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In vitro mitogen responses and lymphocyte subpopulations in cheetahs

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ABSTRACT

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Lack of genetic variability and apparent susceptibility of cheetahs (*Acinonyx jubatus jubatus*) to coronavirus infection has lead to speculation that this species may have immune system deficits. To establish a foundation for evaluation of the immune function, cheetah peripheral blood mononuclear cells (PBM) were stimulated by a panel of six mitogens, and responses compared with those of domestic cat PBM. Individual responses in both species were variable, but evenly distributed throughout the range of stimulation for each mitogen. Proliferation by PBM from domestic cats occurred within the same range as that of the cheetahs. However, a significantly lower response to peanut agglutinin (PNA) was observed with domestic cat PBM.

Although responses varied between animals, certain individual cheetahs were consistent low responders. The decreased values could not be explained by lack of IL-2 responsiveness since exogenous IL-2 significantly enhanced mitogen-stimulated proliferation in 11 of 12 cheetahs tested.

The phenotypic distribution of domestic cat and cheetah lymphocyte subpopulations was similar as assessed by immunofluorescence staining for surface immunoglobulin (sIg) and cytotoxic T (Tc) cells (using a specific monoclonal antibody, FT2). Values for B cells (31.2% sIg+) and Tc (28.7% FT2+) were slightly higher in domestic cats as compared with cheetah PBM (13.3% sIg+; 19.0% FT2+). Even though no species-specific deficits were detected, a significant negative correlation between PHA-stimulated proliferation and percent FT2+ (Tc) cheetah cells was observed. This indicates that proliferation can be used indirectly to assess relative numbers of functional T helper cells in cheetahs. Our studies suggest that these aspects of the cheetah's immune system are comparable with the domestic cat, and establish a basis for in vitro assays evaluating antigen-specific responses.

ABBREVIATIONS

ACD, acid citric dextrose; Con A, concanavalin A; ELISA, enzyme-linked immunosorbent assay; FeLV, feline leukemia virus; FHV-1, feline herpes virus; FIPV, feline infectious peritonitis coronavirus; FITC, fluorescein isothiocyanate; MASH, multiple automated sample harvester; MHC, major histocompatibility complex; PBM, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PHA, phytohaemagglutinin; PMT, photon multiplier tube; PNA, peanut agglutinin; PWM, pokeweed mitogen; SBA, soybean agglutinin; SCM, serum containing medium; WGA, wheatgerm agglutinin.

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INTRODUCTION

Viral diseases are a significant problem in both domestic cat and captive exotic cat populations. Clinical feline leukemia virus (FeLV) (Briggs and Ott, 1986), feline infectious peritonitis coronavirus (FIPV) (Pfeifer et al., 1983), and feline herpesvirus (FHV-1) (Scherba et al., 1988) infections have been reported in cheetahs. During 1982–1983, an epizootic of FIPV infection in a colony of captive-raised cheetahs resulted in over 90% morbidity and 19 deaths (54% mortality) (O'Brien et al., 1985; Evermann et al., 1986, 1988). While coronavirus infection is common in domestic cats, mortality caused by feline infectious peritonitis is low. The apparent susceptibility of cheetahs stimulated speculation that this species' immune system was not competent to defend against specific viral infections, and led to investigations which demonstrated extreme monomorphism at red blood cell and leukocyte isoenzyme loci, as well as major histocompatibility complex (MHC) loci (assessed by allogeneic skin graft survival) (O'Brien et al., 1983, 1985). Since self-restricted, virus-specific cell-mediated cytotoxicity plays an important role in the host's immune response to viral infection, it has been suggested that reduced MHC polymorphism may alter the host's ability to respond to infection and could result in a uniform susceptibility among individuals of a species (Toolan, 1978; McGuire et al., 1985; Watkins et al., 1988). This hypothesis has been suggested as an explanation for the outcome of the FIPV epizootic in cheetahs (O'Brien et al., 1985). However, assays designed to assess the cheetah's general immune function have not been performed.

In this report, we examined the in vitro functional capabilities (mitogen-stimulated proliferation) and phenotype of cheetah PBM to determine whether this species exhibited a uniformly poor immune response that may account for its unique susceptibility to certain pathogens. In addition, these studies lay a foundation for further investigations of virus-specific cell-mediated immune responses, and for possible differences between felids.

MATERIALS AND METHODS

Cats

Adult cheetahs were maintained at the San Diego Wild Animal Park (Escondido, CA), Wildlife Safari (Winston, OR) and Columbus Zoo (Columbus, OH). Blood was drawn from animals under ketamine anesthesia. For comparison, blood samples were obtained from apparently healthy domestic cats, also under ketamine anesthesia, at a local animal shelter in San Diego county. All cats were feline leukemia virus negative as determined by ELISA (Tech America Diagnostics, Elwood, KS).

Reagents

Serum-containing medium (SCM) consisted of RPMI 1640 (Cell Culture Facility-UCSD, San Diego, CA) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES buffer, and 10% fetal calf serum (Hyclone Labs, Logan, UT). Fetal calf serum was heat-inactivated at 56°C for 30 min prior to its addition to SCM. Medium was filter-sterilized using a 0.2 μm millipore filter.

Conditioned medium from a gibbon T cell line, ML 144, was used as the exogenous source of interleukin-2 (IL-2). These culture supernatants have been shown to contain an active factor indistinguishable from human IL-2 and free of other lymphokines and cytokines; i.e. IL-1, IL-3, interferon, B cell growth factor, macrophage-inhibiting factor, macrophage-activating factor, colony-stimulating factor and epidermal growth factor. MLA 144 supernatant was produced in SCM as previously described (Rabin et al., 1981).

Mitogens were prepared as stock solutions of 1 mg/ml from lyophilized samples, filter-sterilized, and diluted in SCM before use. Concanavalin A (Con A) (Calbiochem-Behring Corp., La Jolla, CA), pokeweed mitogen (PWM) (Sigma Chemical Co., St. Louis, MO), phytohaemagglutinin (PHA) (Wellcome Laboratories, Research Triangle Park, NC), soybean agglutinin (SBA), wheat germ agglutinin (WGA), and peanut agglutinin (PNA) (all from Vector Laboratories, Burlingame, CA) were included in the panel of mitogens tested.

Fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ rabbit anti-cat IgG and FITC-affinity purified rabbit anti-mouse IgG were purchased from Cappel Laboratories (Cooper Biomedical Inc., Malvern, PA). A monoclonal antibody, FT2, that identifies feline cytotoxic T cells (Tc) (Klotz and Cooper, 1986), was kindly provided by Dr. Max Cooper (University of Alabama).

Isolation of peripheral blood mononuclear cells (PBM)

Peripheral blood was obtained by venipuncture and collected in acid citric-dextrose (ACD) vacutainers (Beckton-Dickinson, Rutherford, NJ). Whole blood was diluted 1:2 with calcium-free and magnesium-free phosphate-buffered saline (PBS) and layered on Ficoll-Paque (Pharmacia Inc., Piscataway, NJ). After centrifugation for 40 min at $800 \times g$, cells at the interface were removed, washed, and resuspended in SCM. Cells were enumerated and viability judged by 0.2% trypan blue dye exclusion.

Proliferative assays

PBM were diluted to 1×10^6 cells/ml in SCM and 100 μl samples added to 96-well round-bottom microtiter plates (Costar, Cambridge, MA). Mitogens were diluted to the appropriate concentration in SCM and 100 μl added to

quadruplicate sets of wells. In experiments testing the effect of IL-2, MLA 144 supernatant was added at a final concentration of 25% (v/v) with the mitogen.

Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and air for 3 days. Cells received 1 μCi/well ^{3}H-thymidine (ICN Radiochemicals, Irvine, CA) during the last 18 h of incubation. Plates were frozen at −20°C, and thawed just prior to harvesting onto glass fiber filter paper using a multiple automated sample harvester (MASH). Samples were dried, then counted in a liquid scintillation counter. Data are represented as the mean ^{3}H-thymidine incorporation observed in quadruplicate cultures and expressed as counts per minute (cpm) ± standard deviation.

Cell surface staining

Aliquots of $1-2\times10^6$ cells were stained for identification of PBM phenotypes by FACS analysis. Cells were washed with cold PBS + 0.02% NaN_3. Pellets were resuspended in 100 μl of PBS + NaN_3 (controls) or 1:10 dilution of FT2. After incubation on ice for 45 min, cells were washed and resuspended in 100 μl of PBS + NaN_3 (unstained control), or 1:10 dilution of FITC-affinity purified rabbit anti-mouse IgG. Tubes were placed on ice for an additional 45 min incubation, washed and fixed with 0.5 ml of 1% paraformaldehyde. Foil-covered tubes were stored at 4°C until FACS analysis was performed. Surface immunoglobulin was detected using direct immunofluorescence staining with a 1:50 dilution of FITC-F(ab')$_2$ rabbit anti-cat IgG. Cells were handled as described above.

Flow cytometry

Cells were analyzed using a Becton-Dickinson FACS IV (Mountain View, CA) equipped with 2 W argon laser. The fluorescently labeled cells, with a flow rate between 300 and 1000 cells/s, were excited at 488 nm at 400 mW. Fluorescence was detected on a Photo Multiplier Tube (PMT) at 650 V after passing through a 530/30 nm pass filter.

Low angle (forward scattered) light was detected with a photodiode. Orthogonal (90°) scattered light was detected with a PMT at 300 V after passing through a 488/10 nm band pass filter. Two–three $\times10^4$cells were analyzed and data were collected in list mode and processed using the Electric Desk (Becton-Dickinson).

For all the experiments, the operating parameters of the FACS IV were standardized using fluorescent microspheres and the "Calibryte" program.

Statistical analysis

Single comparisons were made using the one-tailed Student's *t*-test.

RESULTS

Response of cheetah PBM to mitogenic stimulation

Optimal conditions for stimulation were determined by incubating 1×10^5 cheetah PBM with varying doses of mitogens (Con A, PWM, PHA) and harvesting daily over a 7-day interval. Maximal ^{3}H-thymidine incorporation occurred on Day 3 or 4 (data not shown), as reported for domestic cats (Cockerell et al., 1975). Therefore, all further mitogen assays were harvested on Day 3.

Several mitogens previously shown to stimulate lymphocytes were chosen to assess in vitro proliferation. In preliminary experiments, stimulation occurred over a wide range of concentrations. Doses chosen to compare optimal responses by individual animals were as follows: 50 μg/ml Con A, 2 μg/ml PWM, 1% PHA, 10 μg/ml PNA, 10 μg/ml SBA and 10 μg/ml WGA. Figures 1 and 2 show results comparing a group of 20 cheetahs and seven domestic cats.

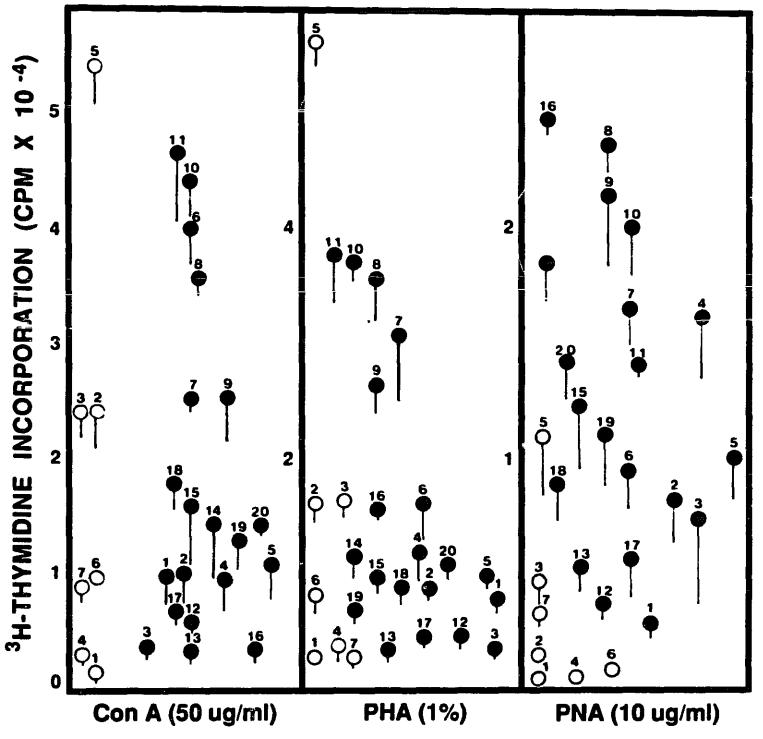

Fig. 1. Proliferative responses of cheetah (solid symbols) and domestic cat (open symbols) PBM to 50 μg/ml Con A, 1% PHA and 10 μg/ml PNA. Cultures were harvested on Day 3 after an 18 h incubation with ^{3}H-thymidine. Mean values of ^{3}H-thymidine incorporation are expressed in cpm ± SD. Proliferation in unstimulated cultures was less than 766 cpm, except Cheetah 11 (control 4909 ± 3214 cpm). Each data point and number represents an individual animal. Data are arranged spatially for easy interpretation and no significance is given to the left-to-right order of data points within each mitogen group.

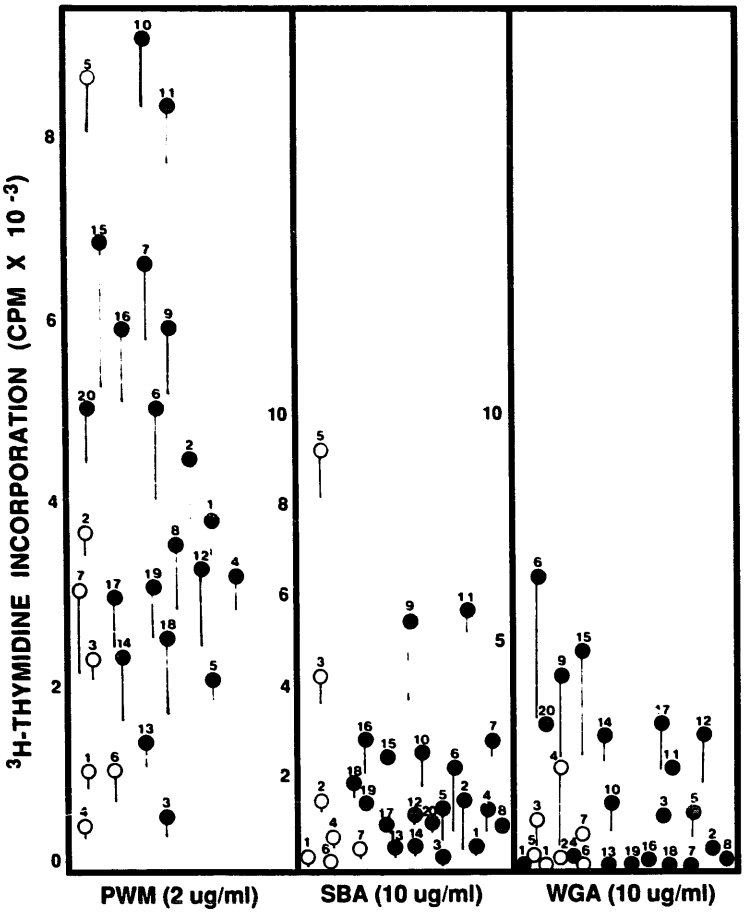

Fig. 2. Proliferative response of cheetah (solid symbols) and domestic cat (open symbols) PBM to 2 μg/ml PWM, 10 μg/ml SBA and 10 μg/ml WGA. See Fig. 1 for culture conditions and control values.

A wide range of responses was observed to each of the mitogens tested, although some mitogens (Con A, PHA, PNA) induced higher levels of proliferation. Proliferation of PBM from domestic cats occurred within the same range as that of the cheetahs and individual responses were evenly distributed throughout this range. However, as a group, domestic cats did not respond as well to PNA (group means: cheetahs, 12, 117 cpm; domestic cats, 3191 cpm: significantly different at $P<0.05$).

Individual variation within species was also evident. Responses of cheetah PBM were as variable as those observed with domestic cat PBM. Cells from animals that were high responders (Cheetahs 8, 10, 11) to one mitogen usually responded well to the rest of the mitogen panel. However, PWM, SBA and WGA were not as potent mitogens as Con A, PHA and PNA. Certain individuals within the population (Cheetahs 12, 13, 17) generally responded less to mitogen stimulation. Previous experiments demonstrated that high

(Cheetah 8) and low (Cheetahs 12 and 13) responders to Con A were consistent over time.

Ability of exogenous IL-2 to enhance mitogenic responses

Investigations in other species have shown that one mechanism of low T cell responsiveness is failure to produce and/or respond to IL-2 (Alcocer-Varela and Alarcon-Segovia, 1982; Gilman et al., 1982). Since IL-2 responsiveness has been correlated with the level of in vitro T lymphocyte proliferation (Ilonen and Salmi, 1982; Miller-Edge and Splitter, 1986), experiments were performed to assess the role of IL-2 in feline mitogenic responses. Preliminary experiments have shown that suboptimal doses of mitogen enhance conditions for detecting responses to exogenous IL-2. Therefore, 0.25% PHA was used in these cultures. Data in Fig. 3 suggest that cheetah PBM were not capable of responding to IL-2 unless stimulated with mitogen to express IL-2

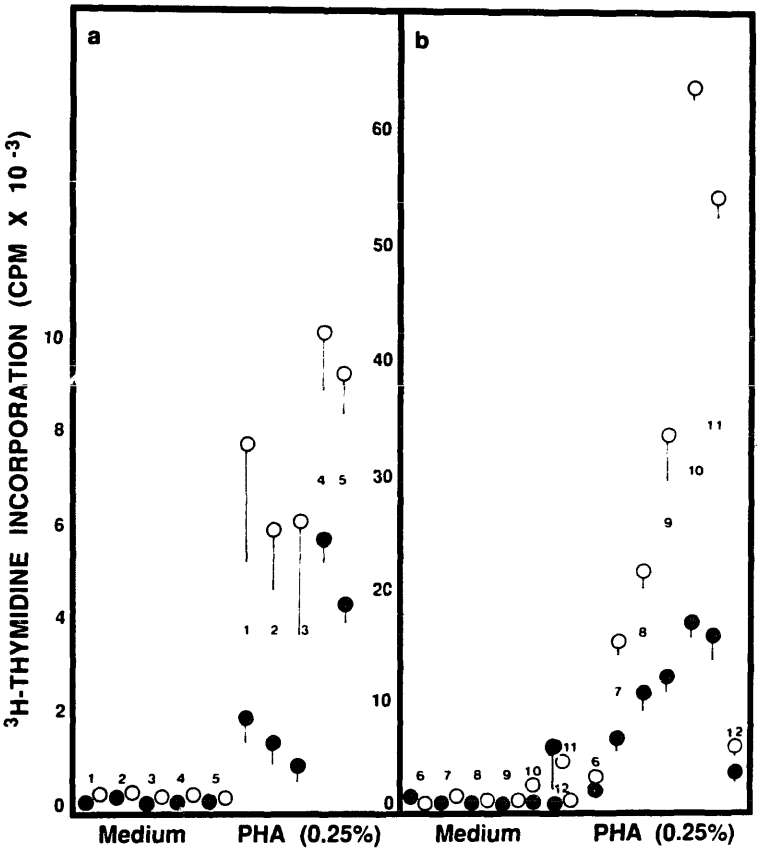

Fig. 3. Augmentation of PHA responses by exogenous IL-2. Cheetah PBM were stimulated with 0.25% PHA in the presence (open symbols) or absence (solid symbols) of exogenous IL-2 for 3 days. Mean values of ^{3}H-thymidine incorporation are expressed in cpm ± SD. Proliferation in unstimulated cultures with (open symbols) or without (solid symbols) exogenous IL-2 is also shown (medium). Panels (a) and (b) represent data from two groups of animals evaluated in separate experiments on different dates.

receptors. Although individual responses varied, most responses were significantly enhanced in the presence of IL-2 (except Cheetah 6; not significant at $P<0.05$). These results indicate that IL-2 responsiveness by cheetah PBM is acquired with mitogenic stimulation and may serve an important role in T cell expansion, similar to other species (Paetkau et al., 1980; Ruscetti and Gallo, 1981). In addition, the lack of responsiveness to IL-2 cannot be considered a likely mechanism for poor responses by cheetah PBM to specific mitogens because exogenous IL-2 augmented responses in the majority of individuals.

Characterization of cheetah PBM subpopulations using flow cytometry

Proliferative responses to B and T lymphocyte mitogens suggested the presence of both populations in cheetah PBM. Altered ratios or the absence of some populations might account for the poor responses in some individuals. In order to characterize the distribution of these populations, fresh PBM were stained with anti-cat IgG and FT2, and FACS analysis performed. Figure 4 shows the typical staining pattern of domestic cat and cheetah PBM with FT2. A summary of results is presented in Table 1. The distribution of sIg+ (31.2%) and FT2+ (28.7%) cells in domestic cats was similar to values previously reported (Klotz and Cooper, 1986; Kuramochi et al., 1987). The number of positively stained cheetah PBM was less than domestic cat. The decreased value may be due to a lower affinity of FT2 antibody for cheetah PBM or fewer cells expressing the surface marker. However, the range of FT2+ cells was similar in both species.

Since FT2+ cells represent the T cytotoxic subpopulation (Klotz and Cooper, 1986), the remaining population consists primarily of B cells, macrophages and T helper cells. Although both subpopulations of T lymphocytes respond to mitogen, the majority of proliferation occurs in the T helper sub-

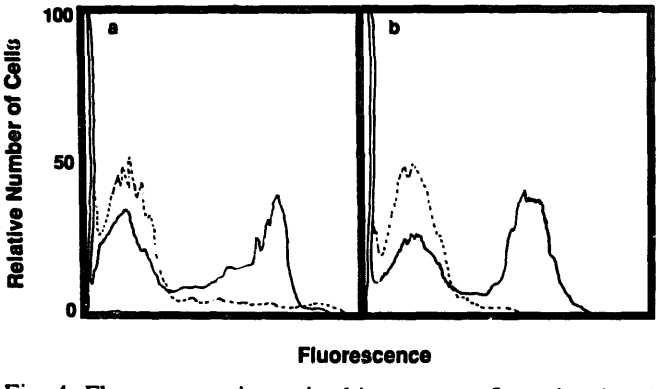

Fig. 4. Fluorescence intensity histograms of unstimulated domestic cat (a) and cheetah (b) PBM. Cells were stained with FT2 antibody and analyzed with a FACS (solid line). The dotted line corresponds to background staining; the X axis indicates fluorescence intensity (log 10 scale) and the Y axis shows relative numbers of cells.

TABLE 1. Surface phenotype of resting feline PBM

Species	*n*	Ig+cells (%)	FT2+ cells (%)[1]
Cheetah	24		
Range		2.6–36.0	1.0–50.8
Mean (SD)		13.3 (9.0)	19.0 (13.8)
Domestic cat	7		
Range		10.8–58.5	16.6–47.9
Mean (SD)		31.2 (17.3)	28.7 (11.4)

[1]Values of FT2+ cells calculated by subtracting percent of background staining by secondary antibody from percent of cells staining with FT2.

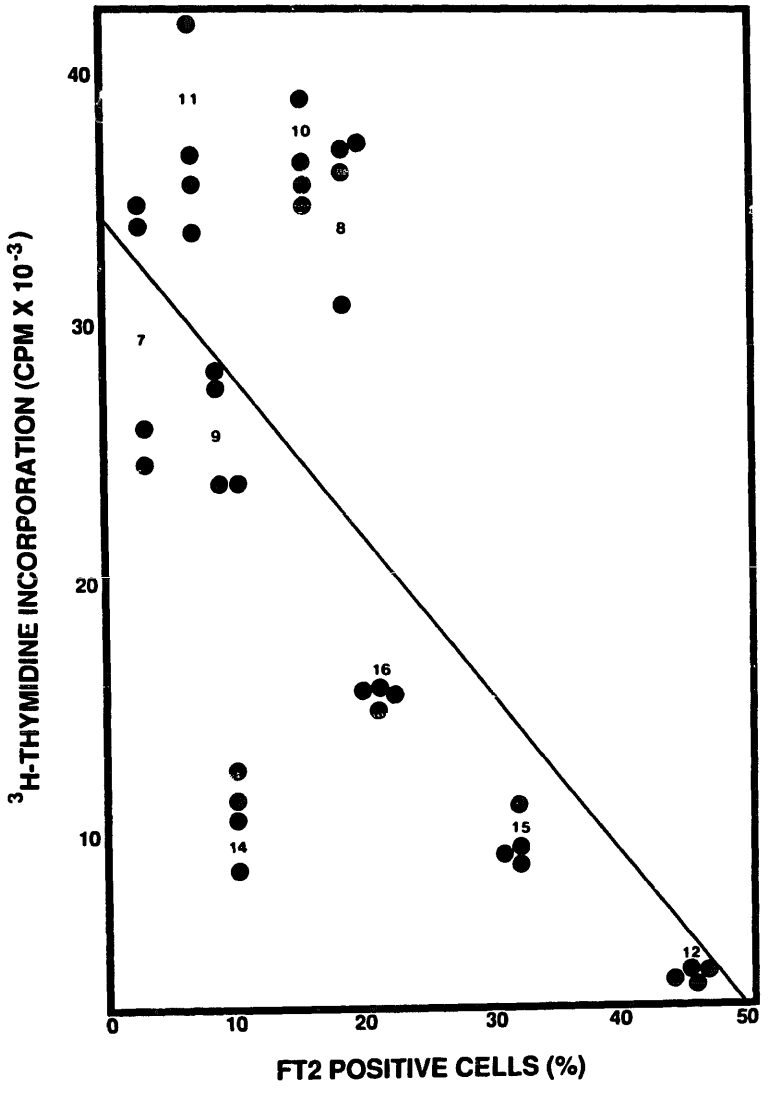

Fig. 5. Correlation between percent FT2+ cells and proliferative responses of PHA-stimulated cheetah PBM. Unstimulated PBM from nine cheetahs were stained with FT2 and analyzed with a FACS. The percent FT2+ cells represent the positive staining cells minus background staining. Cells from the same animals were stimulated with 1% PHA for 3 days and ^{3}H-thymidine incorporation measured (expressed in cpm).

set. Therefore, if the relative numbers of non-T cells remain constant, as T helper cell numbers increase, an increase in proliferation should occur. In order to assess the helper cell subpopulation crudely, the relationship between percentage of FT2+ cells and proliferation to PHA was examined. Figure 5 demonstrates a significant negative correlation ($r = -0.68$, $P < 0.05$), supporting the hypothesis that proliferation can be used as an indirect method of assessing numbers of functional T helper cells. Alternatively, an increase in FT2+ cells may represent an increase in the T suppressor cell population. As previously observed, individuals varied widely in the levels of Tc/Th cells present.

DISCUSSION

In these studies, mitogen-stimulated proliferative responses of cheetah and domestic cat PBM were found to be similar. However, the values tended to be lower than those observed in other species (Larsson, 1981; Smith et al., 1981; Wu et al., 1982). Wide individual variation occurred in both species. Among cheetahs from which serial samples were taken, individuals could be classified as consistently high or low responders. Possible mechanisms accounting for these differences were investigated by examining the response of mitogen-stimulated cells to exogenous IL-2 and phenotypic analysis of the PBM population. The ability of IL-2 supplementation to augment proliferation of both high and low responders indicated that cheetah PBM can be stimulated to express IL-2 receptors, and that both groups have IL-2 responsive cells in their repertoire. Therefore, lack of IL-2 responsiveness probably does not account for the differences observed.

Studies in other species have demonstrated a correlation between level of proliferation and IL-2 production (Lopez-Botet et al., 1982; Miller-Edge and Splitter, 1986; Tompkins et al., 1988). In the light of enhanced responses to exogenous IL-2, insufficient IL-2 production may be an underlying defect in low responding cheetahs. Similar investigations using bovine and porcine PBM have shown that supplemental IL-2 can reconstitute suboptimal responses to maximal levels (Miller-Edge and Splitter, 1986; Stott et al., 1986). Assays to quantitate IL-2 production in cheetah PBM cultures are currently underway.

Differences between animals may be due to variable numbers of IL-2 producing and/or responding cells in an individual's PBM population. Phenotypic comparisons showed slightly higher numbers of SIg+ (B) and FT2+ (Tc) cells in domestic cat PBM versus cheetah PBM. However, the increase may be an artefact of greater affinity for the species to which the antibody was developed (domestic cat). Therefore, the similar phenotypic distribution between species is consistent with the observed similarities in proliferative responses. Interestingly, when examining the relationship between prolifera-

tion and phenotype in individual cheetahs, a significant correlation was observed. For example, Cheetah 12, a low responder in the mitogen studies, had increased numbers of FT2+ cells. The decrease in proliferation with increased numbers of FT2+ cells could be explained by postulating a concomittant decrease in T helper cells or an increase in T suppressor (Ts) cells, as has been demonstrated in humans (Chudwin et al., 1983). Since cytotoxic and suppressor T cells share phenotypic markers in other species, this may be an alternative explanation for the lower response. Some cheetahs may have greater numbers of suppressor cells (possibly FT2+) that upset the immune system's delicate balance. Further studies which examine T cell subpopulation functions such as suppressor assays are necessary to test this hypothesis.

Although cheetahs appear to be more susceptible than domestic cats to coronavirus infections (O'Brien et al., 1985), evidence for species-specific defects in cell-mediated immune function could not be demonstrated using nonspecific in vitro assays. However, if mitogen stimulation is used as a tool for evaluation of CMI, those individuals with low responses may have increased susceptibility to pathogens (Effros and Walford, 1983). Recent studies of immunologic parameters in FeLV-infected domestic cats have linked abrogation of T cell mitogen responsiveness and decreased IL-2 production in vitro with clinical in vivo immunosuppression (Ogilvie et al., 1988). These cats commonly succumb to secondary infections.

The inability to mount an adequate protective response may be virus-specific or may be associated with an inability to recognize foreign antigens because of reduced MHC polymorphism. In order to assess selective deficits in the immune repertoire antigen-specific lymphocyte assays are required.

The increasing availability of feline-specific reagents and characterization of the feline MHC will allow further investigation of immunologic mechanisms in these species. The current studies rule out general immunocompromise in cheetahs as the underlying cause of the species' increased susceptibility to certain viral infections. However, individual animals may exhibit poor responses and be at higher risk. This could be due to insufficient IL-2 production, since supplemental IL-2 enhanced responses. In addition, culture conditions and ranges of expected responses are established for examining antigen-specific T cell function.

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