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IN VITRO CHARACTERIZATION OF CELL-MEDIATED IMMUNE FUNCTION IN NORMAL AND FELINE LEUKEMIA VIRUS-INFECTED CATS
IN VITRO CHARACTERIZATION OF CELL-MEDIATED IMMUNE FUNCTION IN NORMAL AND FELINE LEUKEMIA VIRUS-INFECTED CATS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

by

Mary Irene Stiff, B.Ed., M.S.

* * * * *

The Ohio State University
1982

Reading Committee:
Richard G. Olsen
Ronald St. Pierre
Steve Krakowka
James R. Blakeslee

Approved By

Adviser
Department Veterinary Pathobiology
DEDICATED TO
RYAN STEPHEN TORVIK
ACKNOWLEDGEMENTS

I wish to thank my adviser Dr. Richard Olsen for his encouragement and guidance throughout these studies. I am also grateful to the other members of my graduate committee for the time spent interacting with me and reviewing this research. I would like to thank Melinda Tarr for editing the manuscript, Jennifer Rojko for her collaborative work, and Teri Roberts for her secretarial skills. I also wish to thank my parents, family and close friends for their constant support. A special thanks goes to my husband, Stephen, for his understanding and encouragement throughout the pursuit of this degree.
VITA

June 11, 1952 Born - Toledo, Ohio

1974 B.Ed., University of Toledo, Toledo, Ohio

1976-1978 M.Sc. Department of Microbiology and Immunology, School of Medicine, Wright State University, Dayton, Ohio

1979-1982 Research Associate, Department of Veterinary Pathobiology, The Ohio State University, Columbus, Ohio

PUBLICATIONS

Transfer of Salmonella resistance and delayed hypersensitivity with murine-derived transfer factor. Infection and Immunity (in press).


FIELDS OF STUDY

Major Field: Immunology
   Studies in Cellular Immunology. Professor Randall A. Smith
   Studies in feline leukemia. Professor Richard G. Olsen
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INTRODUCTION

Cell-mediated immunity (CMI) is the most important aspect of protective host immunity against facultative intracellular parasites, including the viruses (1). Besides this protection, cellular immunity plays a role in orchestrating cellular cooperation during an immune response and conducting immunosurveillance mechanisms to rid the host of spontaneous arising tumor cells (2,3). It is also involved in the immunopathological mechanisms of autoimmunity and graft rejection (4,5).

The major lymphoid cell involved in these reactions is the thymus-derived lymphocyte (T-cell). Classically, functional assays of sensitized T-cells have consisted of lymphokine measurement (6), mitogen-induced blastogenesis (7) and measurement of cytotoxicity against specific target cells (8). Transplantation immunology has contributed the mixed leukocyte reaction (MLR) as another correlate of CMI to study immunosurveillance mechanisms (9).

This research is concerned with the cell-mediated reactions found in the cat and the status of these mechanisms during the feline leukemia retrovirus invasion of lymphoid cells.

The feline leukemia virus (FeLV) is a contagious oncogenic RNA virus that has been shown to cause both neoplastic and non-neoplastic diseases in pet cats (10). During viral replication of infected cells, a DNA copy of the viral RNA genome is made and inserted into the DNA-infected cells (provirus) (11). From
there, the integrated provirus can cause cell transformation, viral replication or generation of an oncogenic feline sarcoma virus (FeSV) by recombination with cat cellular genes (12,13). Immunity to this disease process consists of a high titer antibody against FeLV envelope antigens (neutralizing antibody) and FeSV-induced tumor-specific FOCMA (feline oncornavirus membrane antigen) (14,15). Studies of these humoral antibodies have been most successful in delineating the antibody response during FeLV invasion.

Early in the pathogenesis of this disease, cats demonstrate an overall immunosuppressive state resulting in an increase of viral and bacterial diseases leading to the demise of the cat (16). Initially, this suppression was thought to be due to viral destruction of the thymus and paracortical lymph node elements (17,18). Additional investigations demonstrated that infected animals failed to reject skin allografts (19) and peripheral blood lymphocytes (PBL) showed depressed lymphocyte blastogenic transformation (20). Recently, a reduction of lymphocyte membrane con A receptor mobility (capping) was discovered in these cats (21). Finally, viral characteristics were studied and the virion itself was found to contain an immunosuppressive protein (p15E) (22). This purified protein was found to cause suppression of the normal feline LBT (23).

This research examined further mechanisms of feline leukemia immunosuppression. The CMI correlate of the MLR investigates the effects of FeLV on alloantigen recognition. Additional studies with the LBT explore possible defects in the immune modulation mechanism of suppression which may contribute to the depression of the cat's CMI response.
CHAPTER I

FELINE ONE-WAY MIXED LEUKOCYTE REACTION

Introduction

The mixed leukocyte reaction (MLR) occurs between two allogeneic lymphocyte populations in which one cell population (responder) recognizes the histocompatibility antigens of the other cell population (stimulator) and proliferates without previous exposure to the stimulator cell (1). Standard MLR procedures have been studied in most animal species including dog (2), man (3), cow (4), mouse (5), etc., but this technique has not yet been described in cats.

The significance of the MLR was first recognized by Bain et al. (6) and Bach et al. (7) when they mixed leukocytes from two unrelated human donors. The foreign histocompatibility antigens recognized in the MLR by lymphocytes are genetically coded by the major histocompatibility complex (MHC) and are also involved in the cell-mediated immune responses (8). In both the mouse and human systems the MHC has been shown to be composed of different genetic loci coding for lymphocyte defined and serologically defined (SD) cell surface antigens (9). In the human the LD antigens (HLA-D, human lymphocyte antigen-D) are involved in the MLR and are recognized by the responders causing their proliferation, whereas the SD antigens (HLA-A, HLA-B, HLA-C) are involved in the cellular cooperation of effector cell functions such as those in lymphocytoxic reactions (9).
The leukocyte subpopulations involved in the MLR were first demonstrated by use of cells from neonatally thymectomized and burssectomized chickens. No reaction was seen in the thymectomized animals (10), whereas burssectomized animals reacted (10), demonstrating that T-cells are the major cell type in the responder population.

The objective of this study was to explore the parameters of a feline mixed leukocyte reaction as an in vitro correlate of cell-mediated immunity and to demonstrate that histocompatible differences between cats can be demonstrated in vitro.

Materials and Methods

Animals

All cats used in these experiments as donors of peripheral blood cells were from The Ohio State University, Department of Veterinary Pathobiology specific-pathogen-free (SPF) cat colony (11).

Mixed leukocyte culture

Defibrinated blood (20 ml) was collected using aseptic methods. After defibrination the blood was diluted two-fold with L-15 media (Leibovitz Media, Grand Island Biological Co., Grand Island, NY) containing EDTA 0.05% (disodium dihydrogen ethlenediaminetetracetate dihydrate (G. Frederick Smith Chemical Co., Columbus, OH), and supplemented with 1% antibiotics (streptomycin, penicillin and mycostatin). The diluted blood was layered over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) and centrifuged at 400 g for 30 minutes (12). The leukocyte-rich interface was removed and washed twice with
L-15 media. These cells were then placed in RPMI Media 1640 with 25 mM Hepes Buffer (Grand Island Biological Co., Grand Island, NY) supplemented with 1% L-glutamine and 1% antibiotics (streptomycin, penicillin and mycostatin).

Each cell population was diluted in 3 ml, and 2 ml of the cells were treated with Mitomycin C (Sigma Chemical Co., St. Louis, MO, 0.05 mg/10^7 cells) for 30 minutes (37°C with 5% CO₂) after which time these treated cells (stimulators) were washed four times with L-15 media. Differential blood counts were then performed on treated (stimulators) and untreated (responders) cells. The mononuclear leukocytes were subsequently diluted to appropriate numbers in RPMI 1640 with 25 mM Hepes Buffer. Cells were placed in plastic round bottom plates (Linbro Division, Flow Laboratories, Inc., Hamden, CT) and incubated at 37°C for the appropriate number of days. During the final 18 hours of incubation 10 μl of media containing 0.5 μCi of (H³ d-Thd (6.7 Ci/mol, New England Nuclear, Boston, MA) was added. Cells were collected on glass filter paper with a semiautomatic processor (Otto Hiller Co., Madison WI) and assayed for radioactivity by liquid scintillation. Liquid scintillation fluid was a mixture of toluene with Permablend II (Packard, Downers Grove, IL). Counts per minute were collected in Tricarb Scintillation counter (Packard, Tricarb Liquid Scintillation, Spectrophotometer, Downers Grove, IL). Each sample was counted for 5 minutes and net counts per minute (cpm) of triplicate wells were recorded.

Serum supplements

All the serums used were heat-inactivated. Cat serum was obtained from SPF cats and pooled in lots. Rabbit serum was obtained from Flow Laboratories and GIBCO (Grand Island Biological Co., Grand Island, NY). Fetal calf serum was obtained from GIBCO (Grand Island Biological Co., Grand Island, NY).
Results

Serum supplement

Various serum sources were studied in the feline MLR and results indicated the dependence of allogeneic stimulation not only on the type of serum (cat, rabbit) but also on the lot number. In four separate MLRs 5% rabbit serum - Lot #1 gave greatest consistent MLR support (Table 1-A). Cat serum Lot #3 at 20% provided a high allogeneic stimulation in 2 other MLRs (Table 1-B). The one lot of fetal calf serum tried did not support the MLR as well as either feline or rabbit serum. Therefore, cat and rabbit serum were both able to support a mixed leukocyte reaction in cats but maximum stimulation depended on the serum lot and amount used in the assay. Bovine serum albumin (BSA) was also tried as a serum supplement, but failed to support the MLR as well as whole rabbit serum (Figure 1).

MLR time course

The maintenance of mixed leukocyte cultures for various time periods of 2 to 10 days (Figures 2, 3, 4) was performed to determine the appropriate time for maximum stimulation. The data show that in three separate MLRs an eight day incubation period afforded the best stimulation. These figures also illustrated that both rabbit serum Lot #1 (Figure 2) and cat serum Lot #3 (Figures 3 and 4) support maximal MLR on day 8.

Determination of optimal responder-stimulator cell ratio and cell number

Experiments were performed to ascertain the responder to stimulator cell ratio that would yield optimal stimulation (counts per minute) in the MLR assay working with as few cat mononuclear leukocytes as possible in a microtiter
assay. Table 2 shows the results of testing leukocytes from various unrelated cats at responder to stimulator ratios of 1:1 and 1:2. The results uniformly demonstrated that $1 \times 10^5$ responder cells and $1 \times 10^5$ stimulator cells yielded the highest MLR. Increasing the stimulator cells to $2 \times 10^5$ decreased the MLR with responding cells at $1 \times 10^5$. Decreasing the total number of mononuclear cells to $5 \times 10^4$ per well each of responder and stimulator population consistently gave no response in the MLR.

**Tritiated thymidine concentration**

Eighteen hour pulse experiments were done with varying concentrations of ($\text{H}^3$ d-Thd after 8 days of incubation. Tritiated thymidine concentrations of 0.5 $\mu$Ci, 1.0 $\mu$Ci and 2.0 $\mu$Ci per well were studied. Data revealed that 0.5 $\mu$Ci and 1.0 $\mu$Ci afforded sufficient isotope for maximum SI. Since there was no significant difference in the magnitude of response between 0.5 and 1.0 $\mu$Ci per well, 0.5 $\mu$Ci was used throughout the studies (Table 3).

**Family study**

The results of a family MLR along with randomly selected nonfamily members from the SPF colony are presented in Table 4. In this assay, siblings (C and D) were stimulated by nonfamily members (A and B) but reacted poorly to one another and their dam (E). The dam responded vigorously to nonfamily members but somewhat less to her offspring (C and D).
Discussion

The mixed leukocyte reaction is a potentially important histocompatibility test in identifying feline LD antigens in addition to providing an additional assay for studies of cell-mediated immune function.

To override difficulties we encountered with platelet aggregation, we chose to use defibrinated blood. Other important technical requirements for a successful assay were a consideration of the source and amount of serum used, the total amount of incubation time required and the responder to stimulator mononuclear leukocyte ratios.

Within the cat population studied, we were able to find highly reactive pairs of cats along with many low responders. The frequent finding of a fairly low response could be attributed to either a wide variability in species MLR reactivity or, more likely, to the outbred (13) yet closed nature of this cat colony which dates back to 1966 (11). Future studies may include use of conventional cats to help clarify these findings. Nevertheless, highly reactive individuals were found within this colony.

Since a limited amount of blood can be obtained at one time from a cat, a microtiter system using a low number of mononuclear leukocytes was necessary. We found that $10^5$ mononuclear cells of both responder and stimulator populations afforded the best possible stimulation within our obtainable cell range.

Time course studies revealed the requirement of a total incubation time of 9 days with $\text{H}^3$ d-Thd pulse 18 hours before harvesting. Longer incubations at times brought higher cpm, but standard deviations also increased.
Our studies showed that either feline or rabbit serum provided adequate stimulation of the MLR and that the degree of stimulation depended on the particular serum lot and amount. Therefore, serum lots should be screened for supportive capacity and amounts needed and then the same lot should be used throughout a particular series of experiments.

Bovine serum albumin (0.5%) was tried as a serum supplement to reduce background stimulation and serum factor variability, but its use resulted in a much lower MLR in comparison to rabbit serum. This differs from the results of Hsia et al. (14) who used BSA in a serum-free human MLR and found lower nonspecific blastogenesis as well as a more consistent stimulation index throughout time.

The family MLR of all possible combinations between family and nonfamily members helped demonstrate the validity of the feline MLR (Table 4). This is depicted by the ability of a cat's leukocytes (i.e., C) to respond to a nonfamily member (i.e., Am or Bm) and yet have no response to genetically related animals (Dm, a sibling and Em, its dam). In this checkerboard MLR, it is noted that the nonfamily members A and B responded each only to one cat in the family (A with Bm, B with Cm). This can be explained in view of the outbred yet closed nature of this cat colony. Cat members outside of the family were randomly chosen, and therefore it is possible that these cats were related through the male. Litters are recorded associated only with their dams due to a high female to male ratio in the breeding rooms.

We have defined the technical parameters of a feline MLR assay which included a rabbit or cat serum source and a readily obtainable cell population of $1 \times 10^5$ mononuclear cells of stimulator and responder cell populations. Secondly, a family study showed that siblings mounted a low response to their dam while
responding higher to nonfamily members. We have thus described the parameters for a feline MLR assay which can be used in future descriptions of feline LD antigens, histocompatibility studies, and as an \textit{in vitro} correlate to cellular immune function.

**Summary**

The feline one-way mixed leukocyte reaction (MLR) was accomplished by a microtechnique assay using gradient purified mononuclear leukocytes from the peripheral blood of specific-pathogen-free (SPF) cats. An eight day assay was required with an appropriate serum supplement of rabbit or cat serum and obtainable cell concentrations of $1 \times 10^5$ mononuclear cells per well of each responder and stimulator population. This assay was devised as an \textit{in vitro} correlate of cell-mediated immunity and to demonstrate that feline histocompatibility differences can be detected in culture.
Table 1. The effect of various serum supplements and concentrations on stimulation indices of the feline MLR.

### A

<table>
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<tr>
<th>Serum</th>
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<th>SI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MLR</th>
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<td>3.5</td>
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</tr>
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<td></td>
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<td>3.3</td>
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<td>Cat-2</td>
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### B

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<td>Fetal Calf-1</td>
<td>20</td>
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<sup>a</sup> = SI, stimulation index = \( \frac{\text{allogeneic test}}{\text{autologous control}} \)

<sup>b</sup> = Lot number used

<sup>c</sup> = Cat designation number. Also R is a responder cat, S is stimulator cat cells treated with mitomycin C.
Table 2. Comparison of response at various leukocyte stimulator/responder cell ratios.

<table>
<thead>
<tr>
<th>Cats Tested</th>
<th>Mixed leukocyte reaction (cpm(H$_3$)d-Thd)$^a$</th>
<th>Responders at 10$^5$ Cells/well</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cpm</td>
<td>1:1$^b$</td>
</tr>
<tr>
<td>Responder</td>
<td>Stimulator</td>
<td></td>
</tr>
<tr>
<td>895</td>
<td>1595</td>
<td>2958</td>
</tr>
<tr>
<td>895</td>
<td>974</td>
<td>4341</td>
</tr>
<tr>
<td>1595</td>
<td>974</td>
<td>1427</td>
</tr>
<tr>
<td>1595</td>
<td>874</td>
<td>1894</td>
</tr>
<tr>
<td>1209</td>
<td>1181</td>
<td>2700</td>
</tr>
<tr>
<td>199</td>
<td>910</td>
<td>1112</td>
</tr>
</tbody>
</table>

$^a$ cpm = (MLR cpm) - (autologous cntr/cpm)

$^b$ 10$^5$ stimulator cells (mitomycin C treated)

$^c$ 2x10$^5$ stimulator cell (mitomycin C treated)

$^d$ test = autologous control.
Table 3. Effects of varying tritiated thymidine concentration on stimulation indices\(^a\) in the feline MLR.

<table>
<thead>
<tr>
<th>Amount of Tritiated thymidine (µCi/well)</th>
<th>MLR (cat designations)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1172(_R) 984(_S)</td>
</tr>
<tr>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>1.0</td>
<td>1.8</td>
</tr>
<tr>
<td>2.0</td>
<td>0.9</td>
</tr>
</tbody>
</table>

\(\text{R} = \text{responders (1x10}^5\text{ cells/well)\(\)

\(\text{S} = \text{stimulators (1x10}^7\text{ cells/well)\(\)

\(\text{Serum supplement 5\% Rabbit-1.\(\)

\(a\) \text{stimulation index} = \frac{\text{allogeneic test}}{\text{autologous control}}\)
Table 4. Results of mixed leukocyte reaction (MLR) between siblings (C & D),
dam (E), and nonfamily members (A & B); cpm (H\(^2\)) d-Thd.

<table>
<thead>
<tr>
<th>Responding Cells</th>
<th>Stimulating Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Am</td>
</tr>
<tr>
<td>A</td>
<td>160 ± 10</td>
</tr>
<tr>
<td>B</td>
<td>116 ± 22</td>
</tr>
<tr>
<td>C</td>
<td>2552 ± 374</td>
</tr>
<tr>
<td>D</td>
<td>273 ± 7</td>
</tr>
<tr>
<td>E Queen</td>
<td>1259 ± 145</td>
</tr>
</tbody>
</table>

Autologous controls underlined.
Serum supplement 5% Rabbit-Lot #1.
Figure 1. Proliferative responses of allogeneic leukocytes in the MLR between cats 895 x 974 and 895 x 977 using BSA (0.5%), no serum and 5% rabbit serum. Cells \(10^5\) each of responders and stimulators were cocultured for 9 days, pulsed with \(^3H\) d-Thd during the last 18 hours.

\(R = \text{responders. } S = \text{stimulators (mitomycin C treated).}\)
Figure 2. Time course of MLR between cats 984 x 1208 and 984 x 851 along with autologous control. These cats represent a typical time course study using 5% rabbit serum Lot #1. All MLRs were set up at time 0 and harvested on days 5, 6, 7, 8, 9, after pulsing the last 18 hours with $^{3}$H d-Thd.
Figure 2
Figure 3. MLR time course between cats 600 x 1976 and 600 x 1199 along with autologous control on days 5, 8 and 10. The serum supplement used was cat serum Lot #3 at 20%.
Figure 3
Figure 4. MLR time course between cats 1221 and 1181, with autologous control on days 4, 6 and 8. The serum supplement used was cat, Lot #3 at 20%.
Figure 4

\[ \times 10^{-3} \text{ CPM[H}^3\text{]dThd} \]

- ● 1221/1181
- △ 1221/1221

AUTOLOGOUS CONTROL

DAYS

4 6 8
CHAPTER II

EFFECTS OF RETROVIRAL PROTEIN ON FELINE ONE-WAY MIXED LEUKOCYTE REACTION

Introduction

This research examines the effects of UV-inactivated retroviruses on the in vitro cell-mediated immune correlate of the mixed leukocyte reaction (1,2). The feline retroviruses studied are feline leukemia virus (FeLV) which is an oncogenic virus (3) and RD-114 which is an endogenous nondisease-producing cat virus (4).

Several immune parameters of FeLV-infected cats have been examined. Perryman et al. reported that infected animals demonstrated a suppressed allograft rejection (5). Subsequently, Cockerell et al. found that the in vitro lymphocyte blastogenic responses to T-cell mitogens were suppressed in viremic cats even though the T-cell population remained relatively constant (6). To determine whether this suppression in cats was due to specific viral proteins, Hebebrand et al. used UV-inactivated FeLV in in vitro mitogenic studies and noted suppression of normal mitogenic responses with 20 µg per well of viral protein (7). Characterization of a particular immunosuppressive envelope protein (p15E) has been accomplished by Mathes et al. (8,9,10). This research has investigated the possibility that feline leukemia viral proteins may interfere with cellular immunity in cats as evaluated by the MLR.
Materials and Methods

The feline one-way MLR was performed as a modification of the human MLR (11). Defibrinated venous blood was obtained from specific-pathogen-free cats (SPF, greater than 6 months old) (12) using aseptic technique. Diluted blood was layered over Ficoll-paque (Pharmacia Fine Chemicals, Piscataway, NJ) and centrifuged (13). The leukocyte-rich interface was recovered, cells were washed and diluted in RPMI Medium 1640 with 25 mM Hepes Buffer (Grand Island Biological Co., Grand Island, NY). Stimulators were treated with Mitomycin C (Sigma Chemical Co., St. Louis, MO., 0.05 mg/10^7 cells). One-tenth ml of each responder and stimulator cell suspension (10^5 cells) was placed in plastic round bottom microtiter plates (Linbro Division, Flow Laboratories, Inc., Hamden, CT) and incubated for 9 days at 37°C in 5% CO_2, moist air. During the last 18 hr of incubation, 0.5 μCi of (3H)-TdR (6.7 Ci/mol, New England Nuclear, Boston, MA) was added. Cells were collected and assayed for radioactivity by liquid scintillation. Each sample was counted for 5 minutes and net cpm of quadruplicate wells were recorded. All autologous controls were tested along with the allogeneic combinations. Five percent rabbit serum (heat-inactivated) was added as the serum supplement. Viruses used in vitro were the feline leukemia virus (KT strain, propagated from cat cell line FL-74) and RD-114 (from RD-RD114 cell line supplied by Pfizer, Inc., Maywood, NJ). Whole viruses were purified on sucrose gradient, UV-inactivated and dialyzed (7). Viral protein concentrations were placed in twice-concentrated solution and combined in equal volumes of appropriate cell number.
Results

MLRs were done on a number of SPF cats to locate allogeneic combinations which result in stimulation. Initially, dose titration experiments were done using 0, 5, 10 or 20 µg of viral protein per well during the entire incubation period with these allogeneic combinations. The RD-114 viral proteins caused no significant suppression of the MLR at any of these concentrations although slight suppression was noted at 20 µg/well (Figure 5). In comparison, the FeLV proteins caused significant suppression at 5 µg/well (P<0.05, Student's t test), 10 µg/well (P<.005) and 20 µg/well (P<.001) (Figure 6). Viability experiments (trypan blue exclusion) indicated that this suppression was not due to viral-induced cytotoxicity.

No stimulatory allogeneic pairs were studied as controls and neither virus caused significant depression or stimulation of the low MLR (Figures 7 and 8).

Additional studies were done using media (control) or 20 µg per well of each viral protein preparation in the same allogeneic combinations plated at the same time. A significant suppression of the MLR with UV-inactivated FeLV (P<.005) occurred compared to the test without virus, whereas RD-114 did not significantly suppress the response (P<0.1) (Figure 9).
Discussion

In these experiments RD-114, an endogenous cat virus, did cause some suppression of the MLR. It is not known whether this decrease in MLR was caused by an immunosuppressive protein which is less effective than FeLV's p15E or simply the presence of extraneous protein in the reaction system. However, it is clear that FeLV, a leukemogenic agent, causes greater immunosuppression of the cat MLR than an endogenous cat retrovirus (RD-114) which has not been shown to cause any disease (14).

In studies of the murine leukemia viruses, Cianciolo et al. found that the subviral proteins (including p15E) inhibited macrophage function in the inflammatory response (15). Therefore, it is possible that the feline leukemia subviral proteins are interfering with the monocyte's role in the MLR (16).

In addition to immunosuppression of lymphocyte blastogenic transformation (7, 17, 18) antibody production (5), and allograft rejection (5), we find that FeLV viral proteins also suppress the cell-mediated response of the MLR. This data, therefore, suggests that FeLV virus may block recognition of and/or cytotoxic responses to transformed cells, thus aiding the proliferation of FeLV transformed cells.

Summary

The objective of this study was to evaluate the effects of feline retrovirus protein on cat lymphocyte recognition of allogeneic cells in the mixed leukocyte reaction (MLR). Experiments included use of UV-inactivated feline leukemia
virus (FeLV) and RD-114 (a nondisease-producing virus). Zero to twenty micrograms per well of UV-inactivated viral protein from each virus were added to mixed leukocyte cultures. The twenty microgram concentration per well of FeLV-induced significant suppression of the MLR ($P<0.005$). No significant suppression was found using RD-114. Since feline leukemia infection is associated with immunosuppression, the retrovirus proteins may be interfering with the recognition phase of cellular immunity when immunocompetent cells are confronted with an altered host cell.
Figure 5. Effects of UV-inactivated RD-114 virus at concentrations of 0-20 µg of protein per well in the feline MLR.

R = responder

S = stimulator (Mitomycin C-treated)
Figure 5

Micrograms of RD-14 (uv-inactivated) protein / 2 x 10^5 Lymphocytes / well
Figure 6. Effects of UV-inactivated FeLV at concentrations of 0-20 μg of protein per well in the feline MLR.

R = responder

S = stimulator (Mitomycin C-treated)
Figure 6
Figure 7. Effects of UV-inactivated RD-114 virus at concentrations of 0-20 µg of protein per well in allogeneic unreactive feline MLR.
Figure 7

Micrograms of RD-114 (uv-inactivated) protein / $2 \times 10^5$ Lymphocytes / well

$10^{-3} \text{ CPM (} ^3\text{H) TdR}$
Figure 8. Effects of UV-inactivated FeLV at 0-20 μg concentrations per well in an allogeneic unreactive feline MLR.
Figure 8

- Micrograms of FeLV (uv-inactivated) protein / $2 \times 10^5$ Lymphocytes / well
Figure 9. Effects of retroviral UV-inactivated proteins (RD-114 and FeLV) at a concentration of 20 μg/2 x 10^5 cells on an MLR. Cells were incubated in the presence of the proteins for 9 days.

R = responders

S = stimulators (Mitomycin C-treated)

a. Cat designation number.
Figure 9
INTRODUCTION

In the regulation of the immune response, it is now accepted that subpopulations of lymphoid cells exist with helper and suppressor functions (1). The suppressor cell function has been attributed to a negative immunoregulatory influence which is best exemplified by the inhibition of antibody synthesis in some systems by suppressor T-cells to prevent overproduction of antibody by B-cells (1,2,3). Thus, suppressor cells have been shown to play an active role in regulating antibody synthesis (1,2) as well as maintenance of immunological tolerance and cell-mediated reactions (1).

Experimentally, Ferguson et al. (4) and Burns et al. (5) demonstrated that suppressor cells in peripheral blood and spleen were inducible in vitro by appropriate concanavalin A (conA) treatment. Dutton et al. (6) demonstrated that murine inhibitory T-cells are relatively short-lived in culture. Bresnihan et al. (7) subsequently showed that normal human peripheral blood mononuclear cells (PBMC) containing circulating suppressor cells die or cease to function after 24 hr in culture. They found that normal PBMC incubated for 24 hr before
mitogen addition had increased mitogenesis with suboptimal amounts of conA compared to mitogen addition at time 0 (7). These findings suggested that increased blastogenesis at 24 hr was caused by a loss of short-lived suppressor activity. Thus, use of this assay system has been initiated to analyze the presence of this short-lived suppressor effect and its possible role in different immunological dysfunctions. Using this assay developed by Bresnihan et al. (7), other investigators have shown a decrease in suppressor function in patients with systemic lupus (7), certain thyroid dysfunctions (8), and multiple sclerosis (9).

The purpose of this research is to demonstrate this short-lived phenomenon in PBMC of normal healthy animals and to determine whether this effect is present in feline retrovirus-infected (FeLV) cats.

**Materials and Methods**

**Cats**

The specific-pathogen-free (SPF) cats used in the studies reported here were from a hysterectomy-derived (10) breeding colony maintained by the Department of Veterinary Pathobiology, The Ohio State University.

**Viruses**

The Rickard strain of feline leuekmia virus (R-FeLV) (11) was used in these studies. The FeLV-R inoculum was a 20% (wt/vol) homogenate of a lymphosarcoma of the thymus gland and represented the seventh in vivo passage of the original FeLV-R isolate. The inoculum contained $10^5$ focus-forming units ml as
assayed in clone 81 feline cell cultures (12,13). Virus challenge was by oral nasal inoculation of 1 ml on 2 consecutive days (10 cats).

Indirect Immunofluorescent Assay for FeLV Viremia

FeLV group-specific-antigen (GSA) was detected by an indirect immunofluorescence assay modified (14) from that developed by Hardy (15). The primary reagent was goat antiserum against ether-disrupted FeLV; the antiserum was absorbed extensively with normal feline blood cells to remove heterologous and nonspecific reactivity (15).

Preparation of PBMC

Cats were anesthetized with ketamine hydrochloride intramuscularly (Vetalar, Parke, Davis Co., Detroit, MI) and heparinized (10 units/ml) venous blood was obtained. PBMC were separated using a ficoll-paque (Pharmacia Fine Chemicals, Piscataway, NJ) gradient centrifugation technique (16). Three ml of peripheral blood was diluted with 10 ml of L-15 media (Leibovitz Media, Grand Island Biological Co., Grand Island, NY) containing EDTA 0.05% (G. Frederick Smight Co., Columbus, OH) and supplemented with 1% antibiotics (100 ml HBSS, 1.0 g Streptomycin, 2000 U penicillin and 33,000 U of Mycostatin). This diluted blood was underlayered with 10 ml of ficoll-paque and centrifuged at 400 g for 30 min (GLC-2, Sorvall, Newtown, CT). The leukocyte-rich interface was recovered and washed twice with L-15 and finally resuspended in RPMI medium 1640 with 25 mM Hepes buffer (Grand Island Biological Co., Grand Island, NY), supplemented with 1% L-glutamine and 1% antibiotics. Differentials were done and PBMC diluted in media to $1 \times 10^6$ cells/ml.
Cultures were put in 96-well flat bottom microtiter plates (CoStar, Westraco, Watertown, MA). Each well included $10^5$ PBMC in 0.2 ml. Experiments were done in 6 wells per group at $37^\circ$C in 5% CO$_2$ moist air.

At the termination of cultures, 3 days after conA or pokeweed mitogen addition, cells were pulsed with 0.5 $\mu$Ci ($^3$H)TdR (6.7 Ci/mmol, New England Nuclear, Boston, MA) for 18 hr and harvested on glass wool filters on a semi-automated cell harvester (Otto Hiller Co., Madison, WI) and assayed for radioactivity by liquid scintillation (Packard, Tricarb Liquid Scintillation, Spectrophotometer, Downers Grove, IL). Each sample was counted for 2 min and an average cpm of each group of 6 wells was recorded.

Reagents

Phytomitogens, conA (Sigma Chemical Co., St. Louis, MO) pokeweed mitogen (PWM) (Grand Island Biological Co., Grand Island, NY) and phytohemagglutinin-P (Difco Laboratories, Detroit, MI) were diluted as indicated for these studies. ConA, pokeweed, PHA-P and feline serum were used from single lots of material. The serum was obtained from SPF male cats, heat-inactivated and used at a 20% concentration in cell culture.

Suppression Removal Index (SRI)

PBMC were stimulated with conA using the designated doses at the initiation of cell culture (0 hr) and after 24 hr of incubation at $37^\circ$C, 5% CO$_2$ moist air. Cultures were terminated 4 days after addition of mitogen and uptake of ($^3$H)TdR was measured after the last 18 hr of culture. SRI was calculated by the formula (7); (cpm after 24 hr-incubation (with conA) - cpm after 24 hr incubation (without conA))/(cpm 0 hr (with conA) - cpm 0 hr (without conA)).
Both parts (top and bottom) of this equation designate a typical blastogenic experiment, only one is started 24 hr after cells have been incubating before conA addition.

Statistics

The data are given as the mean ± SD. Student's t test was used to determine significance.

Results

Increased Mitogenesis in Staggered LBT

PBMC from normal healthy cats (>6 weeks old) were cultured in a microtiter assay (10^5 cells/well) with addition of 0.1 µg/well conA or 25 ul of PWM (diluted as indicated by manufacturer) or PHA-P (1 µg/well) at 0 and 24 hr incubation.

Increased phytomitogen blastogenic transformation occurred when cultures were allowed to incubate 24 hr before mitogen addition as compared to the LBT at time 0 after leukocyte separation (Figures 8, 9, and 10). This indicated a suppression of conA, PWM, and PHA reactive cells at time 0 that is subsequently lost if cultures are allowed to incubate 24 hr before mitogen addition.

Optimization of SRI: Determination of ConA Dose

Table 5 summarizes the results of experiments to determine the optimum amount of conA which would induce the greatest blastogenic response after the 24 hr incubation. It was found that the conA concentration of 0.1 to 0.5 µg per well per 10^5 PBMC gave the best suppressor removal index values.
Evidence for the Absence of the Accumulation of Stimulation Factors in Culture Medium During 24 Hour Preculture

To demonstrate the possible suppressor cell role in the decreased blastogenesis at 0 hr, experiments were done to rule out the presence of enhancement factors accumulating in preculture. These experiments were accomplished by allowing cells to incubate for 24 hr, washing the cells and adding fresh media along with the conA to stimulate cells. The subsequent cpm were compared to those obtained from cells which received no replacement media. Data from these experiments (Table 6) showed no significant variation between the two sets of 24 hr preculture, thus ruling out the possible accumulation of enhancement factors in the preculture as an explanation for an increase in blastogenesis after 24 hr.

Time of Suppressor Effect Loss (Normal Cats)

Time course studies were performed to determine the appropriate time for optimal blastogenic response to mitogen. Leukocyte cultures were incubated for 0, 2, 6, 18, and 24 hr to measure suppressor activity loss in culture. The beginning of this suppressive effect loss occurred between 6-10 hr of incubation (Figure 11).

Comparison ofSuppressor Function in Normal Versus FeLV-Infected Cats

Table 7 summarizes the data of determining the concentration effects of mitogen in the SRI in two FeLV-infected cats. The SRI remained depressed (~1.0) throughout the various mitogen concentrations. By contrast, healthy SPF
cats exhibited maximum SRI (~3.0) at a conA concentration between 0.1 μg and 0.5 μg.

Table 8 compares CPM and SRI from a group of normal healthy cats with a group of infected cats. Not only is the SRI lower in viremic cats (1.4±0.5 versus 4.1±1.2 in normal cats) but CPM at time 0 are higher. The CPM data, therefore, show that the lower SRI in viremic animals is due to an enhanced conA response at time 0.

To demonstrate this change in conA reactivity with the same cat, assays were set up on cats pre- and post-FeLV challenge. Data in Table 9 shows an increase in blastogenesis at time 0 once cats have become GSA positive. As a result of this increase, SRIs are decreased in cats which became viremic (Table 9).

Overall comparison of SRI between healthy control cats and studied GSA positive cats (Fig. 12) revealed a significant (p<0.005) decrease of SRI (1.1±0.5) from the average SRI of healthy cats (3.8±1.4).

Discussion

The present study indicates that SPF feline PBMC have an increased blastogenesic response to phytomitogens when incubated 24 hr before mitogen addition as compared to blastogenesis at 0 hr. Increased blastogenesis to PHA-P is particularly noteworthy as Cockerell et al. (17) previously reported a poor PHA-P response in the normal cats but with an incubation before mitogen addition, PHA-P response is increased. This 0-hour suppressive effect is lost in vitro between 6 and 20 hr and is greatest at suboptimal doses of conA as
compared to the optimum dose (10 µg/well) used previously for feline LBT (16). This suggests that the optimum dose causes maximum blastogenesis and overrides any suppression of stimulation.

A possible explanation for this increased blastogenesis is the accumulation of blastogenic enhancing factors in the culture medium during the 24 hr before mitogen addition. Experiments using washed cells following incubation revealed the absence of any diffusible factors. The possibility remains that stimulating factors may be cell membrane bound or cytoplasmic in nature and unable to be removed by simple washing. However, a more likely explanation of depressed mitogenesis at 0 hr is hyporeactivity of phytomitogen reactive leukocytes due to a short-lived suppressor cells that dies or is nonfunctional in culture after 24 hr. Other investigators (6,7,8,18,19) have attributed similar findings in humans and mice to short-lived suppressor cells. Studies of autoimmunity in humans (systemic lupus erythematosus and Graves disease) have shown a loss of this suppressor function and subsequent return of activity after treatment (7,8).

In our studies, retrovirus (FeLV)-infected experimental cats demonstrated a defect in their conA suppressive function. Titrations of conA on PBMC showed little change between 0 and 24 hr assays resulting in SRI's of approximately 1.0. Close examination of CPM, at 0 and 24 hr in viremic animals showed hyperreactivity at 0 hr compared to normal cats. Overall comparison of SRI between normal and viremic animals showed a significant decrease (p<0.005) in viremic cats. To examine whether this change occurred in individual cats during FeLV infection, pre- and post-challenged animals were tested. Not only was there a decrease in the SRI in each animal after attaining viremic status, but CPM at 0 hr demonstrated PBMC hyperreactivity to conA as shown previously.
when groups of normal and viremic animals were studied. Therefore, decreased SRI was due to loss of a short-lived suppressor function and not a sustained blastogenic suppression which also would have resulted in an SRI approaching 1.0 by causing a reduced blastogenesis at 0 hr along with a sustained reduction at 24 hr.

Possible interference of suppressor activity by the mere presence of virus in culture is not a likely explanation of this loss of function, as Mathes et al. (18) have shown FeLV to be suppressive, not mitogenic. Our findings using suboptimal doses of conA showed an increased reactivity with viremia at time 0 compared to controls, not a decrease.

SRI ratios in these experiments were dependent on the ability of a cat to respond to conA, on the consistent use of the same stock of feline serum and on the plating of cells within the same designated hours from the time of removal from the cat.

Recently, Rojko et al. demonstrated feline T-cell subsets displaying Fc receptors for IgG (Tg) and IgM (Tm) by differential rosetting techniques and subsequent in vitro mitogenic responses (manuscripts in preparation). In the human system, applying these rosetting techniques along with functional studies, Moretta et al. (20) demonstrated Tg cell suppression of the B cell PWM response.

In conclusion, the data presented here suggest that viremic animals have a defect in this phytomitogen short-lived suppressive function caused either by the immunosuppressive properties of the virus itself (18), by viral invasion of the host which may interfere with immune regulatory mechanisms, or by a lack or presence of a cellular component thereof. If, indeed, FeLV viremia is concomitant with poor immune regulation, this could help correlate with causes
of Coomb's positive anemia along with the occurrence of multiple secondary infections due to overall immunosuppression eventually leading to the demise of these animals.

Future studies with this technique may help elucidate cell types involved as well as a practical way to assess immunoregulatory agents for retrovirus-infected animals.

Summary

Normal feline peripheral blood mononuclear cells demonstrated increased phytomitogen blast transformation after a 24 hr delay in mitogen addition as compared to 0 hr mitogenesis. Increased stimulation after 24 hr which is not due to enhancing factors accumulating in the culture medium has been attributed to a short-lived suppressor cell which loses its effect after 24 hr in culture. This short-lived suppressive phenomenon was studied in normal specific-pathogen-free cats and compared to experimental animals infected with retrovirus (FeLV). Experimental cats demonstrated a subsequent loss of this function following acquisition of a persistent viremia.
Table 5. Determination of optimal SRI with healthy cats: Titrations of conA dose as measured by blastogenesis (uptake in cpm (\(^3\)H)TdR).

<table>
<thead>
<tr>
<th>Time</th>
<th>Cat</th>
<th>0.1</th>
<th>0.5</th>
<th>1.0</th>
<th>5.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>344</td>
<td>16118</td>
<td>32348</td>
<td>82059</td>
<td>67326</td>
</tr>
<tr>
<td>A</td>
<td>2226</td>
<td>20029</td>
<td>44812</td>
<td>56124</td>
<td>46509</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>10221</td>
<td>51556</td>
<td>62281</td>
<td>77966</td>
<td>81308</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>8424</td>
<td>38372</td>
<td>43499</td>
<td>41339</td>
<td>32389</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ0(^a)</td>
<td>A</td>
<td>3.0</td>
<td>3.2</td>
<td>1.9</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>3.7</td>
<td>1.9</td>
<td>1.0</td>
<td>0.7</td>
<td>0.7</td>
</tr>
</tbody>
</table>

\(^a\)Δ0 = cpm 0 hr (with conA) - cpm 0 hr (without conA).

\(^b\)Δ24 = cpm 24 hr (with conA) - cpm 24 hr (without conA).
Table 6. PBMC blastogenesis of washed and unwashed cells after 24 hr preincubation.

<table>
<thead>
<tr>
<th>Time period of preincubation (hours)</th>
<th>Cat</th>
<th>Blastogenesis (cpm)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>A</td>
<td>2,066±583</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>7,116±1,071</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>827±76</td>
</tr>
<tr>
<td>24 unwashed</td>
<td>A</td>
<td>27,369±2,077</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>26,854±3,714</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>6,505±605</td>
</tr>
<tr>
<td>24 washed with fresh media(^b)</td>
<td>A</td>
<td>36,572±10,027</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>26,329±3,715</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>9,459±1,891</td>
</tr>
</tbody>
</table>

\(^a\) 0.1 µg of conA per well.

\(^b\) PBMC after 24 hr incubation were washed with fresh media before the addition of conA.
Table 7. Titration of conA dose with PBMC from FeLV-infected cats as measured by blastogenesis (uptake in cpm \(^{3}H\)Tdr)

<table>
<thead>
<tr>
<th>Microgram concentration of conA/10^5 PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
</tr>
<tr>
<td><strong>Δ0(^a)</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Δ24(^b)</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Δ24/Δ0</strong></td>
</tr>
<tr>
<td><strong>SRI</strong></td>
</tr>
</tbody>
</table>

\(^a\)Δ0 = cpm 0 hr (with conA) - cpm 0 hr (without conA).
\(^b\)Δ24 = cpm 24 hr (with conA) - cpm 24 hr (without conA).
Table 8. Incorporation of (\(^3\text{H}\))TdR of conA stimulated PBMC at 0 and 24 hr with SRI of normal and viremic cats (FeLV).

<table>
<thead>
<tr>
<th></th>
<th>(\Delta 0^b)</th>
<th>(\Delta 24^c)</th>
<th>SRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>2,964</td>
<td>13,276</td>
<td>4.5</td>
</tr>
<tr>
<td>Healthy</td>
<td>1,870</td>
<td>11,275</td>
<td>6.0</td>
</tr>
<tr>
<td>Controls</td>
<td>1,699</td>
<td>6,098</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>1,107</td>
<td>3,872</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>1,449</td>
<td>4,152</td>
<td>2.9</td>
</tr>
<tr>
<td>average SRI</td>
<td></td>
<td></td>
<td>4.1±1.2</td>
</tr>
<tr>
<td>Viremic(^a)</td>
<td>4,924</td>
<td>5,220</td>
<td>1.1</td>
</tr>
<tr>
<td>Cats</td>
<td>2,504</td>
<td>3,137</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>5,849</td>
<td>6,457</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>2,117</td>
<td>4,528</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>4,807</td>
<td>7,097</td>
<td>1.5</td>
</tr>
<tr>
<td>average SRI</td>
<td></td>
<td></td>
<td>1.4±0.5</td>
</tr>
</tbody>
</table>

\(^a\)5-8 weeks post GSA positive.

\(^b\)\(\Delta 0 = \text{cpm 0 hr (with conA)} - \text{cpm 0 hr (without conA)}\).

\(^c\)\(\Delta 24 = \text{cpm 24 hr (withconA)} - \text{cpm 24 hr (without conA)}\).
Table 9. Incorporation of ($^3$H)TdR of conA$^a$ stimulated PBMC at 0 and 24 hr along with SRI on cats before and after FeLV challenge.

<table>
<thead>
<tr>
<th>Cat</th>
<th>State of Viremia (FeLV)</th>
<th>$\Delta 0^c$</th>
<th>$\Delta 24^d$</th>
<th>SRI $\Delta 24/\Delta 0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Nonviremic</td>
<td>2,964</td>
<td>13,276</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>Viremic$^b$</td>
<td>4,924</td>
<td>10,230</td>
<td>2.1</td>
</tr>
<tr>
<td>B</td>
<td>Nonviremic</td>
<td>3,560</td>
<td>9,876</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>Viremic$^b$</td>
<td>5,849</td>
<td>6,457</td>
<td>1.1</td>
</tr>
<tr>
<td>C</td>
<td>Nonviremic</td>
<td>1,795</td>
<td>4,160</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>Viremic$^b$</td>
<td>2,117</td>
<td>3,063</td>
<td>1.4</td>
</tr>
<tr>
<td>D</td>
<td>Nonviremic</td>
<td>1,002</td>
<td>6,302</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>Viremic$^b$</td>
<td>3,280</td>
<td>8,051</td>
<td>2.5</td>
</tr>
</tbody>
</table>

$^a$0.1 μg/well conA.
$^b$2-5 mo. post-GSA positive
$^c$ $\Delta 0 = \text{cpm} 0 \text{ hr (with conA)} - \text{cpm} 0 \text{ hr (without conA)}$.
$^d$ $\Delta 24 = \text{cpm} 24 \text{ hr (with conA)} - \text{cpm} 24 \text{ hr (without conA)}$. 
Figure 10. Blastogenesis (\(^3\)H)TdR) with conA in normal cats at 0 and 24 hr.

ConA = 0.1 μg/well/10^5 PBMC. Each symbol represents a single cat.
Figure 10

$\times 10^{-3}$ counts/min (3H) TdR

Hours of Incubation
Figure 11. Blastogenesis ($^{3}\text{H}T\text{dr}$) with PWM in normal cats at 0 and 24 hr. 
PWM = 25 $\mu$l of stock solution (10 ml of sterile distilled water added to lyophilized PWM). Each symbol represents a single cat.
Figure 11

$\times 10^{-3}$ counts/min. (3H)TdR

Hours of Incubation
Figure 12. Blastogenesis (\(^3\)H)TdR) with PHA-P in normal cats at 0 and 24 hr.

PHA-P = 1 μg/well/10^5 cells. Each symbol represents a single cat.
Figure 13. Rate of loss of suppressive effect in vitro (normal cats). PBMC were cultured (0, 2, 5, 18, and 24 hr) before conA addition (0.1 μg/well).
Figure 14. SRI of PBMC from healthy and retrovirus-infected (FeLV) cats. FeLV viremic cats - 2-5 months GSA positive.
Suppressive Removal Index

Viremic

Healthy Control

Figure 14

1.1 ± 0.5

3.8 ± 1.4
CHAPTER IV

ROLE OF FELINE PERIPHERAL BLOOD MONONUCLEAR GLASS-ADHERENT CELLS IN THE SUPPRESSION OF MITOGEN-INDUCED BLASTOGENESIS

Introduction

Immune homeostasis results from a delicate balance of inducer and suppressor functions (1). Numerous reports indicate that a lack of suppressor function is associated with several disease states including autoimmunity (2,3,4,5). This inhibitory function is found primarily in thymus-derived lymphocytes (T-cells) (1) and cultured adherent immunocytes (6). Adherent suppressor cells possess characteristics of the monocyte/macrophage lineage (6,7). Both T-cells and monocytes are involved in specific and nonspecific immunity and are capable of functioning without new macromolecular synthesis (7).

Several suppressor systems have been characterized in vitro. Investigators report suppressor cells that are concA-inducible (8,9), are age (10,11) and radiation sensitive (12), are separable from leukocyte populations by adherent characteristics (6,7), and produce prostaglandins (13).

The bulk of accumulated data on suppressor cell function has consisted of studying their effects on other cells (14,15). Other methods eliminate these cells...
from established assays to study their effects. Elimination occurs through glass adherence (6,7), aging of cultures (10,11) or radiation treatment (12). Finally, the murine and human systems have contributed the use of antisera to correlate functional capabilities to specific cell subpopulations (16,17).

These studies attempt to characterize the suppressor activity found in the peripheral blood glass adherent cell population of cats. This activity is tested by treatment of adherent cells with mitomycin C (MC) before addition to autologous nonadherent cell LBTs. Along with functional studies, the cell types in the adherent cell population were analyzed by rosetting techniques and nonspecific esterase staining.

**Materials and Methods**

**Cats**

The SPF cats used in these studies were from a hysterectomy-derived breeding colony (18) maintained by the Department of Veterinary Pathobiology, The Ohio State University. The relative age of the animals was >6 months.

**Peripheral Blood Adherent Cell Isolation**

Cats were anesthetized with Ketamine hydrochloride intramuscularly (Vetalar, Parke Davis Co., Detroit, Michigan). A 10% volume of a 4% sodium citrate solution was added to venous blood. Peripheral blood mononuclear cells (PBMC) were separated using a ficoll-paque (Pharmacia Fine chemicals, Piscataway, NJ) gradient centrifugation technique (9). Twenty ml of peripheral blood was diluted with 20 ml of L-15 media (Leibovitz Media, Grand Island Biological Co., Grand Island, NY) containing EDTA 0.05% and supplemented with
1% antibiotics (100 ml HBSS, 1.0 g Streptomycin, 2000 U penicillin and mycostatin 33,000 U). This diluted blood was underlayered with 10 ml of ficoll-paque and centrifuged at 400 g for 30 min (GLC-2, Sorvall, Newtown, CT). The leukocyte-rich interface was recovered and washed twice with L-15 and resuspended in RPMI medium 1640 with 25 mM Hepes buffer (Grand Island Biological Co., Grand Island, NY) supplemented with 1% L-glutamine and 1% antibiotics. The resuspended cells were diluted in 5 ml of medium (10% pooled cat serum) and placed in sterile glass petri dishes (15 x 100 mm) at 37°C for 1 hour. After the nonadherent cells were washed off of the petri dish, 5 ml of cold HBSS without calcium or magnesium (GIBCO) containing 10% pooled cat serum was added to the petri dish and placed on ice for 15 min (20). A rubber policeman was then used to scrape off glass adherent cells and the dish was again rinsed in cold HBSS without calcium or magnesium.

Lymphocyte Blastogenic Transformation

Both adherent and nonadherent cells were washed in L-15 media (as above). The nonadherent cells were diluted in 3 ml of RPMI media and 2 ml were treated with Mitomycin C (Sigma Chemical Co., St. Louis, MO, 0.05 mg) for 30 min at 37°C in 5% CO₂. Adherent cells were diluted in 2 ml and also treated with Mitomycin C (as above). After Mitomycin C treatment, cells were washed three times and all populations were diluted to 1x10⁶ PBMC/ml. The LBT was set up with .1 ml of nonadherent cells with .1 ml of Mitomycin C treated nonadherent cells (at appropriate cell numbers) or with .1 ml of Mitomycin C-treated adherent cells (at appropriate cell numbers). Concanavalin A was added to a final concentration of 10 μg/well. Twenty percent cat serum was added to each test. Cultures were put in 96-well flat bottom microtiter plates (Costar, Westraco, Watertown, MA). Tests were performed in triplicate.
These were 4-day assays with the addition of 0.5 μCi of (H³)TdR (6.7 Ci mmol, New England Nuclear, Boston, MA) for the final 18 hours. Cells were harvested on glass wool filters on a semi-automated cell harvester (Otto Hiller Co., Madison, WI) and assayed for radioactivity by liquid scintillation (Packard, Tricarb, Liquid Scintillation Spectrophotometer, Downers Grove, IL). Each sample was counted for 2 min and an average cpm of each group was calculated.

Percent suppression of the LBT =

\[
100 - \frac{\text{CPM (with conA) nonadherent cells + MC-treated cells}}{\text{CPM (with conA) nonadherent cells}} \times 100
\]

E-rosetting Assay (21)

Heparinized guinea pig blood was collected by cardiac puncture (22). Packed, washed guinea pig erythrocytes (GPE) were treated with 0.143 M aminoethylisothiouronium bromide (AET, Sigma, St. Louis, MO). Treated erythrocytes were washed in PBS without calcium or magnesium until the supernatant was clear. Finally, treated GPE were resuspended in RPMI-1640 as a 10% solution, stored at 4°C and used within 4 days.

For E-rosette assays, 0.1 ml of 2% suspension of treated GPE was mixed with 0.1 ml of PBMC (1-3×10⁶ cells/ml) and centrifuged at 60 g for 5 min at 4°C. Cells were then left at 4°C until gently resuspended. A minimum of 200 cells were counted, using new methylene blue as a differential stain for leukocyte identification. Rosette-forming cells were defined as viable cells of lymphoid morphology with three or more attached erythrocytes.
**Staining for Nonspecific Esterase (Acid α-Napthyl Acetate Substrate)**

Nonspecific acid α-napthyl acetate esterase stain was prepared according to the method of Kosti et al. (23) with some modification. The parasaniline solution was filtered through a 0.45 μ syringe filter prior to mixing with sodium nitrite. This mixture was then added to α-napthyl acetate solution and pH was adjusted to 6.5-6.6 by the addition of 1-2 drops of 4N NaOH. A red-violet precipitate formed and the stain was refiltered into a Coplin jar. Slides were incubated in a 37°C water bath for 2 hours and counterstained for 5-10 min with 0.59 methyl green. The nonspecific esterase-containing cells are easily distinguishable by the presence of intensely red-stained granules in the cytoplasm. Monocyte staining granules are more diffusely distributed in the cytoplasm whereas lymphocyte staining was identified by a single intensely stained granule.

**Reagents**

Concanavalin A (Sigma Chemical Co., St. Louis, MO) was diluted to 200 μg/ml in media and 0.05 ml was add to each culture.

Cat serum was obtained from SPF male cats, heat-inactivated and used at a 20% concentration in cell culture.

**Statistics**

The data are given as the mean ± SD. Student's t test was used to determine significance.
Results

Table 10 summarizes the percent suppression of feline conA mitogenesis in autologous cell mixing experiments. Mitomycin C (MC)-treated adherent cells at a ratio of 1:1 with nontreated nonadherent cells caused an 88.6% suppression of the existing LBT whereas MC-treated nonadherent cells suppressed to a 71% level (p<0.025) (Table 10). With a 1:2 ratio of MC-treated cells to nonadherent cells, suppression was still found to be higher with the adherent cells (62.4%) than with nonadherent cells (37%, p<0.01) (Table 10). Viability remained within the normal range in all groups.

In conjunction with these functional studies, cell types separated by glass adherence were studied by previously characterized feline rosetting and staining techniques of PBMC's. T-cell rosetting demonstrated a higher percentage of T-cells adhering to the glass dish (55%) as compared to the nonadherent group (39%) from the existing 50-60% T-cells found in whole cat blood (Table 11).

Further examination of this cell separation technique by nonspecific esterase stains revealed an enrichment of positive staining monocytes (Table 12) and lymphocytes in the glass adherent cell population.

Discussion

It is evident that to maintain immune homeostasis, a required amount of suppressor and inducer activity must be present in the peripheral blood circulation. In this study, we have begun to characterize the suppressor function in the glass adherent population present in the peripheral blood of normal cats.
PBMC's were separated based on their glass-adherent properties. By nonspecific esterase staining and T-rosetting techniques, cat PBMC's that glass-adhere are enriched cultures of monocytes and esterase positive T-lymphocytes.

In a glass-adherent depleted LBT, significantly more suppression was caused by the addition of MC-treated adherent rather than nonadherent cells. This suppression could be due to the greater than normal distribution of monocytes and/or ANAE lymphocytes in glass adherent populations.

The monocyte has been shown to have pronounced effects on lymphocyte blastogenesis induced by antigens or mitogens in vitro (24). This effect can either promote or inhibit blastogenesis depending on the monocyte cell concentrations. Low concentrations are generally promotive, whereas high concentrations are inhibitory (25,26). Further monocyte purification steps are needed to study the specific effects of the cat monocyte in culture.

Glass-adherent T-cells have also been shown to contain suppressor activity (27). Therefore, interpretation of this data could include suppressor activity from a T-cell.

In the human, differential rosetting techniques of peripheral blood have shown subpopulations of T-cells possessing receptors for the Fc portion of IgG (Tg) and IgM (Tm) molecules (20). Characterization of these two subsets show the majority of suppressor activity in the Tg cells, while inducer activity exists in Tm cells. In the cat, Rojko et al. (21) have demonstrated that these 2 subsets of T-cells exist in cat PBMC's. Further studies of the T-cell subpopulations in adherent and nonadherent cells could be done emphasizing these techniques.
Enzyme markers, such as nonspecific esterase, have been used in human studies of Tg and Tm. It has been shown that Tm possess nonspecific esterase, whereas Tg do not (29). These correlations can be done in the cat along with studies of other enzyme markers used to characterize human Tg and Tm cells (29).

In conclusion, adherent cell populations in the cat are more suppressive than nonadherent cells in a maximum dose conA LBT. Further functional studies, purification steps and specific cell type characterizations must be done before the entire normal suppressor circuit of the cat is understood. Applications can then be used to further delineate the effects of feline leukemia virus on immunocyte differentiation as well as to offer possible specific modes of action for future feline leukemia immunotherapy.

Summary

Cat peripheral blood mononuclear cells were separated based on their glass-adherent properties. Suppressor activity was studied in the two resulting populations of glass-adherent and nonadherent cells. In an autologous conA LBT, significantly greater suppression was caused by the addition of MC-treated adherent cells than nonadherent cells. Characterization of cell types found in these two populations was done by rosetting and nonspecific esterase staining techniques. Glass-adherent cells contained enriched populations of T-lymphocytes and nonspecific esterase-stained monocytes and lymphocytes.
Table 10. Suppression of the conA\textsuperscript{a} response of normal cat nonadherent PBMC\textsuperscript{b} after addition of autologous MC-treated adherent and nonadherent cells.

<table>
<thead>
<tr>
<th>Mitomycin-Treated Cells</th>
<th>Cat Numbers</th>
<th>Average % Suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Adherent cells/well</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 x 10^5</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>0.5 x 10^5</td>
<td>75</td>
<td>78</td>
</tr>
<tr>
<td>Nonadherent cells/well</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 x 10^5</td>
<td>40</td>
<td>80</td>
</tr>
<tr>
<td>0.5 x 10^5</td>
<td>10</td>
<td>56</td>
</tr>
</tbody>
</table>

\textsuperscript{a} 10 pg/well - maximum dose.

\textsuperscript{b} 1x10^5 cells/well.

\textsuperscript{c} p<0.025. Statistical significance of % suppression of concentrations at 1x10^5 cells/well.

\textsuperscript{d} p<0.01. Statistical significant of % suppression at 0.5x10^5 cell concentration.
Table 11. Percentage of T-cell rosettes in adherent and nonadherent cell populations of normal cats.\(^a\)

<table>
<thead>
<tr>
<th>Cat</th>
<th>Adherent PBMC</th>
<th>Nonadherent PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>33</td>
</tr>
<tr>
<td>2</td>
<td>55</td>
<td>33</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>42</td>
</tr>
<tr>
<td>4</td>
<td>55</td>
<td>48</td>
</tr>
</tbody>
</table>

average %   55 ± 4  39 ± 6

\(^a\)T-rosettes in whole blood = 50-60%
Table 12. Percentage of Nonspecific Esterase Stained Monocytes in Adherent and Nonadherent Cell Populations of Normal Cats.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Cat Number</th>
<th>Adherent PBMC</th>
<th>Nonadherent PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>55</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>23</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>8</td>
</tr>
</tbody>
</table>

average % \[31\pm15\] \[9\pm4\]

\textsuperscript{a}Average % in whole blood=10\%
Table 13. Percentage of Nonspecific Esterase Stained Lymphocytes in Adherent and Nonadherent Cell Populations of Normal Cats.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Cat Number</th>
<th>Adherent PBMC</th>
<th>Nonadherent PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>19</td>
</tr>
<tr>
<td>2</td>
<td>26</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>17</td>
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<td>4</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>1</td>
</tr>
</tbody>
</table>

average % 16±8 9±6

\textsuperscript{a}Average % in whole blood=8%
LIST OF REFERENCES

INTRODUCTION


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CHAPTER II


CHAPTER III


CHAPTER IV


