) anchor that secures CD59 is to the cell membrane. I have identified GPI mutants that survive complement and antibody challenge (CD59- phenotype). When stained with CD59 monoclonal antibody, these mutants exhibit variable levels of fluorescence ranging from none to an intermediate level, somewhat below that of wild-type cells. This special class of mutants will be discussed more fully in the next chapter. The ability to readily distinguish between types of mutants is a distinct advantage of flow cytometry.

One of the particularly attractive features of the flow cytometric assay is the ability to rapidly generate mutational spectra. Alterations in A_L mutational spectra have been demonstrated and could provide important mechanistic information for the evaluation of genotoxic compounds. The flow cytometric assay can easily be packaged as a kit including antibodies and standardized analysis software for use by different laboratories with existing flow cytometry equipment. In addition, flow cytometry can be performed on fixed cells, so that samples may be stored and run in batches, to save time. The significance and comparison of mutational spectra is discussed in Chapter 7.

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CHAPTER 5: THE ROLE OF THE GPI ANCHOR IN EXPRESSION OF THE CD59 CELL SURFACE ANTIGEN ON A_L CELLS.

Introduction.

In the last chapter, I described two versions of the A_L mutation assay, which evaluates for mutagenicity at the human *CD59* locus in A_L CHO-human hybrid cells. This assay is an accurate, meaning both sensitive and specific, method for predicting carcinogenicity of chemicals and radiation. I showed that data on the number and types of mutants produced by an agent can also be obtained with a flow cytometric version of the A_L assay and that the flow cytometric assay is faster, less expensive, and produces more information than the original complement-mediated cytotoxicity (CMC) assay.

The main difference between the CMC and the flow cytometric A_L assays is the method for detecting the CD59- phenotype. In the CMC assay, the mutant phenotype is selected for by complement-mediated killing of wild type cells, whereas in the flow cytometric assay, CD59- cells are recognized and quantified by absence of labeling with fluorescent CD59 monoclonal antibody. In the CMC assay, classification of phenotype is purely dichotomous: the cell survives (mutant) or it dies (wild type). In the flow cytometric assay, mutants have been shown to exhibit various degrees of fluorescence based upon the type of mutation. These differences provide information about the nature of the underlying mutation. For example, point mutations may result in intermediate levels of fluorescence: clearly less fluorescent than wild type cells, but more fluorescent than cells that do not express the antigen[1]. Thus, the

flow cytometric assay can provide some information, based upon fluorescent characteristics, about the types of mutations causing the CD59- phenotype. In characterizing A_L *CD59* point mutants using flow cytometry, we have identified a class of A_L mutant that does not express CD59 due to a mutation in the CHO genes coding for the glycosylphosphotidylinositol (GPI) anchor.

The Role of the CD59 Surface Antigen in Health And Disease. The CD59 surface antigen has an immunologic function and is particularly important in preventing immune-mediated disease[2]. In humans, loss of expression of CD59 and other surface antigens is associated with the disease, paroxysmal nocturnal hemoglobinuria (PNH), the pathophysiology of which is still poorly understood[2-4]. In PNH, massive intravascular hemolysis causes the characteristic hemoglobinuria.[2] The underlying genetic defect in people that are afflicted with PNH is a mutation in one of the genes, called PIGA, required for expression of the GPI anchor that is required to attach certain cell surface proteins, including CD59, to the cell membrane{5626}[2]. The CD59 molecule, which is expressed on all cells, protects the cell from lysis by binding components of the complement cascade{5626}{5627}. Therefore, loss of CD59 enhances the sensitivity of cells to killing by complement in immunologic reactions.

The GPI Anchor. Assembly of the glycosylphosphatidyl inositol (GPI) anchor and its subsequent attachment to certain cellular proteins is mediated by the gene products of at least 11 different genes[5]. The anchor is attached to a carboxyl terminus signal sequence when the newly translated protein is processed in the endoplasmic reticulum, before additional modifications in the Golgi apparatus[2,6]. Vesicles from the Golgi apparatus transport the protein to the cell membrane[6]. Mutation in any of the genes responsible for

the GPI anchor synthesis and attachment are not lethal and will result in a lack of CD59 expression. Loss of surface expression of CD59 along with the loss of other GPI-linked membrane proteins, such as DAF and CD90 is characteristic of both PNH patients and GPI mutant cell lines, including CHO[2-8].

We have identified GPI mutant A_L cells generated by various mutagens. When stained with monoclonal antibody against CD59, these mutants display a range of fluorescence. GPI mutants exhibit either no fluorescence or an intermediate level of fluorescence[9]. In this chapter, I will describe the flow cytometric detection of GPI mutant A_L cells.

Materials and Methods.

Mutant Identification. The A_L mutation assays, both CMC and flow cytometric, are described in chapter 4. The mutants (I will call these GPI-mutants) that we present in this section were generated in previous experiments with either UV irradiation or with irradiated histidine treatment. Mutant clones isolated following these treatments were analyzed by PCR and Southern analysis to determine the presence or absence of chromosome 11 genes, as described in chapter 4. .(This analysis was performed by A. Ueno and D. Vannais)

Multiplex PCR. To determine the presence or absence of the four exons of the *CD59* gene, a multiplex PCR procedure has been developed. Primers were designed to amplify the entire first, second, and third exons of CD59 and 400 bp of the fourth exon. This segment of the fourth exon codes for the complement binding site, the epitopes bound by monoclonal antibodies E7.1, MEM43, and H19, as well for the GPI anchor attachment site. (Data provided by D. Vannais)

Sequencing of the Fourth Exon from *CD59* in GPI-mutant AL cells. To evaluate for point mutations in the CD59 gene, genomic DNA was isolated from two A_L mutant cell lines (1039-1 and 1039-4). The fourth exon of the CD59 gene was amplified by PCR. The PCR product was ligated into plasmids and expanded in E. coli. The plasmids were purified from the bacterial culture and sequenced. The sequence was compared to the published sequence of human CD59 using the BLAST software. (This procedure was performed by A. Holmes.)

Cell Fusion Experiments. To determine whether CD59- A_L cells were mutant in the human *CD59* gene or in CHO genes, the mutant cells were fused to GlyB CHO cells. A_L cells and the GlyB cell line are unable to synthesize glycine. These cell lines complement each other, such that fusion of the two restores glycine synthesis. A_L mutant cell lines were fused with GlyB cells and the fused cells were selected for in glycine deficient media. The fused cells were then treated with complement and monoclonal antibody, E7.1, to determine if fusion could restored CD59 expression by complementing a CHO mutation in the A_L cell.

Flow Cytometry. As described in chapter 4, mutant A_L clones were stained with monoclonal antibodies against CD59 (Alexa-conjugated), the GPI-linked CD90 (phycoerythrin conjugated), and the transmembrane protein CD44 (phycoerythrin conjugated). All of these surface antigens are coded for by human chromosome 11 and are expressed on A_L hybrid cells.

To detect the GPI anchor, fluorescent aerolysin (FLAER; Protox Biotech, Victoria, British Columbia, Canada) was used. This protein binds specifically to the GPI anchor and has been shown to allow detection of GPI anchor mutants{5628}. Flow

cytometry was performed on a Coulter® Epics® XL-MCL cytometer. (Flow cytometry experiments were performed by D. Vannais, A. Holmes, and myself.)

Results.

In the course of generating data for mutational spectra, A_L clones were identified that survived complement and monoclonal antibody treatment used in selection of mutants, but revealed an apparently intact chromosome 11 and four normal CD59 exons as determined by PCR. I will refer to this type of mutant as *GPI*-mutants, although we had not yet demonstrated this at the time that the mutants were initially characterized. We hypothesized that these mutants resulted from either very small point mutations in the CD59 gene or from mutation in a CHO locus or loci.

To evaluate for a point mutation in the *CD59* gene, genomic DNA isolated from 2 GPI-mutant clones (1039-1 and 1039-4) and we amplified the fourth exon of the *CD59* gene using PCR. The PCR products were isolated and sequenced. The sequence information corresponded exactly to that published for human *CD59*. The fourth exon codes for the portion of the CD59 molecule that binds to complement and to the GPI anchor and is identified by the monoclonal antibody E7.1 used in the A_L assay. An intact fourth exon is inconsistent with a point mutation in CD59 causing the CD59- phenotype in these mutants.

To determine if the defect in the GPI-mutants was in CHO gene(s), mutant cell lines (1039-1 and 1039-4) were fused with parent CHO cells. When the fused cells were challenged with monoclonal antibody and complement, all were killed whereas the mutant cell lines survived. This shows that CHO genes were able to overcome the defect

in the mutant A_L cells and provides genetic proof that the CD59- phenotype in these particular cells was due to a mutation in a CHO gene.

Next, we set out to determine whether these mutants were, in fact, GPI mutants. We stained a number of mutants with monoclonal antibodies to CD59 and CD90. (Figure 5.1) We analyzed over twenty GPI-mutant cell lines. These A_L clones displayed one of two patterns of fluorescence for CD59 and CD90. They either did not fluoresce at all, as with 1039-18 or exhibited an intermediate level of fluorescence relative to wild type A_L cells, as with 1039-4. The pattern of fluorescence for CD59 and CD90 was the same. In other words, 1039-4 exhibited intermediate fluorescence when stained for either CD59 or CD90 whereas, 1039-18 showed a lack of fluorescence when labeled for these antigens. The GPI-mutants did not differ from wild-type A_L cells in binding to the CD44 surface antigen that represents a transmembrane rather than a GPI-linked protein. Intermediate levels of fluorescence have been described in GPI-mutant cell lines stained for GPI-linked proteins[9]. We hypothesize that in GPI mutant cells, CD59 and other GPI-linked proteins continue to be translated. In some GPI mutants, the protein can be bound by internalized monoclonal antibody, thus leading to diminished (rather than absent) levels of fluorescence compared to cells expressing the protein on the surface.

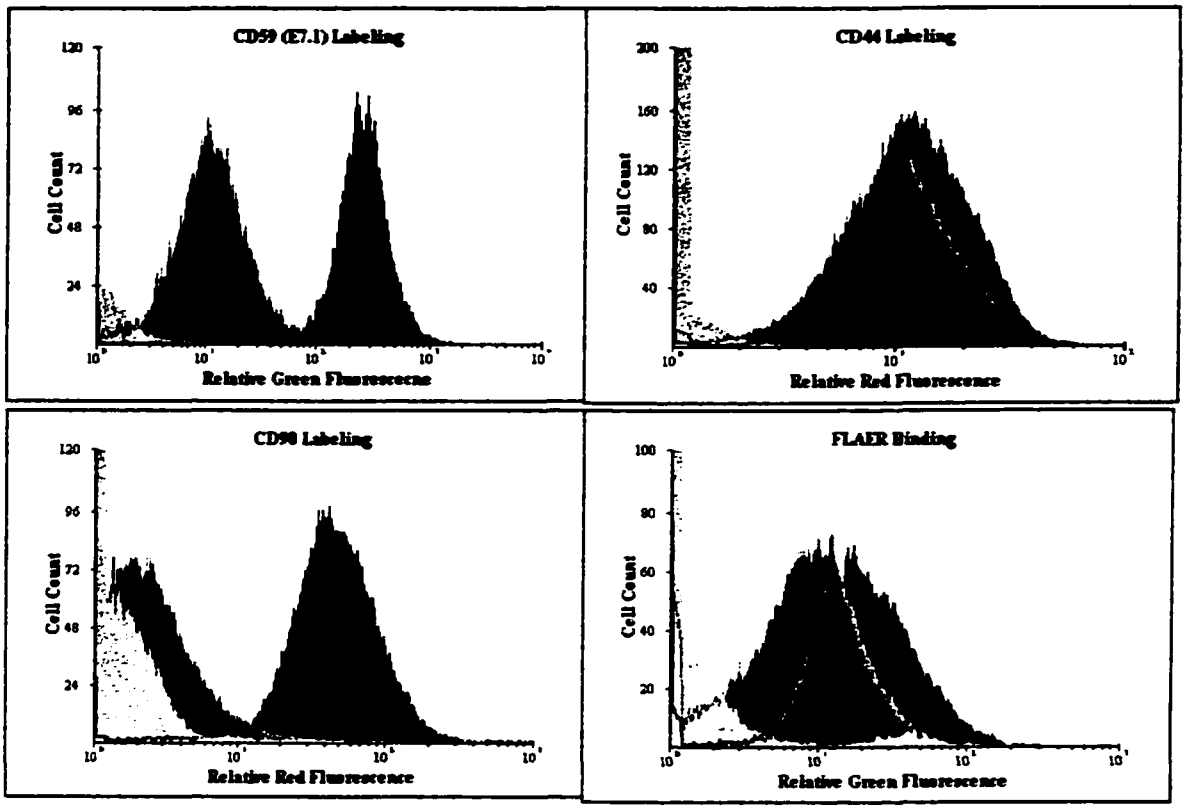

Figure 5.1. Flow cytometric histograms demonstrating labeling of wild-type (black) and GPI-mutant A_L cells 1039-4 (dark gray) and 1039-18 (light gray) with A) Alexa-conjugated CD59 (E7.1 antibody), phycoerythrin-conjugated B) CD44 and C) CD90, and D) FLAER. Both 1039-4 and 1039-18 were identified as mutants using the CMC assay. Both are mutant in CHO GPI anchor genes.

To provide additional means to identify GPI mutant A_L cells, we stained these mutant clones with fluorescent aerolysin (FLAER).(Figure 5.1) This protein binds to the GPI anchor independently of the specific GPI-linked proteins expressed on a cell[10]. FLAER fails to bind strongly to these to GPI-mutant clones further supporting a GPI-associated gene mutation. Still there is variation in the degree of binding of FLAER to various GPI-mutant clones. (Figure 5.1)

Discussion.

The "target" for mutation in the A_L assay, thus includes the *CD59* locus per se along with the GPI anchor-associated CHO genes and is, therefore, larger than we previously thought, involving more than a dozen genes. The large target size may, in part, account for the enhanced sensitivity of the A_L assay compared to the evaluation of other mammalian gene loci such as HPRT, even to so called point mutagens[11,12]. In addition, A_L GPI-mutants represent a class of A_L mutation that seems to be associated with a certain mutagens.

Mutations that are characteristic of a particular treatment could have legal and regulatory significance and provide information regarding mechanisms of DNA damage and mutation. For this reason, there have been numerous efforts to characterize mutational spectra, defined as a collection of mutations resulting from a specific treatment or exposure[13]. Spectra are determined by the genetic locus studied, the type of DNA lesions identified, and the frequency of specific lesions for a specific treatment. Comparison of mutational spectra is discussed in chapter 7. Mutational spectra for the *CD59* locus of A_L cells have been generated for a variety of mutagens.[14-16] These spectra characterize the size of mutations resulting in the CD59- phenotype by determining the presence or absence of genetic markers located on human chromosome 11 of the A_L hybrid. GPI mutants can be determined with flow cytometry and may represent a particularly interesting class of characteristic mutation. This illustrates that flow cytometry provides an improved method of identifying the mutations seen with the CMC assay.

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CHAPTER 6: A SENSITIVE FLOW CYTOMETRIC ASSAY TO DETECT MUTATION AT THE HUMAN *CD44* LOCUS IN HUMAN-HAMSTER A_L HYBRID CELLS.

Introduction.

The CD59 surface antigen requires a GPI anchor for attachment and expression at the cell membrane (see Chapter 5 for a discussion of the GPI anchor) [1-4]. Multiple genes are required for synthesis and attachment of the GPI anchor to CD59[1]. Therefore, absence of CD59 on the cell surface, which is the mutant phenotype selected for in the A_L mutation assay, may result from 1) mutation in the CD59 gene or 2) mutation in any of the GPI genes encoded by CHO.(Figure 6.1) We have identified A_L cells that have mutation in CHO genes that code for the GPI anchor and fail to express CD59. It follows that the A_L assay, although accurate in identifying carcinogens, is not easily used to determine the frequency of mutation at a single genetic locus. An assay evaluating for mutation at the human *CD44* locus of the A_L hybrid cell provides a method to evaluate mutation in one gene.

The CD44 Gene. CD44 is a transmembrane cellular adhesion molecule that is ubiquitously expressed[5-7]. The human *CD44* gene occupies 50-60 kb at 11p13 and has 20 exons[5-7]There are a number of splice variants

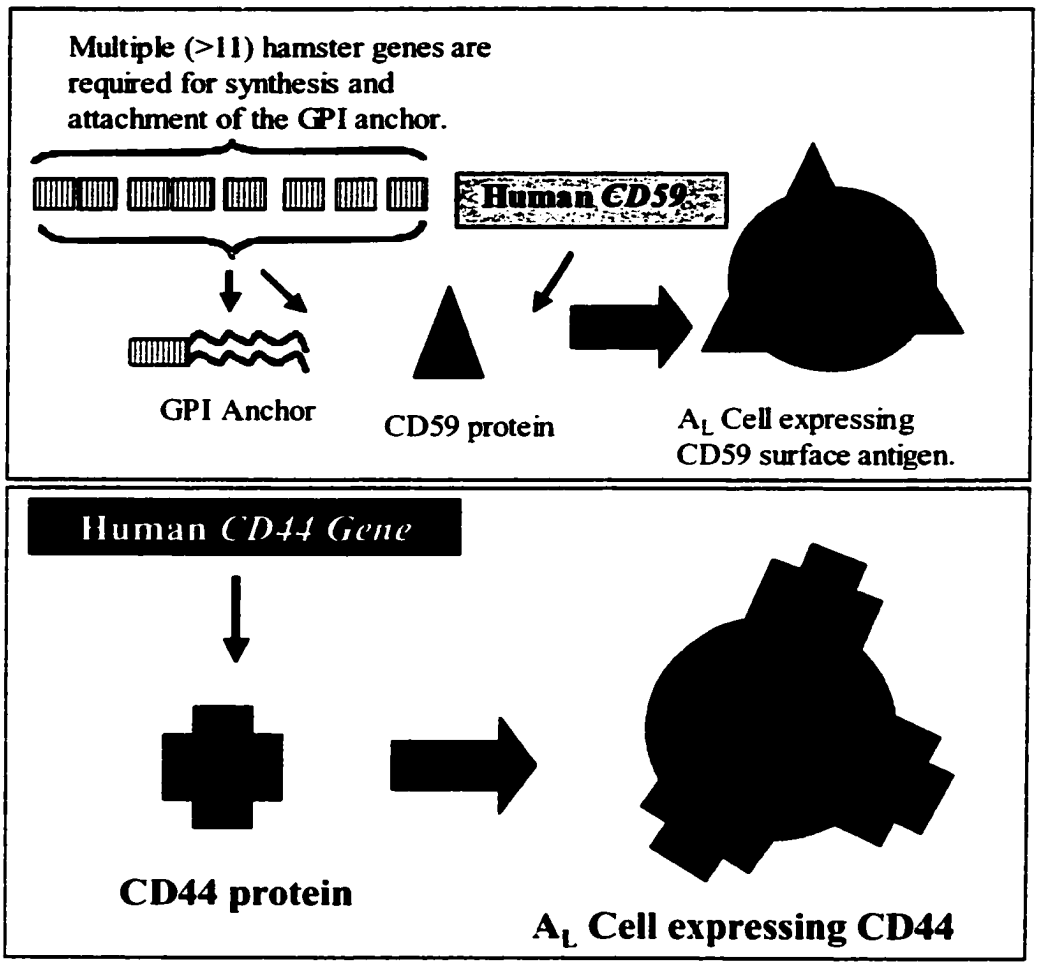
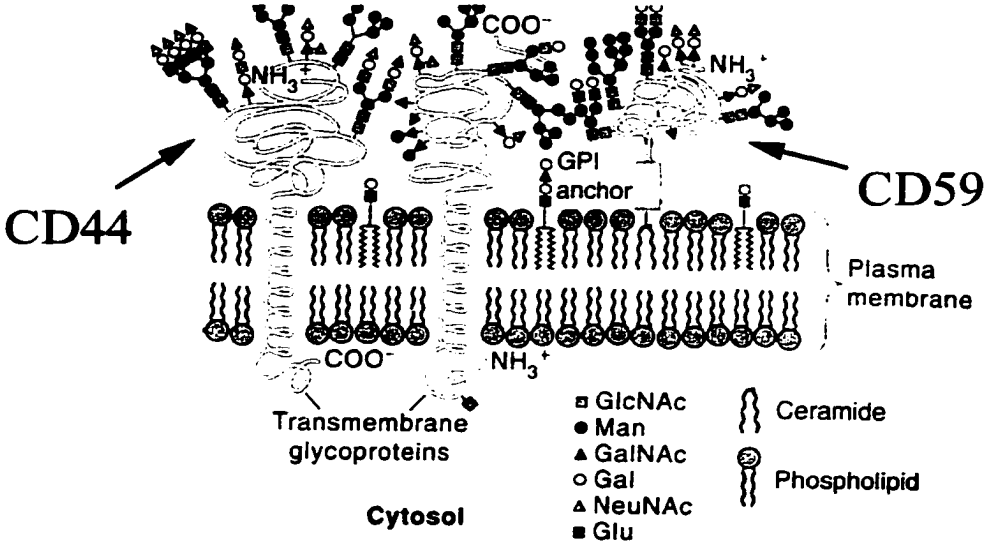


Figure 6.2. A. Multiple genes are required for assembly and attachment of the GPI anchor to CD59. **B.** The expression of CD44 on the cell surface requires only the *CD44* gene.

with a 80-95 kd isoform predominating in normal tissue[5-7]. The protein binds hyaluronic acid and plays a role in lymphocyte and macrophage adhesion and in embryonic development[5-7]. Recent interest in the CD44 protein stems from its association with metastasis and invasion of lymphoma, melanoma, colorectal carcinoma, and a variety of other neoplasms. The CD44 protein is a transmembrane protein, as opposed to GPI-linked, expression in viable A_L cells is likely to result from mutations in the human *CD44* gene and not from mutation in CHO genes.(Figure 6.1 and 6.2) In this chapter, I describe the development of an assay for detection of mutation at the *CD44* gene in A_L cells. This assay provides a flow cytometric method for direct measurement of mutation at a known

single genetic locus. In addition, the simultaneous detection of *CD44* and *CD59* mutation can be performed using flow cytometry of A_L cells. To my knowledge, no other mutagenicity assay allows for the selection of mutants at two distinct genetic loci simultaneously. This information could be useful in determining the likelihood that a mutagenic treatment causes multiple mutations in surviving cells.

Figure 6.1. Illustration of the relationship between surface proteins and the lipid bilayer making up the cell membrane. CD59 is attached by a GPI anchor whereas CD44 is embedded within the membrane



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Materials and Methods.

Reagents. Rabbit serum (Covance, Denver, Pennsylvania); Human Serum (Sigma-Aldrich); FACS buffer (1% Bovine Serum Albumin, 10 mM Na Azide in PBS); 0.5% paraformaldehyde (in PBS); 0.5 mM EDTA (in HBSS); Phycoerythrin (PE)-conjugated

anti-CD44 monoclonal antibody (Pharmingen; San Diego CA). FITC-conjugated anti-CD59 antibody

Cells. Human-hamster hybrid A_L cells (AND-6[8]) were cultured in F12 medium supplemented with 4% heated (56° C for 30 minutes), newborn calf serum, 3% heated (56° C for 30 minutes) fetal calf serum, 1 mM l-glutamine, 50 Units/ml penicillin, 50 ug/ml streptomycin, 20 mM hepes and kept at 37°C in an incubator with an atmosphere containing 5% CO_2 in air. Cells were grown on standard tissue culture plates and harvested with trypsin (0.05%).

Clonogenic Survival Assay. To evaluate ^{137}Cs gamma irradiation for killing, A_L cells were irradiated in suspension and then plated. Plates were incubated for 7-10 days, fixed, stained, and the colonies were counted. Colony counts were adjusted for cloning efficiency determined on untreated control plates.

Standard (CMC) Mutation Assay. (See chapter 4 for detailed description) Sufficient numbers of A_L cells were plated to result in at least 10^5 surviving cells following irradiation. Cells were subcultured every 2-3 days to maintain log phase growth for 12 days. On days 10 and 12, cells were harvested, counted, resuspended in a serum-free medium (Ultraculture®; Biowhittaker; Walkersville , MD) with 1% fetal calf serum, 1 mM l-glutamine, 50 units/ml penicillin, 50 ug/ml streptomycin, and 20 mM HEPES. 2 x 10^5 cells were plated in 100-mm tissue culture plates, and incubated for three hours. Rabbit serum (2% v/v; Covance, Denver, Pennsylvania) and human serum (0.1% v/v; Sigma-Aldrich) as a source of complement and anti-CD59 monoclonal antibody (0.5% v/v; E7.1 clone from mouse hybridoma[9])were added. The plates were incubated for 10 days, fixed, stained, and the colonies were counted. Cloning efficiency (CE) was

determined on plates treated with rabbit serum in the absence of monoclonal antibody (three plates for each treatment). Results were expressed as induced mutants/10^5 surviving cells (induced M_f) and plotted against dose, where:

CE = (average colony counts on plates treated with rabbit serum/number of cells plated);

M_f = Mutant frequency = (10^5)*(number of colonies on treated plates /number of cells plated)/(CE for treated cells);

Spontaneous M_f = (10^5)*(number of colonies on untreated plates /number of cells plated)/(CE for untreated cells);

And Induced M_f = Mutant frequency = M_f - Spontaneous M_f.

The numbers of mutants (M_f) and the number of wild-type cells (Number of cells plated – M_f) was compared for treated and untreated samples by Chi square.

Flow Cytometry. A_L cells were treated and subcultured for 10-12 days as described in the previous section. The cells were then harvested using 0.5 mM EDTA, to prevent trypsin-mediated degradation of surface antigens, and counted. As described in chapter 4, the cells were resuspended in cold FACS buffer, pelleted by centrifugation, and incubated on ice for 30-40 minutes with phycoerythrin-conjugated monoclonal antibody to human CD44. The cells were rinsed twice in cold FACS buffer and resuspended in 0.5% cold paraformaldehyde. After a fifteen-minute incubation on ice, the samples were centrifuged and resuspended in FACS buffer (10^6 cells/ml). Untreated A_L cells were used as the positive control. Mutant A_L cells (128) were used as a negative control. CHO-K1 cells were stained with these antibodies to demonstrate lack of cross-reaction with CHO antigens.

Flow cytometry was performed on a Coulter® Epics® XL-MCL cytometer (Beckman Coulter Inc, Fullerton, California). Samples were "gated" based upon 90° vs forward light scatter characteristics to eliminate cell clumps and debris from the analysis. Voltage across the photomultiplier tubes was adjusted to obtain analyzable histograms with good separation of fluorescent and non-fluorescent cells. Flow rates between 300 and 600 cells per second were used to collect data from about 10^5 cells per sample. List mode files were collected for each experiment. Data analysis was performed with FCS Express© software (Version 1.0; DeNovo Software, Thornhill, Ontario, Canada). Histogram regions were set to enumerate non-fluorescent and fluorescent cells. The percent of cells/events in the nonfluorescent region of untreated wild-type cells was taken as background and was subtracted from the percent of cells in the nonfluorescent region of treated samples. Results were expressed as induced M_f = number mutants/ 10^5 surviving cells.

Results:

In chapter 4, I demonstrated that A_L cells express the human CD44 surface antigen.(Figure 6.3)

Reconstruction experiments. To determine the accuracy of detecting CD44 on A_L cells, mutant cells were mixed with wild-type cells in known proportions (0.1% to 10% mutant cells) and these samples were stained and fixed for flow cytometry. Flow cytometric detection of CD44 antigen was compared to the known proportion of mutant cells. CD44 detection by flow cytometry was able to identify accurately one CD44 – cell in 1000 CD44+ cells.

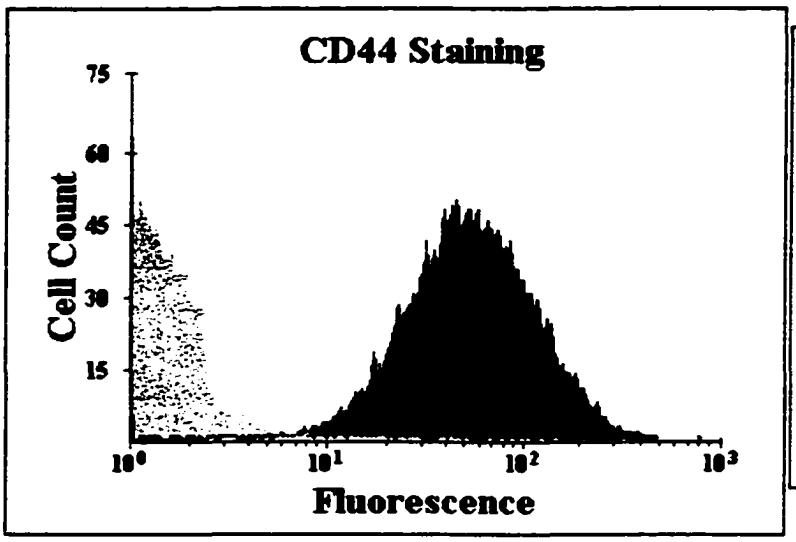

Figure 6.3. Histograms demonstrating flow cytometric detection of CD44 with a phycoerythrin labeled monoclonal antibody to this surface antigen in wild type (CD44+) A_L cells shown in black and mutant (CD44-) A_L Cells (gray).

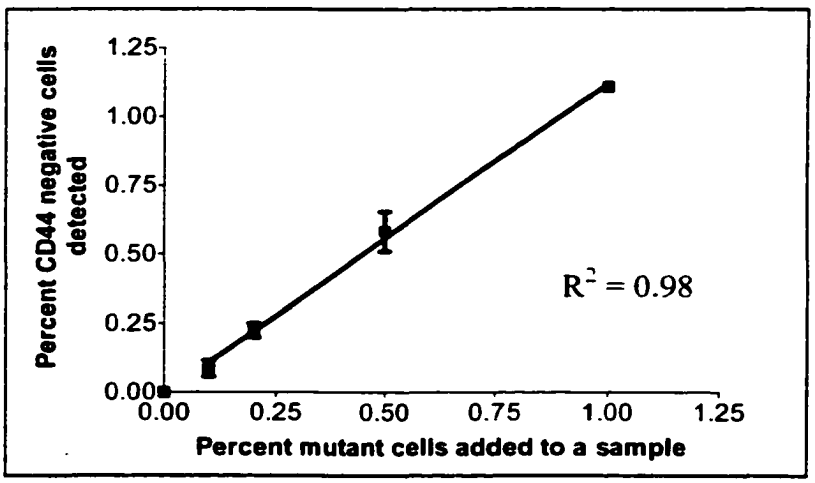

Figure 6.4. Graph demonstrating the accuracy of CD44 detection with flow cytometry. Mixed populations of A_L cells were created by mixing known proportions of mutant and wildtype cells. The cell mixtures were stained for CD44 and run through the flow cytometer. The percent CD44- cells detected by flow cytometry and corrected for background is plotted against the actual proportion of mutants in the mixture.

Correlation with CMC Assay. To see if CD44 detection correlates by flow cytometry correlates with the standard CMC A_L assay, A_L cells treated with ^{137}Cs gamma irradiations were evaluated by both assays.

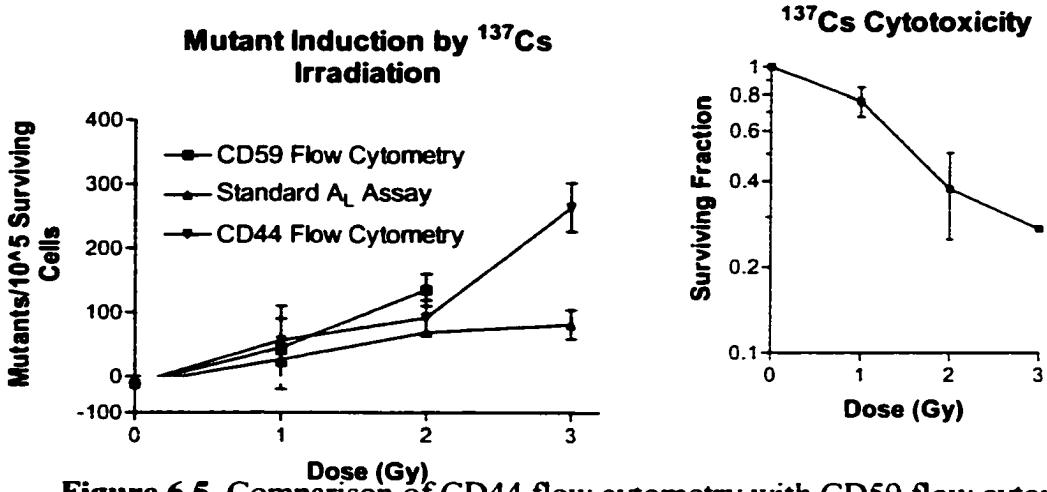

Figure 6.5. Comparison of CD44 flow cytometry with CD59 flow cytometry and traditional A_L assays for detection of mutation induced by ^{137}Cs gamma rays.

Discussion.

The A_L *CD44* assay is comparable to the standard A_L assay in quantifying mutants. An advantage to the evaluation of mutation at *CD44* is the ability to directly determine mutant induction at only one genetic locus compared with the *CMC* assay, which evaluates for mutations in *CD59* and at CHO genes coding for the GPI anchor.

One of the more interesting applications of the CD44 locus assay would be combining it with analysis at the *CD59* or *CD90* loci to evaluate simultaneously for mutation at two independent genetic loci. This is not easily done with other mutagenicity assays, such as TK+/-, HPRT, or APRT. It has been observed through new cytogenetic techniques such as multiplex fluorescence in situ hybridization (M-FISH) that complex genomic rearrangements after treatment with ionizing radiation are far more common

than previously thought[10-13]. Increased levels and complexity of damage may enhance carcinogenic risk from exposure. Evaluation of complex types of mutations may, therefore, be important in safety evaluation and in regulatory procedure. Further study of the combined CD59/CD44 A_L assay is needed to determine the usefulness of this approach. It should also be noted that with the use of X-linked surface antigens, a similar flow cytometric assay could be done in vivo[14].

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CHAPTER 7: COMPARISON OF MUTATIONAL SPECTRA BY A EUCLIDEAN DISTANCE FORMULA

Introduction.

It has been known for almost 50 years that mutations, defined as heritable changes in the genome of any size, are not random, that they can be induced at particular sites by various agents at very different rates and that various agents can induce different, particular kinds of mutations, e.g. [1-3]. While it has been postulated that "signature" mutations can be recovered from cells exposed to a particular agent, the evidence that any agent induces a unique mutation or unique mutational spectrum is not convincing[4-11]. Rather the situation appears to be as described by Muller 60 years ago: "In fact, experience shows that every spontaneous mutation can, if thoroughly searched for, also be found after x-ray treatment" [12]. There is, on the other hand, a substantial body of data indicating that mutations of partilular kinds correlate more or less well with exposure to particular agents or exposure regimens, e.g. [6,7,9,13-21] so that it may be possible in some cases to link an agent to characteristic spectra or semi-"signature" mutations. It would be useful for mechanistic, etiological, epidemiological and legal reasons to be able to prove, unequivocally, that a mutation or collection of mutations (mutational spectrum)[1] resulted uniquely from exposure to a particular agent. Unfortunately, statistical comparison of mutational spectra is complex and uncertain.

Mathematical treatment of mutational spectra. The total data collected for an individual mutagenic treatment may be thought to represent a vector quantity, $A = (a_1, a_2, \ldots a_n)$, where

n = the number of categories of mutation defined, and an entry, a_i = the frequency or percentage of mutations of that type recovered. A collection of spectra may then be thought of as representing a NxM matrix or contingency table of the following type:

$$\begin{pmatrix} n_1m_1 & n_1m_2 & \ldots & & n_1m_M \\ n_2m_1 & n_2m_2 & \ldots & & n_2m_M \\ . & . & & . & . \\ . & . & & . & . \\ . & . & & . & . \\ n_Nm_1 & n_Nm_2 & \ldots & & n_Nm_M \end{pmatrix}$$

Where:

N is the number of categories of mutation.

M is the number of mutagens evaluated.

Each column then represents the mutational spectra for a given treatment.

The entry n_im_j = frequency of mutations of the ith category resulting from treatment with the jth mutagen.

NxM tables have traditionally been compared by the Pearson's chi square test[22,23]. Unfortunately, most mutational spectra have categories that contain very small numbers (low frequency) of mutants. The Chi square statistic is quite inaccurate and inappropriate

when the value of any cell in the table is less than 1 or twenty percent of the values are less than five[22]. The Chi square test has been widely used despite violating these basic statistical requirements.

The hypergeometric test, a generalization of the Fisher's exact test for tables larger than 2X2, has been suggested as a way to overcome the limitations of the Chi square. To obtain level of significance, Adams and Skopek recommend Monte Carlo estimation of the *P* value for this test rather than performing the intensive computation[24]. Although widely accepted, this method is limited by the inability 1) to accurately compare spectra in which some classes contain no mutants in either spectra and 2) to consider all mutation categories simultaneously rather than comparing them class by class.

Multivariate statistics. The explosion, in recent years, of imaging technology has created a need for pattern recognition techniques, which may be applied to data in the form of "spectra". These techniques, which can be referred to as multivariate statistics, are not formal statistical comparisons and do not provide p-values as do regression analysis or ANOVA[25]. Rather, the objective of multivariate analysis is to create logical classifications and generate and test hypothesis by examining interrelationships among variables. Mutational spectra can be thought of as being groups of variables and lend themselves to these types of analyses. In this regard, we, including R.D. Parker, C. Quirk, C. Waldren, and myself have developed an algorithm that employs Euclidean distances and cluster analysis (EDCA) to compare mutational spectra. I demonstrate this method here by comparing mutational spectra induced at the *CD59* locus of human x hamster hybrid cells (A_L cells) by various irradiations. EDCA provides another means with which to evaluate spectral data. Although this technique provide statistical parameters such as *P*

values it is mathematically rigorous and can provide valuable insight into how spectra are related.

Methodology:

A_L Mutation assay and generation of spectra.

Mutational spectra at the *CD59* locus of human x hamster hybrid A_L cells exposed to various irradiations were compared at equitoxic doses[26]. The A_L assay is described in detail in chapter 4.

To generate mutational spectra, individual colonies of surviving mutants are picked, expanded and their DNA is extracted. The spectra here analyzed were obtained by immunologic, isozymic and molecular (PCR and Southern) analysis for the presence or absence of a series of 11 markers mapped to the human chromosome 11 of individual $CD59^-$ mutants[26-33]. The mutations underlying the $CD59^-$ phenotype can range in size from a small, intragenic lesions to deletions of more than 160 Mb. We have defined 43 classes of mutants based on this set of markers. (Table 7.1) The 43rd category, referred to as "complex", consists of a class of genomically unstable mutants[31,34,35].

The radiation treatments included: 1) low linear energy transfer (LET) irradiations (low dose rate and high dose rate gamma irradiation by ^{137}Cs source), 2) High LET irradiations (proton, carbon, nitrogen and iron particle irradiation), and 3) nonionizing ultraviolet irradiation. Various numbers of mutants were analyzed for each treatment ranging from 38 (for UV irradiation) to 153 (for spontaneously occurring mutants). Characteristics of the radiation treatments are given in Table 7.2.

Table 7.1. Mutational Spectra at the CD59 Locus.

		Chromosome 11 markers												No. of Mutants								
Class #	Size (Mb)	RAS	HBE	LDHA	D16	P5	WT	CD59	CAT	ACP	p82H	FTH	APO	Control	UV (24 J/m2)	γ-LDR (3 Gy)	γ-HDR (3 Gy)	Protons (3 Gy)	Carbon (3 Gy)	Nitrogen (1.5 Gy)	Fe (2.75 Gy)	2 Gy/day (10 Gy)
1	3.6	+	+	+	+	+	+	-	+	+	+	+	+	62	17	16	12	1	1	9	13	4
2	4.2	+	+	+	+	+	-	-	+	+	+	+	+	3	12	5	0	0	0	0	2	0
3	5.7	+	+	+	+	-	-	-	+	+	+	+	+	3	0	nd	2	0	0	2	7	0
4	15	+	+	+	+	+	+	-	-	+	+	+	+	10	0	3	2	2	3	0	2	1
5	15	+	+	+	+	+	-	-	-	+	+	+	+	1	0	9	2	0	1	0	0	0
6	17	+	+	+	+	+	+	-	-	-	+	+	+	1	0	0	3	0	0	0	1	0
7	17	+	+	+	+	-	-	-	-	+	+	+	+	1	0	nd	3	1	1	0	7	1
8	17	+	+	+	+	+	-	-	-	-	+	+	+	2	0	5	0	0	0	0	0	0
9	19	+	+	+	+	-	-	-	-	-	+	+	+	6	0	nd	5	1	0	0	11	0
10	21	+	+	+	-	-	-	-	+	+	+	+	+	10	0	5	2	0	0	0	3	2
11	32	+	+	+	-	-	-	-	-	+	+	+	+	5	0	8	9	5	16	2	5	5
12	34	+	+	+	-	-	-	-	-	-	+	+	+	3	0	2	14	5	9	1	20	1
13	36	+	+	-	-	-	-	-	+	+	+	+	+	2	0	2	1	1	0	0	1	1
14	41	+	-	-	-	-	-	-	+	+	+	+	+	0	0	0	0	0	0	0	0	0
15	42	-	-	-	-	-	-	-	+	+	+	+	+	0	0	0	0	0	0	0	0	0
16	42	+	+	+	+	+	+	-	-	-	-	+	+	0	0	0	1	2	0	0	0	0
17	42	+	+	+	+	+	-	-	-	-	-	+	+	2	0	2	0	0	0	0	0	0
18	44	+	+	+	+	-	-	-	-	-	-	+	+	1	0	nd	1	1	0	0	1	0
19	44	+	+	-	-	-	-	-	-	+	+	+	+	0	0	1	3	4	5	2	6	3
20	46	+	+	-	-	-	-	-	-	-	+	+	+	0	0	3	6	11	2	0	7	1
21	51	+	-	-	-	-	-	-	-	+	+	+	+	0	0	0	0	0	0	1	2	3
22	52	-	-	-	-	-	-	-	-	+	+	+	+	0	0	0	0	0	0	0	0	0
23	53	+	-	-	-	-	-	-	-	-	+	+	+	1	0	0	0	1	0	0	0	0
24	59	+	+	+	-	-	-	-	-	-	-	+	+	0	0	0	0	5	2	3	3	3
25	66	-	-	-	-	-	-	-	-	-	+	+	+	0	0	0	0	0	0	0	0	0
26	71	+	+	-	-	-	-	-	-	-	-	+	+	0	0	0	0	2	2	1	1	0
27	78	+	-	-	-	-	-	-	-	-	-	+	+	0	0	0	0	1	0	0	0	0
28	79	-	-	-	-	-	-	-	-	-	-	+	+	0	0	0	0	0	0	0	0	0
29	84	+	+	+	+	+	+	-	-	-	-	-	+	0	0	0	0	0	0	0	0	0
30	84	+	+	+	+	+	-	-	-	-	-	-	+	0	0	0	0	0	0	0	0	0
31	86	+	+	+	+	-	-	-	-	-	-	-	+	0	0	nd	0	0	0	0	0	0
32	103	+	+	+	-	-	-	-	-	-	-	-	+	2	0	0	0	0	0	0	1	0
33	104	+	+	+	+	+	+	-	-	-	-	-	-	1	0	0	0	0	0	0	0	0
34	105	+	+	+	+	+	-	-	-	-	-	-	-	0	0	1	0	0	0	0	0	0
35	106	+	+	+	+	-	-	-	-	-	-	-	-	3	0	nd	0	0	0	0	0	0
36	115	+	+	-	-	-	-	-	-	-	-	-	+	1	0	0	0	0	1	0	0	0
37	120	+	-	-	-	-	-	-	-	-	-	-	+	0	0	0	0	0	0	0	0	0
38	121	+	+	+	-	-	-	-	-	-	-	-	-	2	0	4	5	0	0	0	3	0
39	121	-	-	-	-	-	-	-	-	-	-	-	+	0	0	0	0	0	0	0	0	0
40	133	+	+	-	-	-	-	-	-	-	-	-	-	0	0	0	0	0	1	0	1	0
41	139	+	-	-	-	-	-	-	-	-	-	-	-	0	0	0	0	0	0	0	0	1
42	140	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0	0	0	0	0	0	0
43	?									Complex				31	9	29	2	22	15	27	27	29
										Total =				153	38	95	73	65	59	48	124	55

Table 7.1. Data generated from analysis of *CD59* mutants obtained from populations treated with particular irradiation regimens. The assignment and definition of the 43 mutation categories defined for the A_L system are shown in the first two columns: Class number in the first column, size of the mutation in Mb for the defined class in the second column. Columns 3 through 14 list the chromosome 11 markers that are screened for in the assay. A map of chromosome 11 is provided. The markers are listed from proximal to distal of the CD59 gene. A given class of mutation is defined by the presence (+) or absence (-) of these markers. The 43rd class of mutants is called "complex". These mutants are genomically unstable and show variable presence and absence of the chromosome 11 markers. The last nine columns in the table show the data generated for each of the treatments listed in the first row. The total number of mutant clones analyzed is in the last row of the table. The number of mutant clones analyzed for any given treatment is based upon resources available as no optimum sample number has been established. The rows 2 through 43 show the number of mutants identified in each class.

Table 7.2. Characteristics of Radiation Treatments.

Radiation	LET, keV/μm	D_o, Gy
HZE- ^{56}Fe, BNL,	143	0.6
HZE- ^{56}Fe, LBL, 600 MeV/amu	190	0.6
^{14}C, 290 MeV/nucleon	100	0.8
"	14	1.7
Nitrogen, 32 MeV/amu	126	0.6
Protons (LBL)	2.3	1.5
α-particles, nucleus	90	0.5 (= 4 particles)
CS^{137} γ HDR, 2 Gy/min		1.5 Gy
CS^{137} γ LDR, 3 cGy/h		??? - No killing observed; total dose delivered = 3 Gy
CS^{137} γ Fractionated, 2Gy/day		4 Gy

Table 7.2. Characteristics of the radiation sources used to generate mutant spectra. The first column lists the type of radiation. Iron particle irradiations (HZE- ^{56}Fe) were performed at Lawrence Berkley National Laboratory, Berkley, California (LBL) and Brookhaven National Laboratory, New York (BNL).[36]Carbon (^{14}C) irradiations were performed at the National Institute of Radiobiological Sciences, Chiba, Japan. Nitrogen particle irradiations were performed at LBL.[37] Proton irradiations were performed at LBL.[37] The alpha particle irradiations were performed in the microbeam facility at Columbia University, New York, New York. Gamma irradiations were performed in a ^{137}Cs irradiator at Colorado State University, Fort Collins, Colorado. The second column

provides the LET and the third column provides the D_0, the dose that decreases the surviving fraction by 37% in the log-linear portion of the survival curve, for each treatment. Mutants analyzed here were induced by 3 Gy of HDR CS^{137} γ rays; 10 Gy for fractionated CS^{137} γ rays; 2 Gy for other radiations. These doses are nearly equitoxic.

Euclidean Distance Calculations. The Euclidean distance formula is commonly used in two-dimensional space, where a point consists of an ordered pair of numbers (coordinates) that describe its location in Cartesian space, or in three-dimensional space to determine distances between points. In two-dimensional space, the Euclidean formula is the basis for the Pythagorean theorem: the length of the hypotenuse of a right triangle is equal to the square root of the sum of the squares of the sides. (Figure 7.1) The Euclidean distance algorithm used here was adapted from that applied by Parker to read

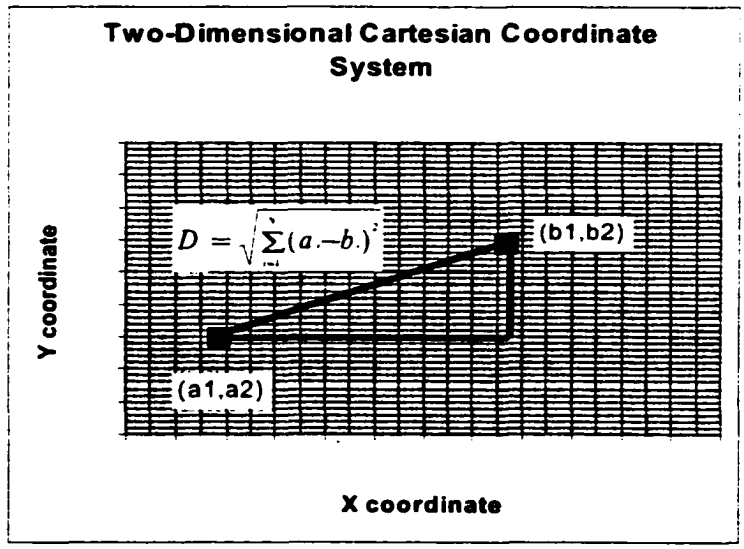

Figure 6.1. Diagram illustrating the principle of the Euclidean distance function in 2-dimensional space. This concept is generalized to n-dimensional space for application to mutational spectra.

characters in Banctec Corporation Scanning Equipment. (See report #89-04 of the Operations Research Group Report, Johns Hopkins University entitled 'Calculating the weights of a mask for character recognition', which may be obtained from the authors.) The formula may be generalized to n-dimensional space where n = 2 to infinity. Given two spectra A = (a_1, a_2, ... a_n) and B = (b_1, b_2, ... b_n), the Euclidean distance, D, between these spectra is defined by,

$$D = \sqrt{\sum_{i=1}^{N}(a_i - b_i)^2}$$

where n = the number of mutation categories or classes defined and an entry, a_i = the frequency (percentage) of mutants of that mutation class following the treatment. The frequency of mutants within a class is defined as the percentage of mutants analyzed belonging to that class. For instance, the frequency of spontaneous mutants of the 43rd class is (31/153) x 100 = 20.3. (Table 7.1)

Euclidean distances between mutational spectra created by various irradiations were calculated using a Visual Basic® program used with a Microsoft® Excel (Microsoft Corporation, Redmond, WA) spreadsheet.

Cluster analysis. A simple form of cluster analysis was performed using Euclidean distance to place spectra into groups. To do this, the nearest neighbor algorithm was employed to find and diagramatically illustrate the smallest distance between spectra. The procedure is as follows:

Let the matrix of Euclidean distances between spectra be represented by:

$$\begin{pmatrix} D_{1,1} & D_{1,2}\ldots & \ldots D_{1,n} \\ D_{2,1} & D_{2,2}\ldots & \ldots D_{2,n} \\ . & . \quad . & . \\ . & . \quad . & . \\ . & . \quad . & . \\ D_{n,1} & D_{n,2}\ldots & \ldots D_{n,n} \end{pmatrix}$$

Where n = the number of spectra.

$D_{i,j}$ = euclidean distance between the ith and the jth spectra.

For i = 1 to n, Let $m_i = \min (D_{i,j})$ for j = 1 to n and $j \neq i$

Then let N(i) = the jth spectra.

Then we can form the order pairs of spectra (i, N(i)) corresponding to each m_i.

Such that: $(1,N(1)) = m_1, (2,N(2)) = m_2, \ldots (n,N(n)) = m_n$.

We may rank the m_i from smallest to largest and diagram the ordered pairs in a cluster diagram in order of the corresponding m_i.

Other Statistical Tests. Mutational spectra have traditionally been compared with the chi square test and the hypergeometric test with Monte Carlo estimation of the *P* value. To compare these tests with the EDCA method, we applied them to pairs of mutational spectra produced by irradiation. Chi square and the hypergeometric test with Monte Carlo estimation of the p-value were performed using SAS (chi square) and software developed by Cariello et al (hypergeometric test http://metalab.unc.edu/dnam/mainpage.html)[24,38,39].

Results.

Mutational spectra generated at the CD59 locus by equitoxic doses of various irradiations are given in Table 7.1. It is difficult to discern differences in these spectra,

with the exception of the spectra produced by UV irradiation. UV irradiation obviously produces smaller mutations than the other radiations, but all treatments produce some small mutations. The logical conclusion is that when spectra are in toto, there is little difference between spectra generated by various ionizing radiations and the spontaneous spectra. This has important implications for the notion of the existence of unique "signature" mutation spectra.

Euclidean distances between pairs of mutational spectra are given in Table 7.3. These distances range from 0 to 63. The smallest, nonzero, distance between spectra is 15, which represents the distance between fractionated gamma irradiation and nitrogen particle irradiation. This is surprising considering that fractionated low LET radiation is expected to produce smaller damage and allow for repair compared to high LET nitrogen particle irradiation, which is expected to produce large clusters of damage with no time to repair during administration of the dose. The maximum distance is between UV and carbon particle irradiation. The distances between UV and all other irradiation treatments are relatively large ranging from 42 to 63.

Table 7.3. Euclidean distances between radiation induced mutational spectra.

	Control	UV	Cs*	Cs**	Protons	Carbon	Nitrogen	Iron
Control	0	32	29	38	47	50	44	36
UV		0	42	55	59	63	53	51
Cs*			0	36	28	32	31	26
Cs**				0	40	34	59	24
Protons					0	27	35	25
Carbon						0	45	29
Nitrogen							0	40
Iron								0

Control = untreated cells, UV = ultraviolet irradiation, Cs* - Low dose rate Cs gamma irradiation, Cs** - High dose rate Cs gamma irradiation.

A cluster diagram was created by representing the minimum distances between spectra determined from the nearest neighbor algorithm. (Figure 7.2.) In addition, the distances less than 32 units (the largest minimum distance) and between 32 and 40 units are also shown. UV is relatively distant from the other spectra except for the spontaneous spectrum. The UV spectrum is distinctly separate from other irradiations. The other

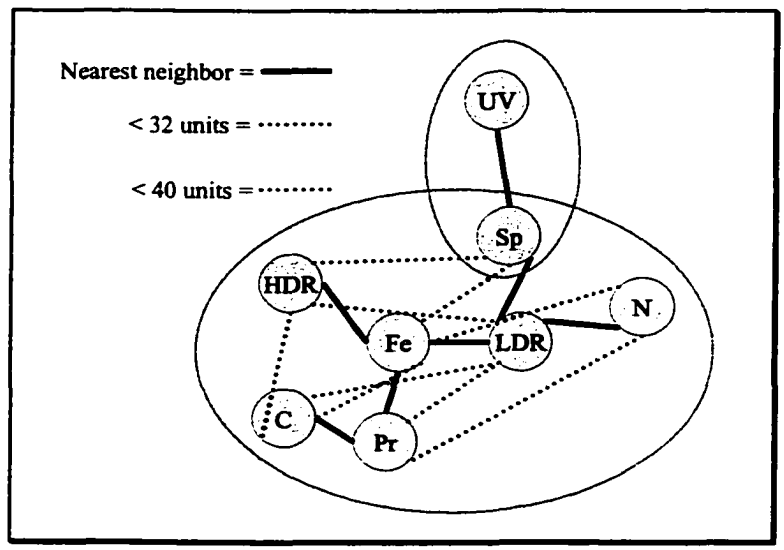

Figure 7.2. Cluster diagram illustrating the relative Euclidean distances between mutational spectra (green circle). C = Carbon spectra, Fe = Iron spectra, HDR = high dose rate gamma spectra, LDR = Low dose rate gamma spectra, N = Nitrogen spectra, Pr = Proton spectra, Sp = spontaneous spectra, UV = UV spectra.

irradiation spectra are relatively close and seem to fall into a single cluster. The nitrogen and high dose rate gamma spectra are somewhat more distant from other spectra than are carbon, low dose rate gamma, iron, and proton spectra.

When the irradiation spectra are compared with Chi square and the hypergeometric test, all are considered statistically different with $P < 0.05$.

Discussion:

Mutational spectra have been compiled for various treatments at a variety of loci. These include bacterial and mammalian somatic cell systems evaluating mutation in genes such as *hprt, aprt*, and the thymidine kinase locus, in addition to *CD59*, as used here. The *CD59* gene and adjacent loci on human chromosome 11 are nonessential to cultured CHO cells, which allows detection of very large and very small mutations. This greatly increases the sensitivity of the A_L assay compared to many other mutation assays. Mutational spectra also exist for cancer related genes identified in tumors. The *p53* gene in particular has been frequently evaluated to determine types of mutations in tumors. Several statistical approaches have been applied to decide if different treatments induce the same or different spectra at a genetic locus [23,24,38-41,41-44,44,45,45,46]. Application of these tests has pointed to the existence of characteristic mutations and mutational "hot spots" in various genes after *in vitro* or *in vivo* exposure to such agents as cigarette smoke[47], radon [48], other ionizing radiations[46], sunlight [49] and heterocyclic amines found in cooked foods [17,50]. But these analyses appear slanted towards detecting unique mutations in ways that are not statistically rigorous.

Because mutation underlies carcinogenesis from the initiating step through development of a malignant phenotype, it is essential that we understand the mechanism by which a genotoxic exposure can induce mutations particularly in cancer related genes. The study of mutational spectra in *in vitro* systems and in tumors could refine criteria for

determining occupational exposure and risk and legal criteria for causation of tumors and other genetic disease. A stumbling block to the study of mutational spectra has been the lack of adequate, mathematically rigorous, statistical methods to analyze these types of data.

To be maximally informative, a method for comparison of mutational spectra should be applicable to categorical data even with few or no mutants in a category. Further, this method should involve a global parameter that takes into account all categories simultaneously, rather than being limited to category by category comparisons. Other comparison methods, as used, do not meet these criteria and may, we suggest, lead to incorrect conclusions. Although there is always some level of subjectivity in deciding the usefulness of different methods, we believe that the Euclidean distance calculation with cluster analysis offers advantages over existing methods including that (1) classes containing few mutants are taken into account. (2) The entire spectrum is compared simultaneously, not class by class.

Whereas, other methods of comparison found differences between all spectra, our algorithm highlighted certain differences as being particularly prominent. We found that the mutational spectrum induced by UV differed from all other radiation induced spectra, but did not differ from the spontaneously occurring mutational spectrum. Upon inspection of the data, this difference is attributable to a predominance of small mutations in the UV spectra although these mutations occur with the other irradiations. It is also likely to be significant that most of these spectra were ***not*** different, being composed of similar sets and proportions of mutations. This finding casts doubt on the existence of

unique, signature mutations being generated by various ionizing radiations as compared to spontaneous spectra.

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PART 3: FELINE NORMAL AND TUMOR CYTOGENETICS

CHAPTER 8. CULTURED FELINE LYMPHOCYTES EXHIBIT HYPERSENSITIVITY TO APOPTOSIS

Introduction.

Comparative Genomics. Comparative genomics is crucial to understanding the structure and function of the genome and to the isolation and characterization of new genes[1]. Genes and genomic structure are highly conserved across species allowing for extensive interspecies extrapolations. Yet there are pronounced species differences in cellular regulation and response. These instances of variability may be particularly useful in understanding gene structure and function. Investigating species variations is analogous to the study of mutant cell lines or knock-out mice to discover the function in the wild-type.

In recent years there has been significant progress in characterizing the genome of companion animals[1-21]. Genetic diseases in pets have been developed as models for disease in humans such as narcolepsy, progressive retinal atrophy, Niemann-Pick disease among others[21-31 2221}32]. Tumors in pet animals may also be useful for the study of the genetics of cancer[33]. The genetics of spontaneously occurring cancer in animals is virtually unexplored, but may provide insight into the pathogenesis of human cancer, particularly genetic susceptibility to cancer and the relationship to environmental exposures.

Cancer Susceptibility. There is a great deal of evidence that certain cancers in animals have a heritable basis. Certain breeds of dogs, such as boxer and Golden retriever, have

higher incidence of cancer than other breeds. In addition, specific tumors occur more frequently in some breeds such as lymphoma in Bull Mastiffs, malignant histiocytosis in Burnese Mountain dogs and histiocytic neoplasms in flat coated retrievers[34-38]. Recently, a genetic locus that confers susceptibility to a cancer syndrome in German shepherds has been mapped. This autosomal dominant syndrome, RCND, is associated with multifocal renal cystadenocarcinoma, nodular dermatofibrosis (multiple fibrous skin nodules) and uterine leiyomyoma in affected females[33].

Interestingly, most heritable tumors in dogs are not 'pediatric' tumors, but occur in adult dogs, and are not accompanied by other medical problems. Therefore, cancer predisposition in dogs, with the exception of RCND, represents a process distinct from the rare cancer predisposition syndromes identifiable in human populations such as retinoblastoma, Li-Fraumeni, Wilm's tumor, or Xeroderma pigmentsoum. The genes responsible for cancer predisposition in pets are likely to be of the less penetrant variety which have greater impact on the population. Unlike most humans, there is a wealth of information regarding the heritage of purebred pets. In addition, early breeding age and multiparous births allow for large numbers of related animals. The pedigree information available in animals can be exploited to better understand the genetic component of cancer risk. Further, genetically informative matings can be carried out. Finally, genetic screening programs may be easily implemented in animals without the controversy that exists in people[39-41]. Pets can serve as a preclinical testing population to establish reliability of screening techniques prior to use in people.

Purebred cats are much less common than purebred dogs, so breed predisposition for cancer is not well established, but environmental susceptibilities are recognized and some

feline tumors may have a heritable basis[42-44]. Feline models may be particularly helpful in understanding the role of host factors in the development of skin cancer, the hormonal aspects of breast cancer, the pathogenesis of oral cancer, and the role of retroviral infection in carcinogenesis[45-48]. The study of the genomics of feline tumors should be particularly interesting, in that the organization of the genome is highly conserved between humans and cats[1,6].

Significance of Tumor Cytogenetics in the Study of Carcinogenesis. Thirty years of karyotypic studies have identified a plethora of chromosome abnormalities in human tumors. The identification of consistent, recurrent aberrations in tumors and the association of these rearrangements to cancer related genes and tumor biologic factors such as tumor stage and response to therapy have established cytogenetics as a critical area of clinical and basic cancer research[49-52]. Cytogenetic study has been instrumental in the identification of a variety of cancer related genes that drive carcinogenesis and tumor progression[49-52]. Examples of genes that were identified largely based upon specific cytogenetic changes include RB, WT1 and 2, and DCC[49,53]. Identification of loss of heterozygosity (LOH) in tumors may be particularly helpful in identifying recessive or suppressor genes that are important in tumor susceptibility[51,52,54]. LOH is frequently associated with a large deletion readily identifiable with cytogenetic techniques. The introduction of molecular cytogenetic techniques including chromosome and gene specific fluorescence in situ hybridization, comparative genomic hybridization (CGH), and multiplex FISH (M-FISH) has greatly enhanced the resolution and specificity of tumor cytogenetics[49,51,52].

Lymphocyte Culture. It was my intention to develop techniques for feline cytogenetics to characterize genetic alterations in feline tumors particularly feline vaccine-associated sarcoma. Feline cytogenetic studies have been relatively uncommon. (See chapter 9 for a discussion of feline cytogenetics) The first step in cytogenetic evaluation is the preparation of metaphase chromosomes for study. Because peripheral blood lymphocytes (PBL) are convenient to collect and culture, at least in humans, I selected these cells for my research. Techniques for mitogen stimulation of human PBL's to produce metaphase preparations for karyotyping have been applied successfully for nearly 40 years[55,56]. Culture and stimulation of PBL's from nonhuman animals is described for some species and it is known that PBL's from different animals respond differently to the plant lectins that are used as mitogens[57]. Despite using mitogens based on blastogenesis studies, my yield of metaphase cells for cats was poor compared to humans, dogs, and a variety of other species. I have discovered that this is due to the hypersensitivity of feline lymphocytes to apoptosis in culture. I also have demonstrated that this process can be modulated with appropriate culture conditions.

Methods.

Cells. Blood was collected by jugular venipuncture of cats that were clinically normal and tested ELISA negative for feline leukemia virus and feline immunodeficiency virus. Blood samples were anticoagulated in sodium heparin collection tubes (Vacutainer®, Bectin Dickinson; Franklin Lakes, NJ).

Lymphocyte and Whole Blood Concentration. For flow cytometry, lymphocytes were prepared with separation media (density = 1.077), stained with nuclear isolation medium (0.6% Nonidet P-40, 50ug/ml Propidium iodide) and counted with a hemocytometer.

Separated lymphocytes were cultured at a concentration of 4-5 x 10^5 cells/ml. For preparation of metaphase spreads, whole blood was added to cultures (125 ul, 250 ul, 500 ul, or 1 ml of blood was added to a 5-ml culture).

Culture Media. Cat lymphocytes were cultured in RPMI 1640 with 5%, 10%, or 20% heat inactivated fetal calf serum, 4mM l-glutamine, 50 Units/ml penicillin, 50 ug/ml streptomycin, 20 mM HEPES buffer. Any adverse effect of serum was evaluated by comparison with serum free media (Ultraculture®, BioWhittaker, Wakersville, MD).

Incubation Conditions. Cells were incubated at 37° C or 39°, to account for body temperature of cats (38-39° C[58]), in a humidified, 5% CO_2 incubator.

Mitogens. Various plant lectins were evaluated as mitogens. Concanavalin A (ConA) (Sigma, St. Louis, MO) was evaluated at 75 ug, 125 ug, 250 ug, 375 ug, and 500 ug per 5 ml culture, Pokeweed mitogen (PW) (Sigma, St. Louis, MO) at 5 ug, 25 ug, 50 ug, 100 ug, 200 ug per 5 ml, and phytohemagluttinin (M form; Gibco BRL) at 100ug per 5ml culture.

IL-2. Human and mouse recombinant IL-2 (Roche Molecular Biochemicals, Indianapolis, Indiana) were evaluatred at 10, 50, and 100 units per 5-ml culture.

Metaphase Preparation. Metaphase slides were prepared on days 3, 4, or 5. Colcemid (0.1 ug/ml;GibcoBRL, Grand Island, New York) was added for the last 0.5 – 2 hours of

culture. Cultures were centrifuged for 10 min at 1200 RPM in a standard clinical centrifuge. Cell pellets were resuspended in warm, hypotonic (0.075M) KCl and incubated for 15 minutes at 37° C then centrifuged. The pellets were resuspended in 10 mls of 3:1 methanol: acetic acid and incubated at room temperature for at least 20 minutes. The methanol:acetic acid treatment was repeated until a clear supernatant and clean white pellet was obtained (twice for separated lymphocyte cultures; 3-4 times for whole blood cultures.) Nuclei were dropped on clean microscope slides and stained with 4',6-diamidino-2-phenyl-indole (DAPI, 5 ug/ml) in antifade buffer (SlowFade, Molecular Probes, Eugene, OR) for morphologic evaluation.

Flow Cytometry. Cell cycle analysis of feline lymphocytes was performed with flow cytometry, Separated lymphocyte cultures were centrifuged and the cell pellets were fixed in 50% cold citric acid buffer (250 mM sucrose, 40 mM trisodium citrate, 5% DMSO, pH 7.6) + 50% cold ethanol. Fixed cells were stored at 4° C until flow cytometry could be performed. Cells were centrifuged and resuspended in cold propidium iodide solution (50 ug/ml in PBS), a dye intercalating dye that can be used to evaluate DNA content, and incubated at 4° for at least 20 minutes. Cells were analyzed to determine cell cycle progression using a flow cytometer (EPICS V; Beckman Coulter Inc., Fullerton, CA) equipped with a 488-nm laser to excite propidium iodide. Laser light was blocked with a 515-nm long pass filter. Emitted light was split with a 590-nm dichroic filter and directed through a 630-nm long pass filter before detection by a photomultiplier tube.

Preparation of Apoptotic DNA Ladders. Apoptotic ladders were prepared as described.[59] Separated feline lymphocytes (10^7 cells) were cultured in the presence of PW (50 ug per 5 ml culture) for 5 days, Con A (350 ug per 5 ml culture) for 3 days, or PHA (100 ug per 5 ml culture) for 6 days. Cultures were centrifuged as above and washed once in cold Hank's buffered saline solution (HBSS). The cell pellets were resuspended in 250 ul of TE buffer (10mM Tris HCl, 1 mM EDTA pH 7.4). 250 ul of lysis buffer (5 mM Tris HCl pH 8.0, 20 mM EDTA, 0.5% Triton-X 100) was added and the samples were incubated for 30 minutes at 4° C. The samples were centrifuged at 13,000 g for 15 minutes. The supernatant was discarded and the pelleted DNA was precipitated by adding 50 ul of 5 M NaCl and 1 ml of cold ethanol. The DNA was precipitated at –70° C overnight and collected by centrifugation at 13,000 g for 15 minutes. The DNA pellets were dissolved in 500 ul of TE buffer and 2 ul of RNAase (2.5 mg/ml RNAase A, 25,000 Units/ml RNAase T1) was added. The samples were incubated at 37° C for 30 minutes. DNA was extracted with 500 ul of phenol:chloroform:isoamyl alcohol (25:24:1). The samples were vigorously vortexed and centrifuged at 13,000 g for 5 minutes. The aqueous phase was transferred and extracted with 500 ul chloroform:isoamyl alcohol (24:1). Vortexing, centrifugation, and transfer of the aqueous phase were repeated. DNA was precipitated by adding 50 ul of 5 M NaCl and 1 ml of cold ethanol and incubated overnight at –70° C. The DNA was collected by centrifugation. The pellets were washed with 70% ethanol, dried briefly, and dissolved in 20 ul of TE buffer.

Isolation of Genomic DNA from Uncultured Lymphocytes. 10^7 separated feline lymphocytes were collected by centrifuging at 1200 RPM for 10 minutes then treated

with lysis buffer (10 mM Tris HCl, 400 mM NaCl, 2 mM EDTA, pH 8.2) at 37° C overnight. Cellular debris was precipitated with 6M NaCl at 4° C for 15 minutes. The sample was centrifuged and the supernatant was collected. DNA was precipitated by addition of ethanol then collected by centrifugation at 19,000 g for 5 min. The pellet was washed with 70% ethanol, dried briefly and resuspended in TE buffer. RNAase was added and the sample was incubated for 1 hour at 37° C. DNA was re-precipitated with 5 M ammonium acetate and ethanol, washed with 70% ethanol, and resuspended in TE buffer.

Results.

Metaphase Yield From Mitogen Stimulated Feline Lymphocyte Cultures. Based upon blastogenesis studies and the few existing cytogenetic reports Con A is highly mitogenic to feline lymphocytes in peripheral blood with optimum[57,60-64]. Although ConA has been used in previous cytogenetic studies of feline lymphocytes, I obtained a very low (0-3%) mitotic index after 2 hours in colcemid on days 3 and 4 of culture and no mitotic figures on day 5[61-64]. Con A stimulated cultures did not yield any metaphases unless at least 75 ul was added to a 5-ml culture. Higher concentrations of Con A were of no additional benefit. Metaphase preparations from Con A stimulated whole blood cultures had abundant background debris due to red cell agglutination. No metaphases could be collected from PHA stimulated cultures. PW stimulated cultures yielded less than 2% mitoses on day 3 and 2-4% mitoses on days 4 and 5 of culture. Based upon these results, PW is the mitogen of choice for metaphase collection from feline lymphocytes. Addition of 50 ul of PW to a 5-ml culture was selected as a starting concentration based

on our experience with canine lymphocytes. Varying the concentration of pokeweed did not significantly improve the mitotic index.

Cell Cycle Progression in Mitogen-Stimulated Feline Lymphocytes. Flow cytometric studies were undertaken to determine the optimum time to harvest PW-stimulated feline lymphocytes to maximize recovery of mitotic lymphocytes. The initial results are given in figure 8.1. These results show that while the G2/M peak increases minimally, a sub-G1 peak becomes increasingly evident with time. These results suggest that stimulation of feline lymphocytes with PW results in more apoptosis than cell proliferation. Unstimulated feline lymphocytes as well as those stimulated by Con A and

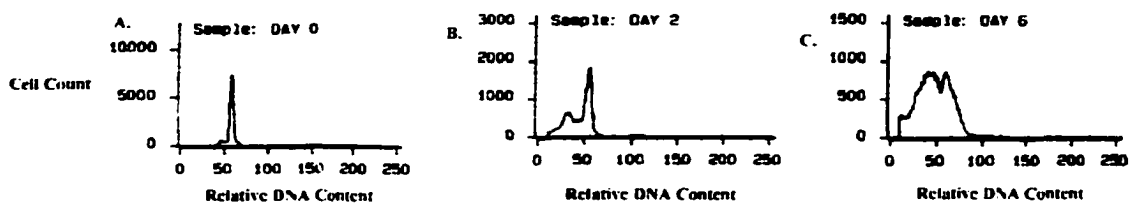

Figure 8.1. DNA histograms generated by flow cytometry of PI stained feline lymphocytes. Feline lymphocytes were cultured in the presence of PW. The cells were fixed for flow cytometry and stained with PI a) before culturing (day 0) and daily for 1 week. The histograms from PW stimulated feline lymphocytes cultured for b) 2 days and c) 5 days are shown.

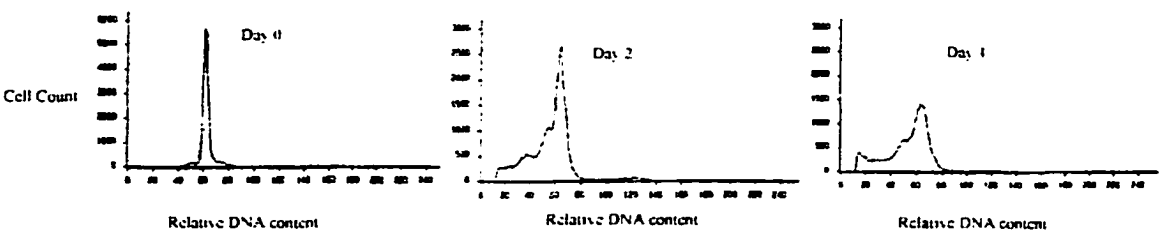

Figure 8.2. DNA histograms generated by flow cytometry of PI stained feline lymphocytes. Feline lymphocytes were cultured in the presence of Con A. The cells were fixed for flow cytometry and stained with PI a) before culturing (day 0) and daily for 1 week. The histograms from Con A stimulated feline lymphocytes cultured for b) 2 days and c) 6 days are shown.

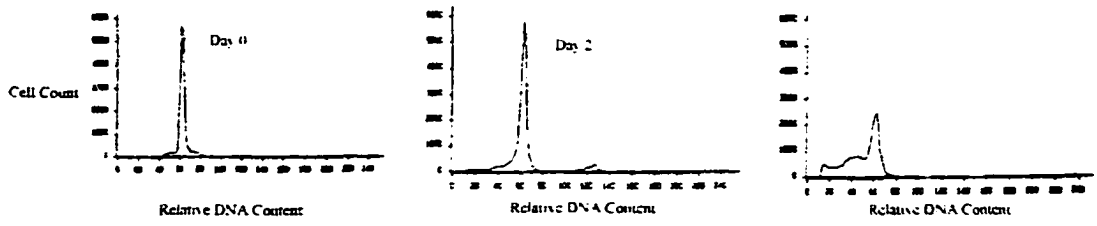

Figure 8.3. DNA histograms generated by flow cytometry of PI stained feline lymphocytes. Feline lymphocytes were cultured in the presence of PHA. The cells were fixed for flow cytometry and stained with PI a) before culturing (day 0) and daily for 1 week. The histograms from PHA stimulated feline lymphocytes cultured for b) 2 days and c) 6 days are shown.

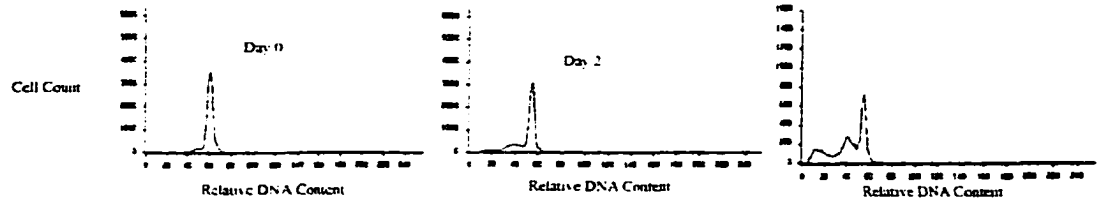

Figure 8.4. DNA histograms generated by flow cytometry of PI stained feline lymphocytes. Feline lymphocytes were cultured without a mitogen. The cells were fixed for flow cytometry and stained with PI a) before culturing (day 0) and daily for 1 week. The histograms from unstimulated feline lymphocytes cultured for b) 2 days and c) 6 days are shown.

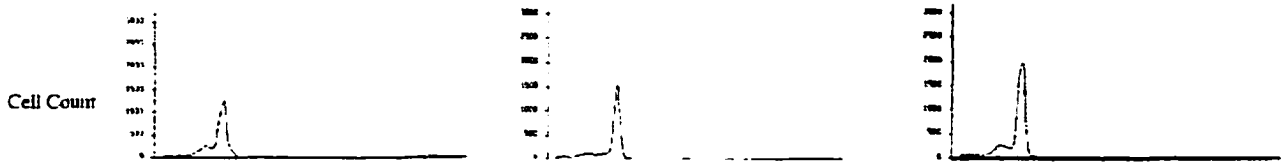

Figure 8.5. DNA histograms generated by flow cytometry of PI stained feline lymphocytes. Feline lymphocytes were cultured in the presence of a) 5 ul, b) 25 ul, and c) 50 ul of PW per 5-ml culture. The cells were fixed for flow cytometry and stained with PI before culturing (day 0) and daily for 1 week.

PHA also undergo apoptosis readily in culture. (Figures 8.2-8.4). The kinetics of the development of the subG1 peak differs with mitogen. Further, induction of apoptosis appears to have little dependence upon dose within the range studied. (Figure 5)

Confirmation of Massive Apoptosis in Feline Lymphocytes. A subG1 peak is consistent with, but not direct proof of apoptosis. To determine if apoptosis was indeed occurring in stimulated feline lymphocytes, genomic DNA was isolated and evaluated by agarose gel electrophoresis. DNA from mitogen stimulated feline lymphocytes clearly shows the 200 bp ladder which is characteristic of apoptosis. (Figure 8.6) Furthermore, when metaphase slides were examined with fluorescence microscopy, nuclei forming apoptotic bodies are visible with DAPI staining. (Figure 7) Therefore, the subG1 peak identified by flow cytometry in feline lymphocyte cultures indicates an apoptotic cell population.

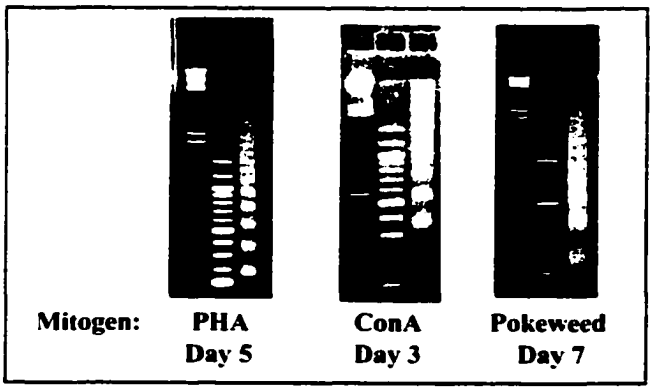

Figure 8.6. Apoptotic DNA ladders isolated from mitogen stimulated feline lymphocytes that were cultured in the presence of a) PW, b) Con A, or c) PHA for a length of time sufficient to assure that the subG1 peak in the DNA histogram would encompass most of the cells. The apoptotic DNA was isolated and run on an agarose gel by electrophoresis.

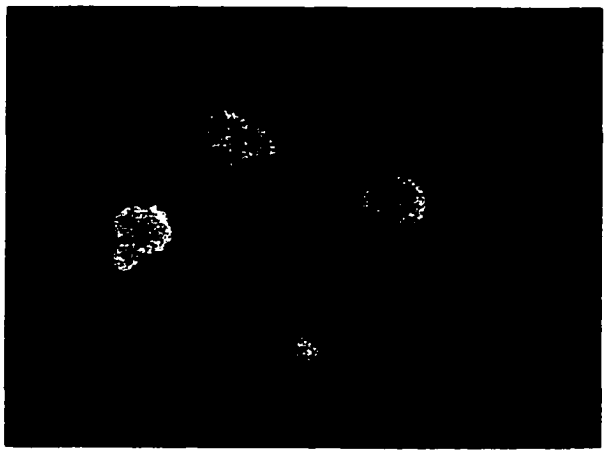

Figure 8.7. Apoptotic bodies visualized with Dapi-staining of feline lymphocyte metaphase preparations. These particular cells came from a culture stimulated for 5 days with PW. Apoptotic bodies are the morphologic equivalent of membrane encompassed DNA fragments produced during apoptosis.

Modulation of Apoptosis to Improve Mitotic Recovery from Feline Lymphocyte Cultures. To enhance my ability to stimulate feline lymphocytes and thus to collect metaphases, it was necessary to inhibit the apoptotic response. Because serum contains immunologic proteins capable of inducing apoptosis, I evaluated the effect of eliminating serum and varying the concentration of heat inactivated fetal bovine serum on mitotic yield from whole blood cultures. These efforts failed to improve mitotic index in the cat.

A high concentration of ZnS04 in culture media is reported to inhibit apoptosis[65]. I confirmed a pronounced inhibition of apoptosis with ZnS04. Unfortunately, lymphocytes failed to proliferate and no mitotic cells could be collected.

Cytokines, particularly IL-2, are known to modulate apoptosis in lymphocytes[66-70]. Feline recombinant IL-2 is not commercially available, so we evaluated the effects of human and murine recombinant IL-2 on cat lymphocytes. Mitotic indices from 5-day cultures treated with pokeweed alone or pokeweed and murine IL-2 were 3.2% and 3.6%, respectively. Cultures treated with human IL-2, however, had a mitotic index of 6.8%.

Discussion.

Human peripheral blood lymphocytes (PBL's) have been used in genetic studies for 40 years[55,56]. These cells are particularly convenient with respect to collection and culture. Another useful feature is the ability of mature lymphocytes to be stimulated from the quiescent G0 state, when collected, into the cell cycle. This particular attribute reflects the lymphocyte's function as an immune cell. When a lymphocyte is appropriately stimulated by an antigen for which it has specificity, clonal expansion ensues. This process allows for development of a specific immune response. Just as

stimulation is an important characteristic of lymphocytes, control of inappropriate stimulation of lymphocytes is equally important. This is accomplished through the exquisite sensitivity of lymphocytes to apoptosis and is an important mechanism for self-tolerance.

Normal lymphocytes from healthy humans are resistant to apoptosis in the absence of stimulation[65]. It has been reported that unstimulated cat lymphocytes from specific pathogen free cats are relatively sensitive to spontaneous apoptosis in culture[65]. Our findings show that both stimulated and unstimulated normal feline lymphocytes are highly susceptible to apoptosis. Disease states, particularly viral infections are known to increase susceptibility of lymphocytes to apoptosis. Sensitivity of lymphocytes to apoptosis in HIV infected individuals contributes considerably to the pathogenesis of AIDS[71]. Feline immunodeficiency virus infection in cats is an important animal model for HIV infection. It has been reported that the lymphocytes of FIV infected cats are more susceptible to apoptosis than normal cats[72,73]To my knowledge, the extreme sensitivity of normal cat lymphocytes to apoptosis in culture that results in low blastogenic response and mitotic index, has not been specifically reported.

Modulation of apoptosis in PW stimulated feline lymphocytes is possible in culture with the use of human recombinant, but not murine IL-2. This suggests that human IL-2 has adequate homology with feline IL-2 to bind and activate cell receptors whereas mouse IL-2 does not. It has been previously reported that both human and feline recombinant IL-2 are capable of stimulating feline lymphocytes[67]. Feline IL-2 fails to stimulate human lymphocytes[67]. I were unsuccessful in promoting proliferation and inhibiting apoptosis with other means including use of different mitogens at various

concentrations, varying serum concentration or eliminating serum, raising the incubation temperature. I obtained the best mitotic yield and metaphase quality when feline whole blood was culture in the presence of PW and IL-2 for 4-5 days with colcemid added for the last ½-2 hours of culture.

We have also observed increased sensitivity to apoptosis in the lymphocytes from turtles, but not from dogs or cattle. This suggests that sensitivity to apoptosis is species dependent. In contrast to our experience with cats, turtle lymphocytes are best cultured in serum free media, suggesting that apoptosis in these cells was triggered by immunologic proteins present bovine serum.

I do not know the mechanism for increased sensitivity of feline lymphocytes to apoptosis. Because culture condition such as incubation temperature and serum concentration failed to inhibit apoptosis, it is likely that the sensitivity to apoptosis reflects a cellular mechanism in cats, rather than culture conditions. It has been suggested that spontaneous apoptosis in feline lymphocytes results from high constitutive expression of MHC II antigen on unstimulated feline lymphocytes whereas human lymphocytes do not express this antigen without stimulation[65]. Apoptosis is a highly regulated cellular process coordinated by a variety of gene products including inducers such as p53 and inhibitors such as Bcl-2. The pathways controlling apoptosis from initiating signal to nuclear fragmentation are under intensive investigation as evidenced by a search of the PubMed database yielding over 800 references for apoptosis and signal transduction this year (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). It is possible that the feline proteins, integral to the apoptotic pathway, are modified in such a way as to confer hypersensitivity to apoptosis.

There is abundant evidence that mitochondrial derived reactive oxygen species (ROS) are important in signaling apoptosis in lymphocytes[74-76]. Interestingly cats are sensitive to oxidants. Heinz bodies, red blood cell lesions resulting from the oxidation of hemoglobin, are considered a normal finding only in cats and may be found in seemingly excessive numbers in healthy cats[77,78]. Cats are can become severely intoxicated from minimal exposures to oxidant toxins such as onions or onion powder in baby food, propylene glycol present in some pet foods, and acetominophen[79-83]. Oxidant toxicity in the cat is characterized by excessive Heinz body formation and hemolytic anemia. Cats may have low levels of tissue antioxidants compared to other species[84]. It is possible that enhanced sensitivity to apoptosis in cats is related to their tolerance of oxidative damage. The impact of enhanced lymphocyte apoptosis in vivo, particularly on the prevalence and course of disease such as immune-mediated illness and cancer, would be worthy of investigation.

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CHAPTER 9: ESTABLISHING THE FELINE KARYOTYPE FOR NORMAL AND TUMOR-BEARING CATS USING COMPUTER ENHANCED FLUORESCENT R-BANDING

Introduction.

Cancer Genetics and Predisposition. Cancer is a genetic disease.[1-4] Understanding the genes involved in carcinogenesis from the initiating event to the development of an increasingly malignant phenotype is critical to detection, prevention, and treatment of tumors. It is clear, that individuals vary in their susceptibility to the development of specific tumors. There are two categories of genes conferring genetic susceptibility to cancer: 1) The highly penetrant, recessive genes. Mutations in these genes are associated with greatly increased risk of cancer to the individual, but are rare in the population and do not have a significant impact on the general population. These genes are responsible for familial retinoblastoma, Li-fraumeni syndrome, and Wilm's tumor, for example. 2) The genes that have low penetrance, but are very frequent in the population. These genes minimally increase risk to the individual, but greatly increase the population attributable risk.[5] The impact of the low penetrance genes on cancer risk is modified by lifestyle factors, exposures, and other genetic traits.[5] These traits may be very important in the occurrence of 'sporadic ' as well as inherited cancer in humans.

3. Cats developing vaccine-associated tumors are younger than cats developing soft tissue sarcomas that are not vaccine associated. As with people, developing a tumor as a child or young adult strongly suggests a predisposition[9].
4. The latency period, between vaccination and tumor development, is short. Reported latency periods range from a few weeks to two years. This suggests that tumorigenesis is accelerated in these individuals[6,8].
5. There are reports vaccine associated tumors in siblings (littermates) suggesting an inherited genetic component to tumorigenesis[10].
6. Some cats develop multiple primary tumors. This is reported at CSU and by other veterinary institutions[11].

Although some of this evidence is informal and anecdotal, the suggestion of susceptibility remains and is worthy of further exploration. To investigate the genetics of feline vaccine associated, I set out to develop cytogenetic techniques for use in cats. My intention was (and is) to define chromosomal loci that are important in carcinogenesis and may provide means of preventing and diagnosing feline vaccine-associated sarcoma as well as targeting therapy. The first step in this process was to define normal feline karyotypes.

Feline Cytogenetics. The normal G-banded karyotype has been established for the domestic cat. Cats possess 36 autosomes and two sex chromosomes. The autosomes are arranged into 6 groups (A through F) according to chromosome size and centromere position[12-16]. Comparative mapping studies have demonstrated a great deal of conservation between the human and feline Karyotypes[17-19]. Nine out of 20 cat chromosomes are entirely labeled by a single human chromosome painting probe and 14

of 23 human chromosomes are entirely labled by a single cat chromosome painting probe[19]. There is significant conservation of gene location within chromosomal segments[17,20,21]. "Only seven chromosome breaks, one intrachromosomal inversion and 11 simple whole chromosome fusions are necessary to 'convert' the human karyotype to that of the cat[19]."

There are only a handful of reports describing cytogenetic aberrations in feline tumors. These include studies of lymphoma, mammary tumors, and three fibrosarcomas[22-33]These reports substantiate that aberrations are commonly associated with feline tumors, but important, recurrent aberrations have not been identified or characterized.

In this chapter, I will demonstrate the normal feline karyotype from lymphocytes using a novel form of fluorescent R-banding that we developed. In addition, I have karyotyped peripheral blood lymphocytes from five cats with vaccine-associated sarcoma to evaluate for chromosome abnormalities that might be associated with tumor susceptibility.

Materials and Methods.

Blood Collection. Blood was collected by jugular venipuncture of client owned cats presenting to the Veterinary Teaching Hospital at Colorado State University. All cats tested ELISA negative for feline leukemia virus and feline immunodeficiency virus and were either clinically healthy or had feline vaccine-associated sarcoma. Blood samples were anticoagulated in sodium heparin collection tubes (Vacutainer®, Bectin Dickinson; Franklin Lakes, NJ).

Metaphase Preparation from Lymphocytes. One ml of heparinized whole blood was added to 10 mls of media (RPMI 1640 containing 20% fetal calf serum, 4 mM l-glutamine, Penicillin, streptomycin, and hepes). Pokeweed mitogen (10 ug/ml) and human recombinant IL-2 (10 Units/ml) were added and the flasks were incubated for 5 days at 37° C in 5% CO2. The cell pellet was then collected and treated with warm, hypotonic KCl (0.075 M) and fixed with 3:1 methanol: acetic acid. The fixed nuclei were dropped on clean microscope slides and dessicated for at least 1 week.

Metaphase Preparation from AK-D Cell Line. I prepared metaphase slides of the cell line with a standard technique[34]. Briefly, Colcemid (0.1 ug/ml final concentration) was added when cultures were 70-80% confluent. Cells were harvested at 30 minutes, 1 hour, and 2 hours following addition of colcemid. I varied the time in colcemid to optimize degree of condensation of metaphase chromosomes for the purpose of karyotyping. Cells were trypsinized, centrifuged, and the pellet was resuspended in warm hypotonic (0.075 M) KCl for 15 minutes. Following a second centrifugation, cells were fixed in 3:1 methanol: acetic acid. The cell pellet was diluted in the fixing solution to achieve an appropriate cell concentration and to allow for spreading of chromosomes on microscope slides. Slides were dried and aged for at least one week.

R-Banding. A novel method of computer enhanced fluorescent R-banding (D/C R-banding) was used for karyotyping. For this procedure, metaphase preparations were 4', 6-diamino-2-phenyl-indole (DAPI), which binds A-T rich regions of DNA and with chromomycin A3, which binds preferentially G-C rich regions. I captured 2 gray scale images of metaphase nuclei using a fluorescent microscope equipped with a filter wheel containing 350 nm and 405 nm filters, and a cooled CCD camera. Using a computerized

image analysis system (Metamorph®) the images were normalized for intensity to account for variability in the staining, the 350-nm filtered image was arithmetically divided by the 405-nm image yielding a high-resolution R-banded pattern unique to each chromosome.

Karyotyping. Chromosome number was determined from 20 spreads from each cat. Five spreads were karyotyped from each cat. Chromosomes were arranged in pairs examined for banding pattern aberrations.

Results.

Figure 8.1. Computer enhanced D/C R-banded normal feline metaphase spread.

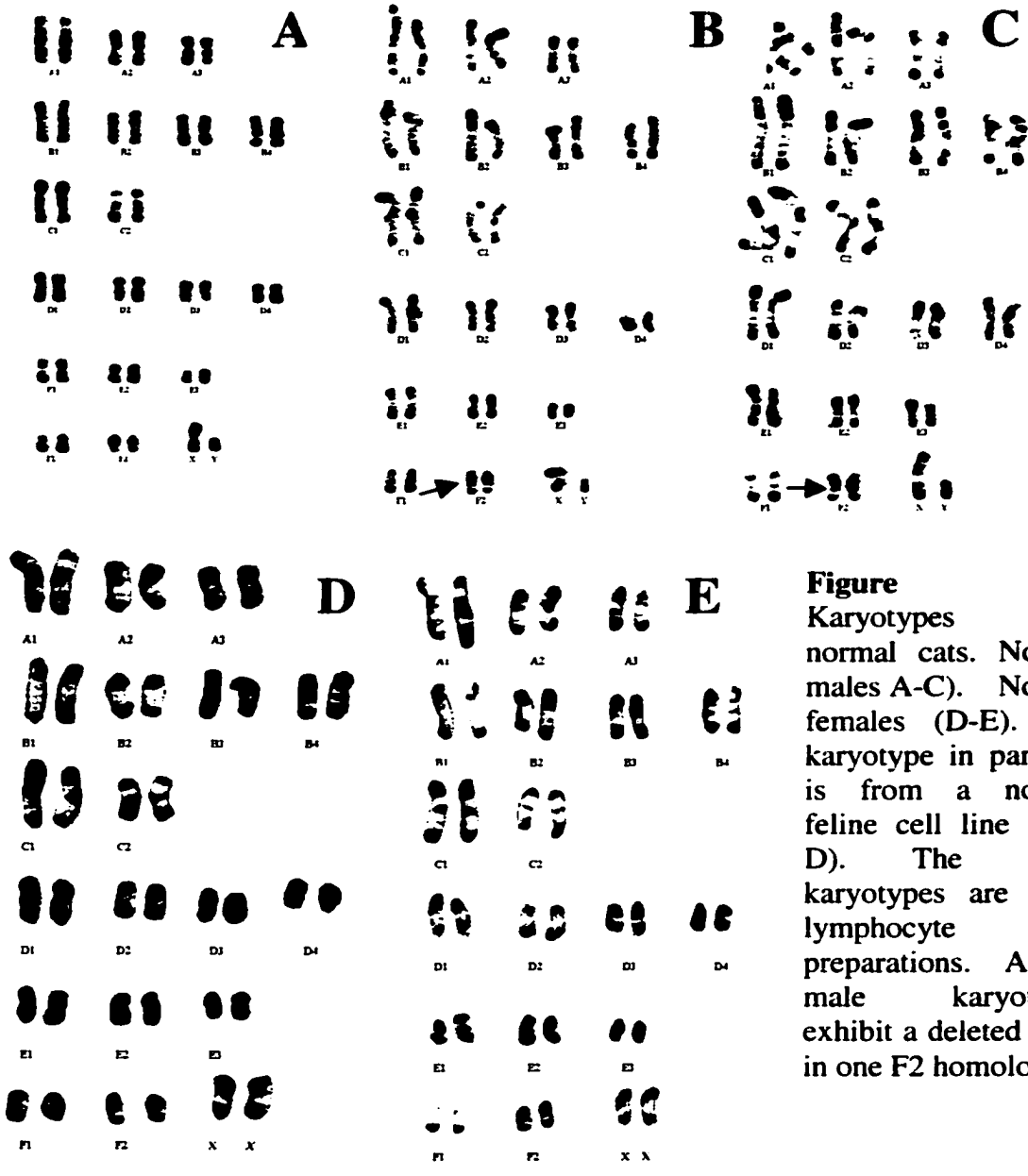

Figure 9.2. Karyotypes from normal cats. Normal males A-C). Normal females (D-E). The karyotype in panel C is from a normal feline cell line (AK-D). The other karyotypes are from lymphocyte preparations. All 3 male karyotypes exhibit a deleted band in one F2 homologue.

Normal R-Banded Karyotypes. High quality banded metaphase spreads were obtained with D/C R-banding technique. (Figure 8.1) Five different, apparently normal feline karyotypes are shown as examples of this technique and to demonstrate the characteristics of the feline R-banded karyotype. (Figure 8.2 A-E) As expected, the modal number was 38. A deleted band in the F2 chromosome was detected in two normal cats and in a normal feline cell line (AK-D). The lymphocyte karyotypes were

obtained from two healthy adult male cats ("Bartholomew" McNiel and "Mungo Jerry" LaRue. Both have never shown signs of disease. Although the significance of this

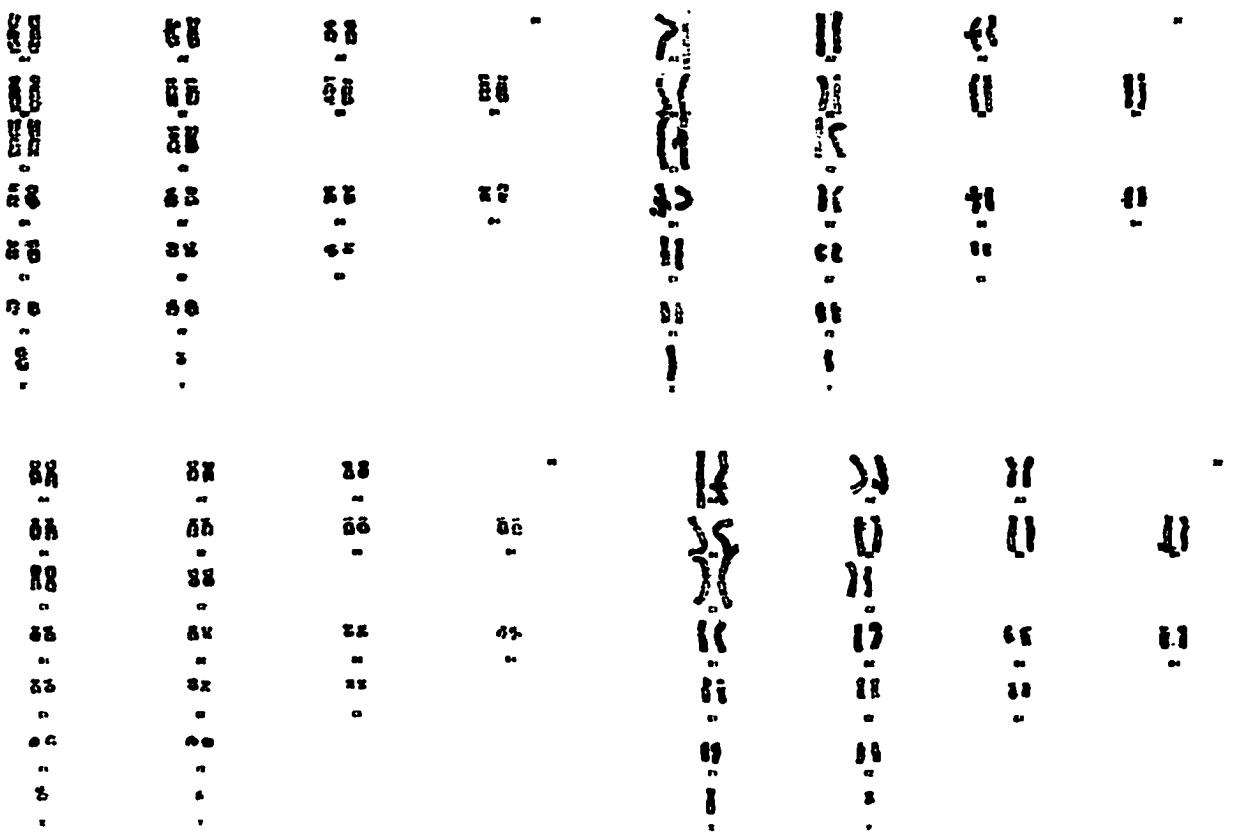

Figure 8.3. Karyotypes of cats with feline vaccine associated sarcoma. (lymphocyte preparations). All karyotypes are normal.

abnormality is unknown, it may be a normal variation in the feline karyotype.

Karyotypes of Cats with Feline Vaccine-Associated Sarcoma. Certain hereditary diseases are associated with chromosomal aberrations. To determine whether a cytogenetic lesion is associated with cancer predisposition in cats that develop vaccine-associated tumors, I karyotyped 5 cats with feline vaccine-associated sarcoma. These karyotypes revealed no significant abnormalities (Figure 8.5 A-D).

Discussion.

I have established a database of normal feline karyotypes using a novel form of fluorescent, computer enhanced R-banding. This database will facilitate further banding and molecular cytogenetic studies of the feline genome. Besides producing high quality banding patterns, fluorescent R-banding can be performed simultaneously with fluorescent in situ hybridization techniques such as comparative genomic hybridization (CGH) and multiplex fluorescence in situ hybridization. Previously the performance of FISH techniques has relied on a relatively indistinct DAPI banding patterns.

Another feature of the R-banding technique is the associated "linescan" patterns that are unique to particular chromosomes and can be used in automated karyotyping. I have worked to establish linescan databases and am developing computer software to carry out chromosome identification.

Cats with vaccine-associated sarcoma have an apparently normal karyotype in peripheral blood lymphocytes. The resolution of detailed banding studies is limited to aberrations ? in size, however. It is possible that the inherited genetic defect that confers cancer susceptibility is smaller than this. Tumor studies are likely to be helpful in finding recurrent aberrant loci and determining which loci occur early in tumorigenesis.

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CHAPTER 10: FELINE TUMOR CYTOGENETICS.

Introduction.

Molecular Cytogenetics. Although chromosomes have been studied for 100 years, limitations in microscopy and staining techniques restricted the information that could be derived from cytogenetic evaluations of tumors[1]. However, the first recurrent tumor associated aberration described, the so-called "Philadelphia chromosome," in chronic myelogenous leukemia (CML) was identified even before the development of banding techniques[1-3]. This particular abnormality exemplifies all of the useful aspects of tumor cytogenetics: including 1) providing a prognostic and diagnostic marker and 2) localizing the position of cancer associated genes, in this case, the bcr-abl rearrangement that codes for an oncogenic fusion protein[1-3].

Chromosome banding was first reported in 1970 by Casperson[4] and the subsequent improvement and application of these staining techniques were critical to the identification of recurrent abnormalities in tumors. Most of the progress over the next 20 years was in the characterization of hematopoietic tumors which have rather simple karyotypes compared to solid tumors[4]. A variety of cancer-associated genes and chromosomal loci have now been identified through the application of cytogenetic techniques.

In the last 10-15 years, the introduction of molecular cytogenetic techniques including chromosome and gene specific fluorescence in situ hybridization, comparative genomic

hybridization (CGH), and multiplex FISH (M-FISH) has greatly enhanced the resolution and specificity of tumor cytogenetics.[3,5,6] These advancements are particularly important for the study of the complex karyotypic rearrangements found in solid tumors. In the last decade, the number of reports of abnormal karyotypes from solid tumor cases has exploded primarily due to the development of CGH and M-FISH[1,3,5,6]. CGH is a method for detecting numerical chromosome changes in tumors that involves the simultaneous hybridization of tumor and normal DNA, labeled with 2 different fluorochromes, onto a normal metaphase spread. Computer image analysis is then able to find differences in the fluorescent intensity of the two fluorochromes indicating gains or losses in the tumor at particular chromosomal loci.

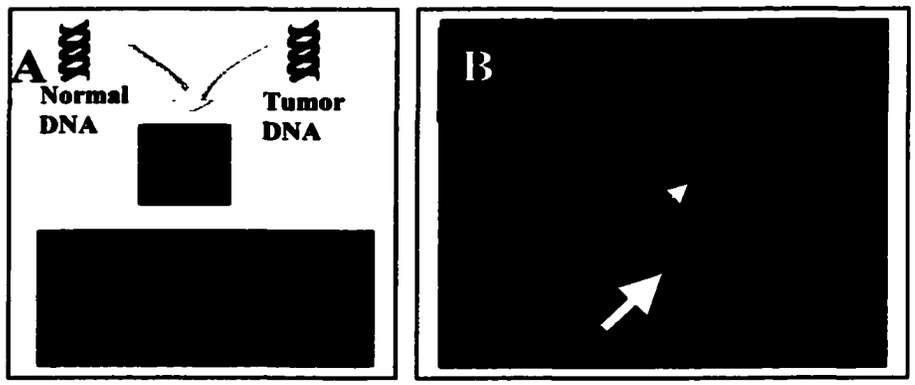


Figure 9.1. Comparative genomic hybridization involves the hybridization of normal DNA labeled with a red fluorescent dye and tumor DNA labeled with a green fluorescent dye onto normal chromosomes. Areas where the Green to red fluorescent ratio is high indicates amplification. Where it is low indicates deletion. A) Diagram of the process. B) CGH of a human tumor showing multiple abnormalities including amplifications (large arrow) and deletions (small arrow).

M-FISH allows for the detection of both numerical and exchange type chromosomal aberrations (translocations). M-FISH involves the simultaneous hybridization of whole

chromosome painting probes for each chromosome onto a tumor metaphase spread. The whole chromosome painting probes are labeled with different combinations of fluorochromes, one unique combination for each chromosome. Different chromosomes can be readily identified by a unique fluorescent signal using a computer image analysis system. Solid tumors account for a considerable portion of human cancer incidence and mortality. The genetic study of solid tumors may be of particular importance in the fight against cancer.

In this chapter I demonstrate the use of classical and molecular cytogenetic techniques to characterize chromosomal aberrations a feline tumor cell line.

Materials and Methods.

Cell Line. SPARKY, a feline bronchoalveolar carcinoma cell line produced from a pleural effusion of a neutered male cat (generously provided by Sanford Barsky MD, PhD, UCLA Medical Center, Los Angeles, California), was cultured in alpha-MEM (15% fetal calf serum, 1 mM l-glutamine, 50 Units/ml penicillin, 50 ug/ml streptomycin, and 20 mM HEPES buffer) in a humidified incubator at 37° C with an atmosphere of 5% CO_2 in air.

Metaphase Slide Preparation. I prepared metaphase slides of the SPARKY cell line with a standard technique[7]. Briefly, colcemid (0.1 ug/ml final concentration) was added when cultures were 70-80% confluent. Cells were harvested at 30 minutes, 1 hour, and 2 hours following addition of colcemid. I varied the time in colcemid to optimize degree of condensation of metaphase chromosomes for the purpose of karyotyping. Cells were trypsinized, centrifuged, and the pellet was resuspended in warm hypotonic (0.075 M) KCl for 15 minutes. Following a second centrifugation, cells were fixed in 3:1 methanol:

acetic acid. The cell pellet was diluted in the fixing solution to achieve an appropriate cell concentration and to allow for spreading of chromosomes on microscope slides. Slides were dried and aged for at least one week.

Confirming Feline Origin. To demonstrate that SPARKY was derived from feline tissue, I performed fluorescence in situ hybridization with FITC labeled feline genomic DNA hybridized onto human, feline, and SPARKY metaphase slides. I extracted DNA from peripheral blood lymphocytes of a "normal" cat and used the genomic extract to create a probe using degenerative oligonucleotide-primed polymerase chain reaction (DOP-PCR) with digoxigenin-conjugated dUTP(Boehringer-Mannheim, Indianapolis, IN)[8,9]. Labeled DNA was mixed with 50% formamide, 2X SSC, and 10% dextran sulfate, denatured for 10 minutes at 72°C, and preannealed at 37°C for 45 minutes. No blocking DNA was used. I then allowed the probe to hybridize for 24 hours at 37°C to denatured metaphase slides from i) the SPARKY cell line, ii) normal feline lymphocytes (positive control), and iii) normal human lymphocytes (negative control). The slides were washed at 45°C in 1) 50 % Formammide/2 X SSC, 2) 2X SSC, 3) 0.1X SSC for 10 minutes each. Slides were then rinsed in room temperature PBD. Slides were treated with FITC-conjugated anti-digoxigenin antibody (Boehringer-Mannheim, Indianapolis, IN). Slides were visualized using a Zeiss Axioskop fluorescent microscope (Carl Zeiss Inc., Thornwood, NY).

R-Banding and Karyotyping[10]. Chromosomal abnormalities were characterized using D/C R-banding as described in Chapter 9. I enumerated chromosomes in 20 tumor metaphase spreads and fully karyotyped 4 metaphases.

Comparative Genomic Hybridization. ***DNA isolation:*** Tumor DNA was isolated from cells in culture. Lysis buffer was added to a confluent,100-mm tissue culture plate which was incubated briefly. Lysis buffer containing cell debris was transferred to a conical centrifuge tube and incubated at 37° C overnight. Saturated sodium chloride was added and the tube was refrigerated for twenty minutes, centrifuged, and the supernatant was transferred to a high speed centrifuge tube. Ethanol was added to precipitate the DNA. The DNA was collected by centrifugation and treated with RNAase for 1 hour. The DNA was once again precipitated using Ammonium acetate and ethanol, collected by centrifugation, rinsed with 70% ethanol, and dissolved in TE buffer. Normal DNA was isolated from whole blood samples obtained from healthy cats, as described.

CGH was performed using a previously reported technique.[11,12] SPARKY DNA was labeled with FITC dUTP (green fluorescence) (Boehringer-Mannheim, Indianapolis, IN) and Normal DNA will be labeled Texas Red® dUTP(NEN® Life Science Products, Boston, Massachusetts) (red fluorescence) using Degenerative Oligonucleotide Primed Polymerase Chain reaction (DOP-PCR), as described[9,13]. Briefly, universal primers and low annealing temperature cycles were used to uniformly amplify all sequences in each DNA sample. Labeled nucleotides were incorporated into the amplified product by the Taq polymerase. Labeled tumor and Normal DNA were precipitated and dissolved in hybridization buffer (Hybrisol® VII, Intergen, Purchase, New York) and heated to 72° C for 10 minutes. The DNA probe was cooled to 37° C, incubated 1 hour, and transferred to denatured normal feline metaphase slides. The slides were dipped in Chromomycin A3, counterstained with DAPI in antifade solution and examined under a fluorescent microscope. Images were captured by a cooled CCD camera in conjunction with a

computerized image analysis system (Metamorph®). Metaphase spreads were karyotyped using the computer enhanced R-bands obtained from DAPI and Chromamycin A3 images. The fluorescent intensity of the two fluorochromes (FITC and Lissamine) was examined.

Results.

SPARKY is feline. (Figure 10.5)

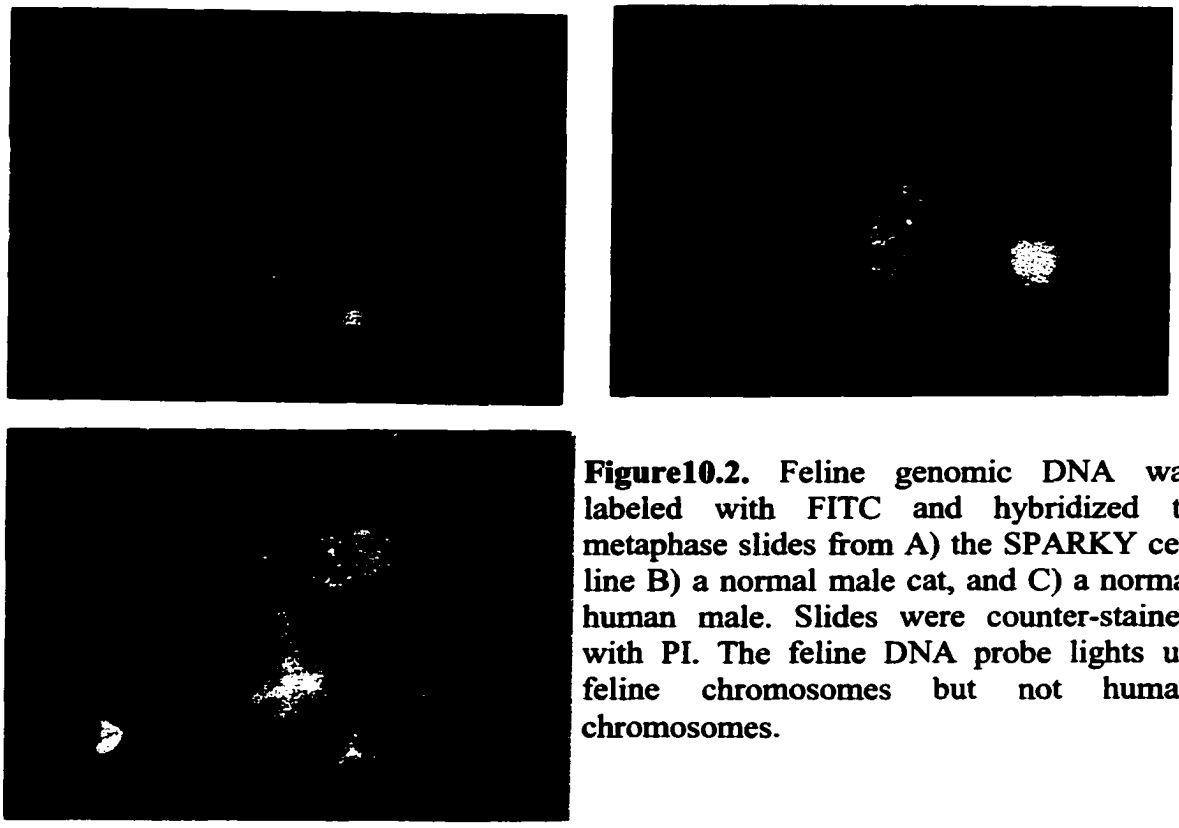


Figure10.2. Feline genomic DNA was labeled with FITC and hybridized to metaphase slides from A) the SPARKY cell line B) a normal male cat, and C) a normal human male. Slides were counter-stained with PI. The feline DNA probe lights up feline chromosomes but not human chromosomes.

SPARKY Karyotype. The chromosome number for 20 metaphase spreads from the SPARKY cell line ranged from 54 to 68 with a mode of 66. Three karyotypes are presented in figures 10.3 A-C.

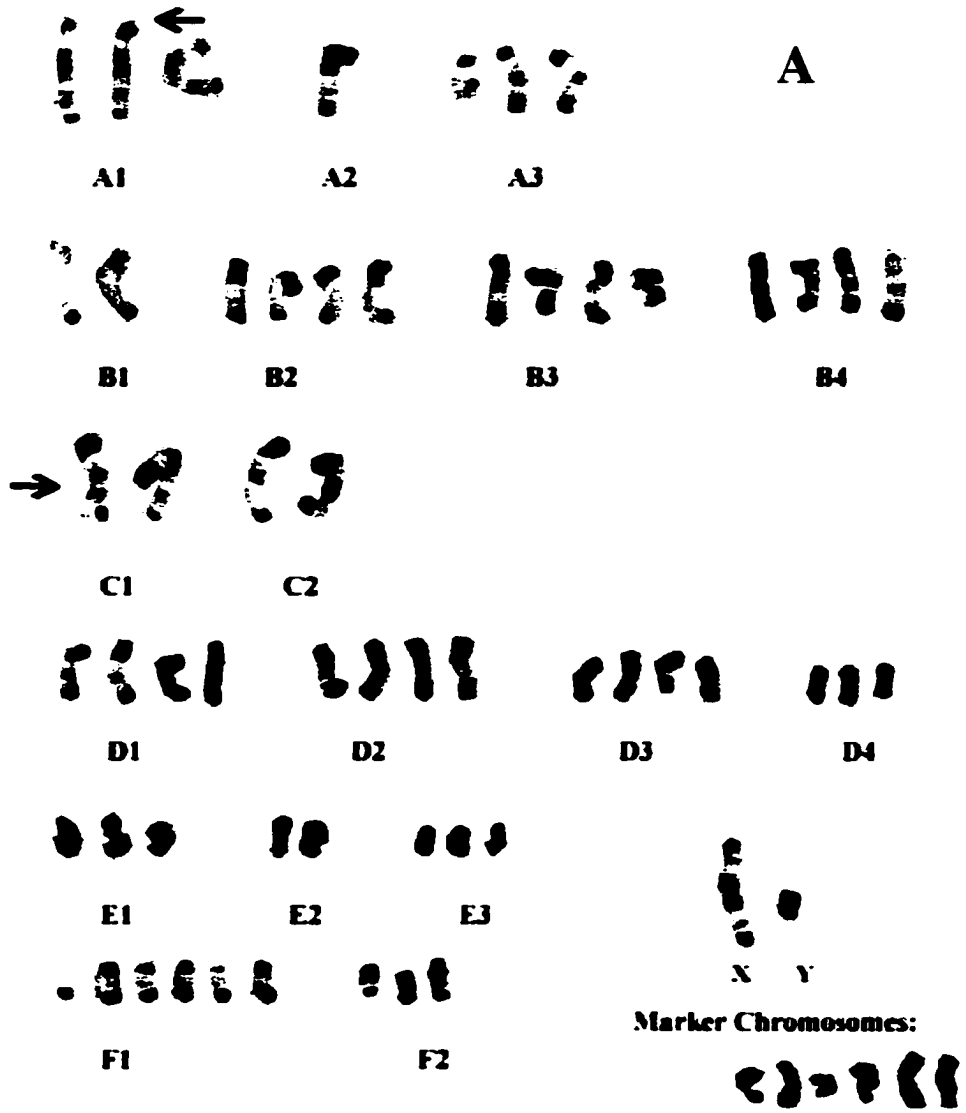
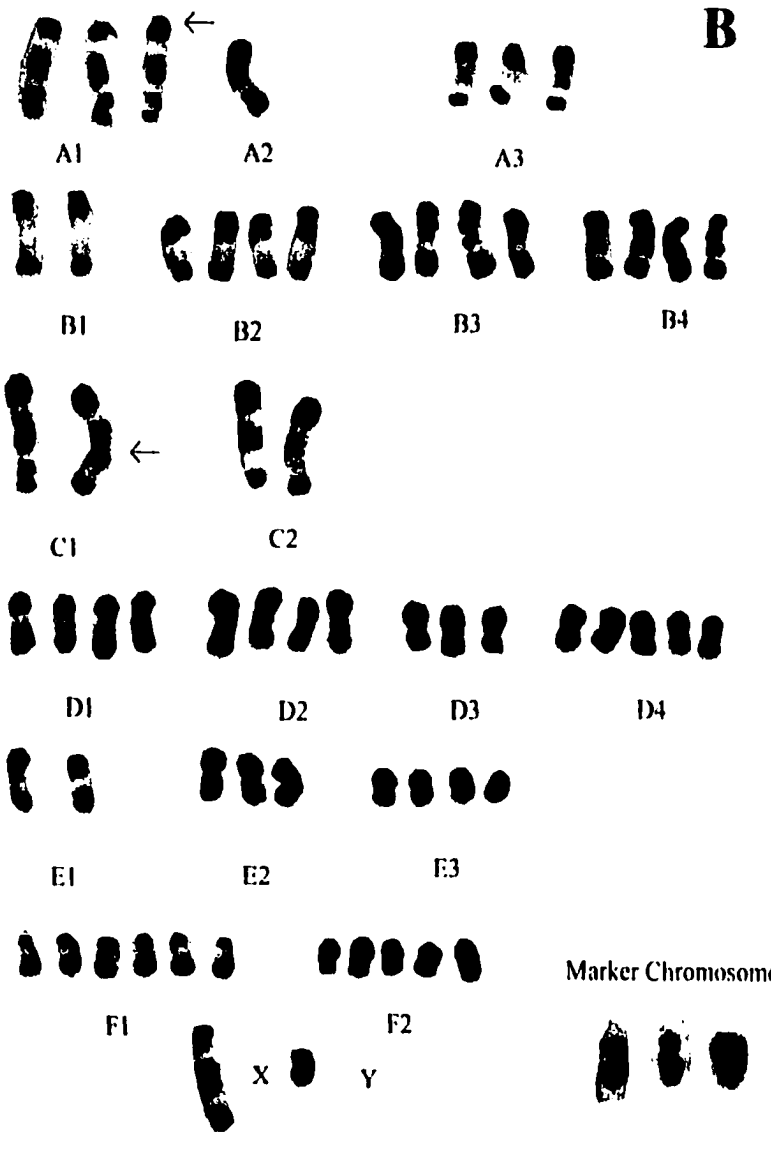
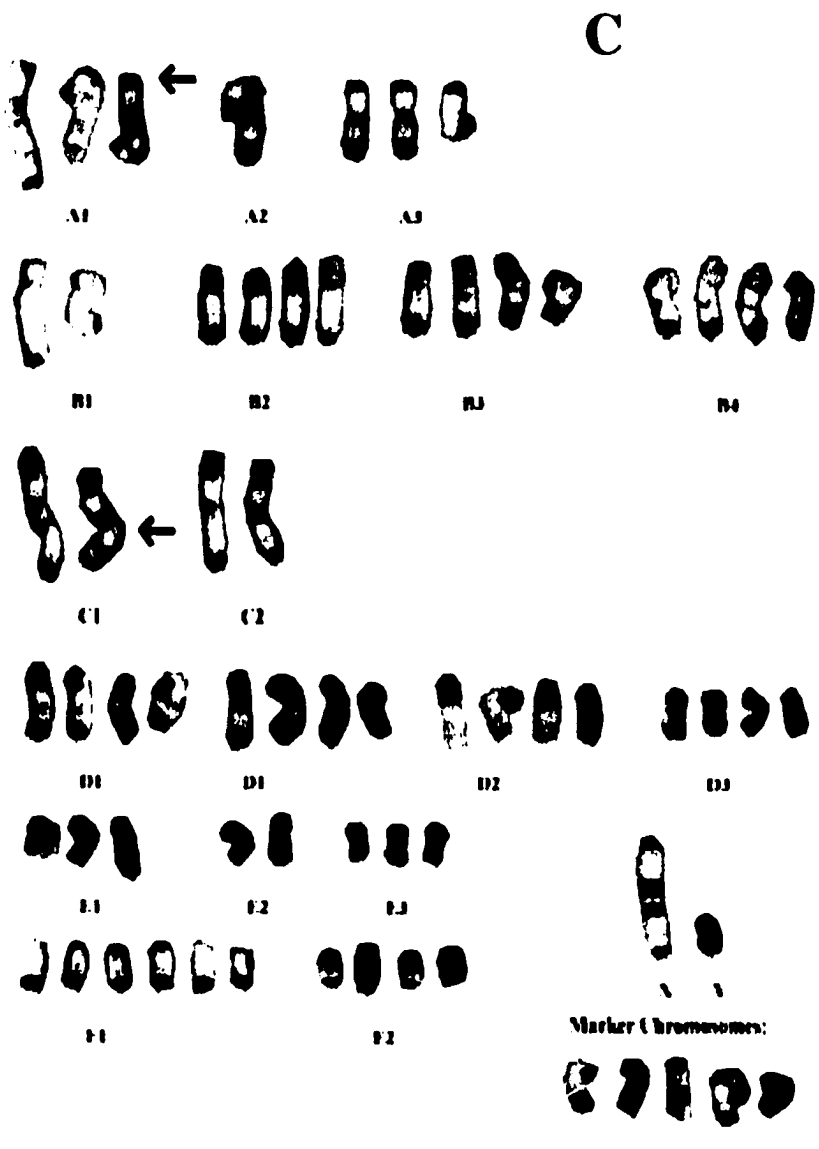


Figure 10.3. D/C R-Banded Karyotypes of the SPARKY cell line. There are multiple copies of most chromosomes and some structural abnormalities including the unidentifiable marker chromosomes. Structural alterations (indicated by arrows) include an addition onto one A1 homologue, deletions in the C1 homologues, and deletions in some of the F2 copies.and the arrows indicated by arrows.





CGH. Comparative genomic hybridization confirms the numerical aberrations identified in the R—banded karyotype. (Figure 10.4A-C)

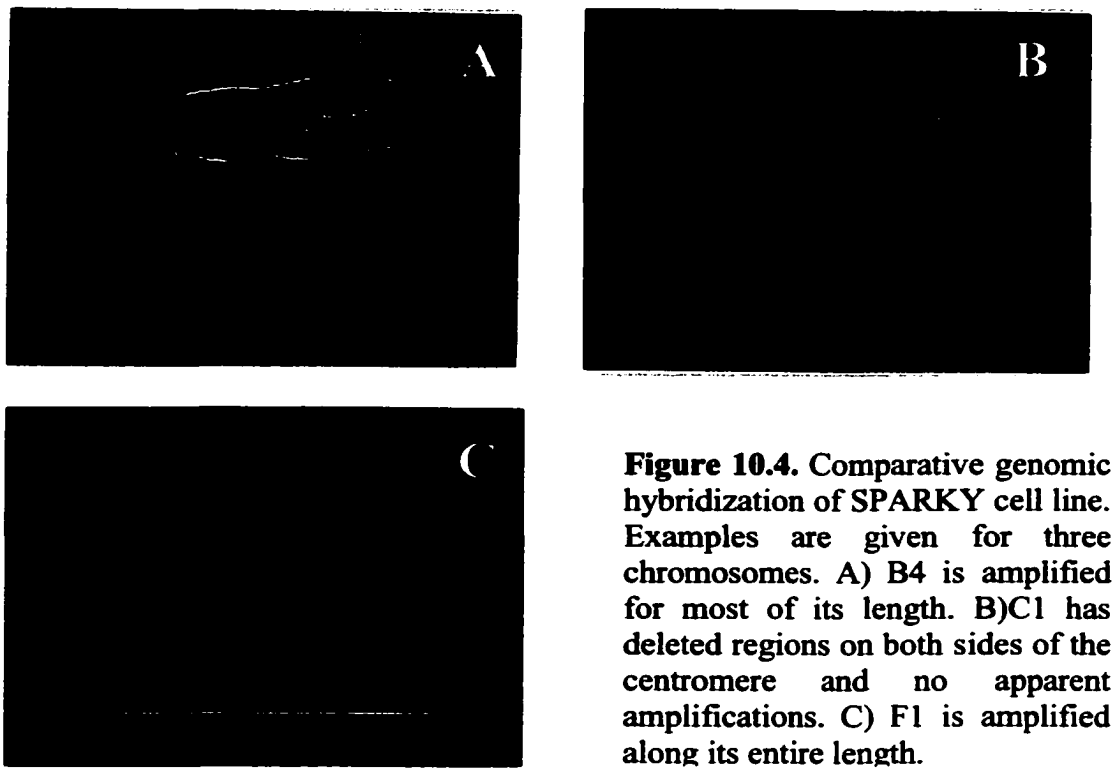


Figure 10.4. Comparative genomic hybridization of SPARKY cell line. Examples are given for three chromosomes. A) B4 is amplified for most of its length. B)C1 has deleted regions on both sides of the centromere and no apparent amplifications. C) F1 is amplified along its entire length.

Discussion.

The study of naturally occurring pet animal tumors can advance the understanding of tumor biology and the development of therapeutics for animal and human use. Characterization of specific chromosomal alterations in specific tumors is crucial to the discovery of genes important in carcinogenesis[2,3,5,6]. These aberrations also have diagnostic and prognostic value[2,3,5,6]. I am, therefore, developing techniques to evaluate feline tumors and have applied these techniques to the analysis of a feline tumor cell line. I have found that the D/C R-banding technique facilitates tumor karyotyping. Numerous

abnormalities were identified in the Feline BAC using this technique. Further, I have adapted FISH techniques such as comparative genomic hybridization (CGH) for use in the cytogenetic study of feline tumors. The results of CGH are consistent with the R-banded karyotype. I am continuing to develop this technique and have recently begun to work with a computer system specifically designed for CGH, which is greatly facilitating my efforts. I intend to continue characterizing feline chromosome aberrations in tumors including the feline vaccine-associated sarcoma.

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CHAPTER 11: CONCLUSIONS

The three parts of this dissertation have a common purpose: to gain a better understanding of carcinogenesis. From my results, I can draw a few significant conclusions relevant to vaccine-associated sarcoma in cats. However, I believe that the development of tools for research is equally important in this work. My research, as with biologic investigation in general, is often driven by technologic development rather than by hypothesis testing. Although it is the essential biologic questions that provide the significance to research, it is the schemes for answering these questions that often provides the challenge. The development of polymerase chain reaction and the subsequent flood of molecular genetic research exemplifies this process. Although I ask the question, "what causes cancer?", I find that I spend a great deal of time developing tools rather than collecting observations and data needed to answer this question. As I continue my work as an independent researcher, I will be able to refine these tools and apply them to other research problems.

Tools for Research.

Cancer in Pets. The first and I think the most valuable tool that I bring with me is the pet animal model for carcinogenesis. This model is potentially informative yet virtually uninvestigated. I have focused on the study of feline vaccine associated sarcoma. This tumor is important as it provides an excellent model for carcinogenesis, tumor progression, and genetic susceptibility. As I continue my research, I hope to explore a variety of animal cancers. Of particular interest to me is cancer predisposition. Genetic

susceptibility seems to be a frequent and important component in cancer in dogs. Cancer prone families of dogs should be easier to identify and study than humans. **The A_L Assay.** The second tool that I have gained is the A_L mutation system. The flow cytometric variations of the original A_L assay have three unique and useful features: 1) the assay is high-throughput and very accurate for identifying carcinogens 2) the assay allows for rapid characterization of the types of mutants (deletion maps, point mutants, GPI mutants) and the generation of mutational spectra. 2) mutation can be assessed at two independent loci simultaneously. I intend to seek validation of this assay and promote its use in drug and chemical testing.

Cytogenetics. Finally, I have made a great deal of progress in the development of cytogenetic techniques for cats. I am now prepared to apply classical and cytogenetic techniques to the study of feline tumors.

Vaccine-Associated Carcinogenesis.

Application of this set of tools has allowed me to reach three important conclusions and these will provide a refined hypothesis for the carcinogenesis of feline vaccine-associated sarcoma and disease in cats.

1. Adjuvanted feline vaccines are mutagenic in vitro.
2. These vaccines induce ROS, which mediates mutation.
3. Feline lymphocytes are exquisitely susceptible to apoptosis.

Both vaccines and the inflammatory response induced by vaccines induce ROS. Oxidative stress may have two cooperating carcinogenic mechanisms: 1) DNA damage is induced by ROS and 2) ROS is involved in mitogenic signaling suggesting a tumor promotional effect. There is evidence that oxidative stress is an important carcinogenic

mechanism[1-3]. Although controversial, some have proposed that the underlying cause of most cancer is oxidative damage[4]. Feline vaccine-associated sarcoma may be the only animal model of oxidative stress induced cancer.

Further, if ROS plays a significant role in the induction of vaccine-associated tumors, sensitivity to ROS may indicate a mechanism for susceptibility in cats that develop this cancer. Cats, in general, are sensitive to oxidants.

Another interesting aspect of feline disease models relates to the sensitivity of feline lymphocytes to apoptosis. Apoptosis is an important regulatory event and loss of control results in disease such as cancer. Mitochondrial ROS are believed to be involved in signaling apoptosis. It is possible that sensitivity to apoptosis and susceptibility to cancer are linked in some way. I am currently conducting studies to evaluate the antioxidant capacity and ability to repair oxidative DNA lesions in cats with and without tumors.

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