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In vitro responses of cheetah mononuclear cells to feline herpesvirus-1 and *Cryptococcus neoformans*

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ABSTRACT

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In vitro T cell function by domestic cats and cheetahs to two common pathogens, feline herpesvirus-1 (FHV-1) and *Cryptococcus neoformans*, was assessed. Peripheral blood mononuclear cells (PBM) were stimulated with two strains of UV-inactivated FHV-1, whole heat-killed organisms or capsular antigen of *Cryptococcus neoformans*, and proliferative responses measured. As a group, cheetah PBM responded significantly poorer than domestic cat PBM when cultured with FHV-1. However, individual cheetah responses varied widely. Supplementation of cultures with exogenous interleukin 2 (IL-2) significantly increased the level of response of individual cheetahs to both strains of FHV-1. Cheetah sera contained slightly higher neutralizing antibody titers to FHV-1 than did domestic cat sera, suggesting that B cells function adequately in cheetahs. When stimulated with *Cryptococcus neoformans*, both species had similar incidences of positive proliferative responses.

These data demonstrate that cheetahs exhibit heterogeneous responses to specific antigens, similar to domestic cats. However, a lower group response to FHV-1 in cheetahs suggests species differences occur. In addition, level of variability in major histocompatibility complex (MHC) class I-like genes, as determined by Southern blot hybridization, does not appear to correlate with a uniform response in in vitro functional assays. Therefore, additional mechanisms influence the final outcome of the immune response.

INTRODUCTION

Captive cheetahs appear to have increased susceptibility to specific viral diseases (Briggs and Ott, 1986; Evermann et al., 1988; Scherba et al., 1988; Heeney et al., 1990). This apparent sensitivity has stimulated speculations that this species' immune system is not competent to defend against infections, possibly due to lack of genetic variation (O'Brien et al., 1985). Previous studies suggest that the cheetah is genetically monomorphic, based on lack of polymorphism at red blood cell, leukocyte isoenzyme, and MHC loci

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as well as delayed rejection in limited allogeneic skin grafting experiments (O'Brien et al., 1983, 1985; Yuhki and O'Brien, 1990). Disease susceptibility/resistance and antigen responsiveness have been associated with particular allotypes in several species such as mouse, chicken, and human (Pazderka et al., 1975; Batchelor and McMichael, 1987; Leist et al., 1989). Thus, a population that exhibits MHC monomorphism might be expected to be uniformly susceptible or unresponsive to certain antigens.

In this report, we investigated the *in vitro* proliferative responses of cheetah peripheral blood mononuclear cells to feline herpesvirus-1 (FHV-1) and *Cryptococcus neoformans* to assess the uniformity and level of immune function. A wide variation in immune responses was observed. Level of proliferation did not correlate with specific polymorphic patterns at MHC class I-like loci; therefore, genetic factors alone could not directly predict which cheetahs would be high and low responders to particular antigens. Other possible mechanisms accounting for the differences were examined, including response to exogenous interleukin 2 (IL-2) in low responders.

MATERIALS AND METHODS

Animals

Blood was obtained from adult cheetahs maintained at the San Diego Wild Animal Park (Escondido, CA), Wildlife Safari (Winston, OR), Fossil Rim Wildlife Center (Glen Rose, TX), Columbus Zoo (Columbus, OH), and Phoenix Zoo (Phoenix, AZ). For comparison, blood samples were taken from apparently healthy domestic cats at a local animal shelter in San Diego county. Both species were under general anesthesia (ketamine hydrochloride) during blood sampling. All cats were negative when tested for feline leukemia viral antigen and serum antibody to feline immunodeficiency virus using the combination CITE kit (Agritech, Portland, ME).

Reagents

Serum-containing medium (SCM) consisted of RPMI 1640 (J.R. Scientific, Woodland, CA) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 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.

Recombinant IL-2

Human peripheral blood mononuclear cells were cultured in RPMI 1640 supplemented with 1000 U/ml human recombinant IL-2 (Hoffman La Roche, Nutley, NJ). Conditioned medium, harvested at 48 h, was used as the source of exogenous IL-2 (kindly provided by Dr. Jackie Hank, University Hospital,

University of Wisconsin-Madison, Madison, WI). Supernatants were clarified by ultracentrifugation and filter-sterilized. Conditioned medium was diluted in SCM to a final IL-2 concentration of 100 U/ml.

Mitogens and antigens

Concanavalin A (Con A) (Calbiochem-Behring Corp., La Jolla, CA) was prepared as a stock solution of 1 mg/ml from lyophilized samples. Stock was diluted in SCM and filter-sterilized before use. Previous studies have shown optimal response at a Con A concentration of 10 μ g/ml.

Stocks of feline herpesvirus-1, pathogenic and vaccine (temperature-sensitive mutant) strains, were obtained from the American Type Culture Collection (Rockville, MD). Virus was grown in Crandell feline kidney cells (CRFK). Cultures were subjected to three freeze-thaw cycles after extensive cytopathic effect (CPE) was observed in the monolayer. Supernatants were centrifuged to remove cellular debris, then tested in a plaque assay on CRFK cells to determine virus titer. Virus was UV-inactivated by placing supernatant in plastic Petri dishes and irradiating for 15 min. Virus thus treated was rendered noninfectious. Mock-infected culture supernatants were processed as above and appropriate dilutions used as background controls in proliferative assays.

Cryptococcus neoformans cultures were kindly provided by Dr. Charles Davis and Mr. Phil Payne (University of California, San Diego Medical Center, San Diego, CA). Sabouraud's broth cultures were inoculated and flasks incubated at 35°C. Organisms were heat-killed by subjecting cultures to 90 min in a 55°C waterbath. Tissue culture fluids were centrifuged and the pellets washed and resuspended in phosphate-buffered saline (PBS). Presence of capsule was determined using India ink stain. A final concentration of 1×10^6 organisms/ml was used in culture wells.

Capsular antigen was prepared according to the method of Murphy and Cozad (1972). Briefly, Sabouraud's broth was inoculated with a *Cryptococcus* colony and incubated at 35°C for 72 h. Organisms were killed by adding buffered formalin to a final concentration of 2%. Cell debris was pelleted and the supernatant extensively dialyzed against PBS. This crude antigen supernatant was concentrated using an XM-50 membrane in an Amicon ultrafiltration system, and filter-sterilized with a 0.45 μ m millipore filter. Dose response experiments were performed to determine the optimal dilution for stimulation (final dilution in wells was 1:50). Approximate protein concentration of stock was 8 mg/ml.

Isolation of peripheral blood mononuclear cells (PBM)

Peripheral blood was obtained by venipuncture and collected in acid-citric-dextrose (ACD) vacutainers (Becton-Dickinson, Rutherford, NJ). Whole blood, diluted 1:2 with calcium-free, magnesium-free PBS, was layered on

Ficoll-hypaque (1.077 g/cm^3). After centrifugation for 40 min at 800 g, cells at the interface were removed, washed, and resuspended in SCM. Cells were enumerated and viability judged by eosin dye exclusion.

Proliferative assays

PBM were diluted to 2×10^6 cells/ml in SCM and 100 μl samples added to 96-well round-bottomed microtiter plates (Corning, Newark, CA). Mitogens and antigens were diluted to twice the indicated final concentration in SCM and 100 μl added to replicate sets of six wells. Due to the variable number of cells available for analysis, not all forms and/or concentrations of antigen were tested in each individual. In experiments testing the effect of IL-2, conditioned medium was added to a final IL-2 concentration of 100 U/ml at the initiation of cultures.

Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and air for 4 (mitogen) or 7 (antigen) days. Plates received 1 $\mu\text{Ci/well}$ ^3H -thymidine (ICN Radiochemicals, Irvine, CA) during the last 24 h of incubation. Cells were harvested onto glass fiber filter paper using a multiple automated sample harvester (PHD, Cambridge Technology, Watertown, MA). Samples were dried, then counted in a liquid scintillation counter. Data are represented as the difference in mean ^3H -thymidine incorporation between stimulated and control wells, and expressed as counts per minute (CPM). Single paired comparisons were made using the one-tailed Student's *t*-test.

Virus neutralization assay

Neutralization titers were obtained by preparing two-fold serial dilutions of serum in 96-well flat-bottomed microtiter plates. All serum was heat-treated for 30 min in a 56°C waterbath to inactivate complement and filter-sterilized. FHV-1 (pathogenic strain) was added at 50 plaque-forming units (PFU)/well and incubated with serum samples for 1 h at 37°C . CRFK cells were added to each well at this time. Plates were incubated for 3 days, then monolayers fixed with formaldehyde and stained with crystal violet. Titers are expressed as the highest serum dilution that resulted in total inhibition of CPE.

Southern blot analysis

DNA was extracted from cheetah peripheral blood mononuclear cells as described by Alexander et al. (1987). DNA samples (10 μg) were doubly digested at 37°C with 20 units each of BamHI and EcoRI (Boehringer-Mannheim Biochemicals, Indianapolis, IN).

Horizontal slab gels of 1.0% agarose (BRL, Gaithersburg, MD) were used in a 20×30 -cm electrophoresis chamber (C. Scott, Del Mar, CA). Wells were loaded with the entire digested sample, along with HindIII-digested lambda

DNA as molecular weight markers. Gels ran at 20 V for 95–113 h, with daily changes of buffer.

Gels were prepared and transferred to Hybond-N membrane (Amersham, Arlington Heights, IL) as described by Maniatis et al. (1982). At the end of the transfer, membranes were exposed on a UV lightbox for 5 min, then pre-hybridized in 50% de-ionized formamide, $5\times$ Denhardt's solution (0.1% Ficoll, 0.1% polyvinyl-pyrrolidone, 0.1% bovine serum albumin fraction V), $5\times$ standard sodium citrate (SSC) (0.75 M sodium chloride, 0.075 M sodium citrate), 0.2% sodium dodecyl sulfate (SDS), 20 mM sodium phosphate, and 10 $\mu\text{g}/\text{ml}$ salmon sperm DNA at 42°C overnight. The HLA class I cDNA probe, HLA-B7 (Sood et al., 1981), was labelled with ^{32}P -dCTP and ^{32}P -TTP (Amersham) to a specific activity of 5×10^8 CPM/ μg of DNA using a random-primed labelling kit (Boehringer-Mannheim). Radiolabelled probe was added to the blot and incubated for another 22–25 h at 42°C. Membranes were washed twice in $2\times$ SSC/0.1% SDS for 5 min, then twice in $1\times$ SSC/0.1% SDS for 30 min at room temperature. Membranes were exposed to Kodak X-Omat RP film using an intensifying screen at -70°C .

RESULTS

Proliferative responses to mitogen

Peripheral blood mononuclear cells from a number of captive cheetahs have been tested for their *in vitro* ability to respond to mitogens (Miller-Edge and Worley, 1991), an assay used in other species to measure general immune

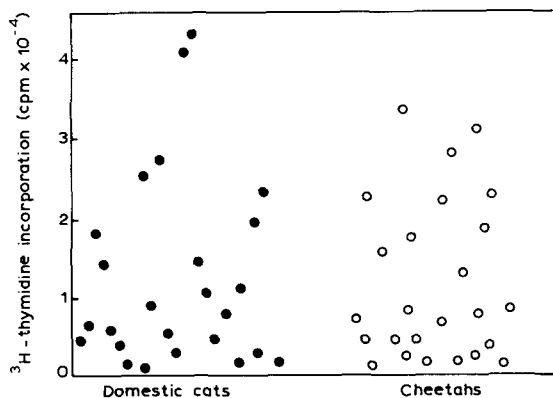


Fig. 1. Proliferative responses of Con A-stimulated cheetah (open symbols) and domestic cat (solid symbols) PBM. Cells were cultured with 10 $\mu\text{g}/\text{ml}$ Con A for 4 days. Net values of ^3H -thymidine incorporation in replicate wells are expressed in counts per min (CPM), and calculated as mean CPM in stimulated wells minus mean CPM in background control wells. Each data point represents an individual animal. There is no significance to the horizontal spatial relationship of data points within each group.

capability (Cockerell et al., 1975; Kateley and Bazzell, 1978). Because in vitro functional assays give relative values, data from the cheetah population were compared with results from a group of domestic cats. As previously shown, a wide range of individual variation occurred both within and between species; however, the mean responses of the two species to Con A were similar (domestic cats — $12\,528 \pm 11\,872$ CPM; cheetahs — $11\,636 \pm 10\,435$ CPM; $P > 0.25$) (Fig. 1). These results suggest that cheetahs as a group are not immunologically impaired or identical.

Proliferative responses to antigen

Although the species' mitogen responses were comparable, antigen-specific assays were performed to evaluate potential differences in individual and species' responses to pathogens. In order to eliminate possible low response artifacts due to immunologic naiveté, only individuals with known vaccination histories were included in the analysis. Animals were vaccinated with commercial feline vaccines containing FHV-1, with the last immunization 1–13 months prior to sampling. There was no correlation between level of FHV-1-stimulated proliferation and interval after last vaccination.

Feline herpesvirus-1 (FHV-1) infection has been associated with clinical disease in cheetahs (Scherba et al., 1988; San Diego Wild Animal Park medical records, unpublished observations, 1989) and is routinely included in vaccination programs. Two different concentrations of a pathogenic isolate and a temperature-sensitive vaccine strain of FHV-1 were used in stimulation assays. As shown in Fig. 2, both strains of UV-inactivated virus were immunogenic for domestic cat and cheetah PBM and resulted in a wide range of responses. However, when comparing species, cheetah PBM had a significantly lower mean response ($P < 0.05$) to all strains and concentrations used (i.e., mean net responses to 10^6 PFU/ml pathogenic strain: domestic cats — 1822 ± 2835 CPM, $n=9$; cheetahs — 215 ± 1006 CPM, $n=25$). Therefore, cheetahs, as a group, appeared to have reduced capacity to recognize and respond to a specific pathogen (FHV-1).

Interestingly, approximately 40% of this cheetah population (10 animals) have had documented clinical problems with rhinotracheitis, conjunctivitis/keratitis, and/or herpetic skin lesions. None of the vaccinated domestic cats had similar medical histories. Due to individual variation and the low number of animals in each group, no statistical differences in responses between cheetahs with or without a history of disease were seen.

Similar studies were performed to evaluate responses to *Cryptococcus neoformans*. Therefore, PBM from cheetahs and domestic cats were incubated with whole heat-killed organisms or a crude capsular preparation. Since *Cryptococcus* is a ubiquitous fungus in the environment and assessment of previous exposure is difficult, both herpesvirus-vaccinated and unvaccinated animals were screened (Fig. 3). Although domestic cats tended to have slightly

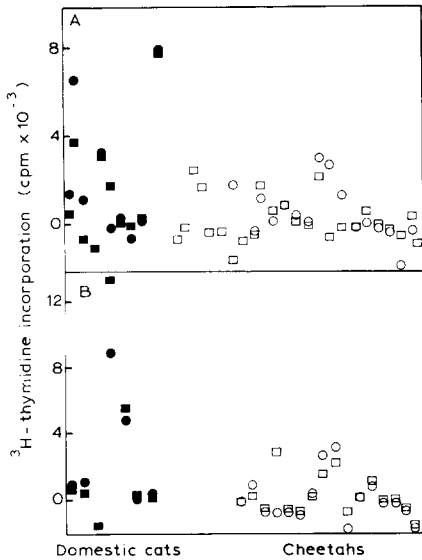


Fig. 2. Proliferative responses of FHV-1-stimulated cheetah (open symbols) and domestic cat (solid symbols) PBM. Cells were cultured with 10^6 (squares) or 10^7 (circles) PFU/ml UV-inactivated pathogenic (A) or temperature-sensitive (B) strains of FHV-1 for 7 days. Net values of ^3H -thymidine incorporation are expressed in CPM and calculated as described in Fig. 1. Symbols representing responses of a single individual to both concentrations of virus are aligned vertically; however, there is no significance to the horizontal spatial relationship of data points within each group. Not all individuals were tested with both strains and concentration of virus.

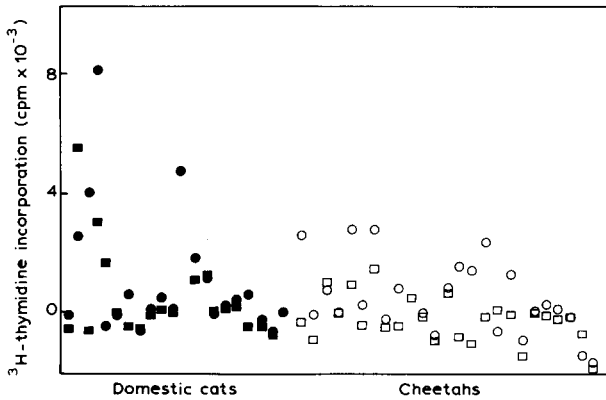


Fig. 3. Proliferative responses of cheetah (open symbols) and domestic cat (solid symbols) PBM to *Cryptococcus neoformans*. Cells were cultured with capsular antigen (squares) or whole heat-killed organisms (circles) for 7 days. Net values of ^3H -thymidine incorporation are expressed as CPM and calculated as described in Fig. 1. Symbols representing responses of a single individual to both antigen preparations are aligned vertically; however, there is no significance to the horizontal spatial relationship of data points within each group.

higher responses to both antigens, the differences between species were not significant ($P > 0.05$). Comparison of responses to the two preparations showed no significant differences ($P > 0.05$). Because responses to mitogen and cryptococcal antigens were similar in both species, the data suggest that cheetahs are generally immunocompetent. The low level of feline herpesvirus-stimulated proliferation may represent an antigen-specific difference in recognition and/or response between species.

Exogenous IL-2 supplementation of antigen-stimulated cultures

One possible explanation for low response to FHV-1 may be insufficient production of interleukin 2 (IL-2), an antigen-nonspecific lymphokine required for T cell proliferation (Smith, 1988). In order to assess the role of IL-2 in FHV-1 responses, domestic cat and cheetah PBM were stimulated with virus in the presence or absence of supernatant containing human recombinant IL-2. When cheetahs were ranked according to response to FHV-1, the low responders tended to show the greatest enhancement of proliferation in the presence of IL-2 (Fig. 4). As a group, exogenous IL-2 significantly increased the mean proliferative responses of cheetah PBM to both strains of virus ($P < 0.025$). However, mean domestic cat PBM responses did not significantly change with addition of IL-2, except in individual cases, where both enhancement and suppression were observed (data not shown). These results support the hypothesis that inadequate levels of IL-2 may be at least partially responsible for low proliferative responses by cheetah PBM. Once optimal

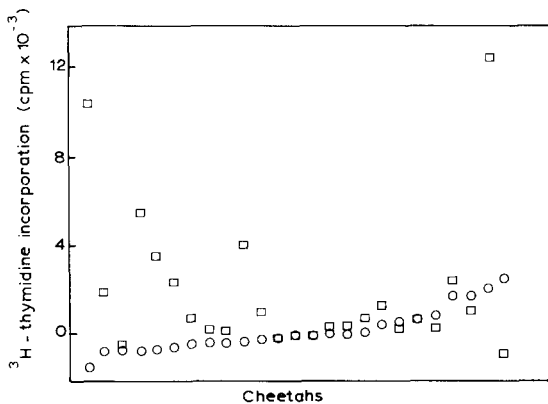


Fig. 4. Augmentation of FHV-1-stimulated responses by exogenous IL-2. Cheetah PBM were stimulated with UV-inactivated pathogenic strain of FHV-1 (10^6 PFU/ml) in the absence (circles) or presence (squares) of 100 U/ml IL-2 for 7 days. Net values of ^3H -thymidine incorporation are expressed as CPM and calculated as described in Fig. 1. Controls included unstimulated cultures supplemented with exogenous IL-2. Individual FHV-1-stimulated responses (in the absence of IL-2) are ranked from lowest (left) to highest (right).

conditions for stimulation exist, additional IL-2 may not augment the response (high responders). When mean responses of FHV-1-stimulated domestic cat PBM were compared to the mean cheetah response after IL-2 supplementation, there was no statistical difference ($P > 0.05$) (cheetah vs. domestic cat: pathogenic strain — 1992 vs. 1822 CPM, respectively, vaccine strain — 2359 vs. 2606 CPM, respectively); thus, cheetah PBM appear capable of responding to the same degree as domestic cat PBM under optimal conditions.

Humoral immune response to FHV-1

Since both branches of the immune system play a role in host defense against viral infection, an evaluation of humoral immunity was performed using viral neutralization assays. This method is commonly employed to quantitate the relative functional concentration of circulating antibody. Neutralizing antibody titers to FHV-1 were not significantly different ($P > 0.05$) between vaccinated domestic cats (mean titer 1:47; range 1:8–1:256) and cheetahs (mean titer 1:64; range 1:8–1:512). Therefore, it appears that cheetah humoral immune capabilities are comparable to those of domestic cats, and the difference in response to FHV-1 is restricted to cell-mediated immune function.

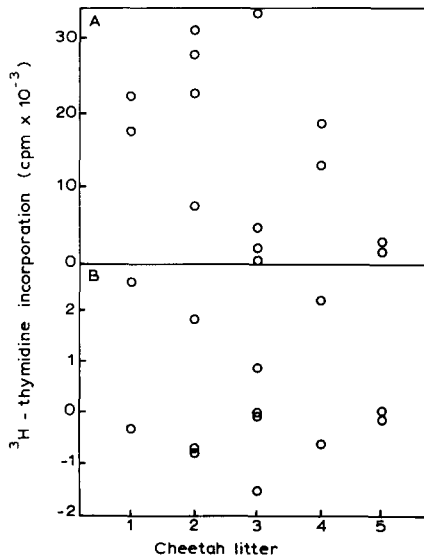


Fig. 5. Comparison of proliferative responses among full-sibling cheetahs. PBM from 14 individuals in five litters were cultured with $10 \mu\text{g/ml}$ Con A (A) for 4 days, or 10^6 PFU/ml pathogenic strain of FHV-1 (B) for 7 days. Net values of ^3H -thymidine incorporation are expressed in CPM and calculated as described in Fig. 1. Each data point represents an individual animal.

Proliferative responses among genetically similar cheetahs

To evaluate the genetic contribution to the level of in vitro immune response, proliferation of PBM from cheetah littermates was compared. Fig. 5 shows that individual variation occurred among full-sibling cheetahs when stimulated with either Con A (panel A) or FHV-1 (panel B). Analysis of MHC class I-like genes in cheetahs using Southern blot hybridization suggests that diversity exists even between full-siblings (manuscript in preparation). Because of the low number of full-sibling cheetahs in this study (12 animals), no significant correlation ($P > 0.05$) was apparent between level of response to either mitogen or antigen and specific patterns of restriction fragment length polymorphisms (RFLP) detected by Southern blot (data not shown). Therefore, although genetic factors influence immune response, other mechanisms play a role in determining the ultimate host response.

DISCUSSION

Our studies have shown that in vitro mitogen- and antigen-stimulated cheetah PBM exhibit a wide range of responses, similar to other outbred species (Cockerell et al., 1975; Kateley and Bazzell, 1978). The relatively poor T cell responses of cheetahs to feline herpesvirus appear to be antigen-specific, since responses to mitogen and *C. neoformans* were similar in both species. Therefore, low responses were not due to general immunosuppression or compromise. In addition, the presence of antibody titers indicated that antigen was delivered effectively during immunization.

In vitro assays detect secondary antigen-specific responses and have been used to measure cell-mediated immune response to other herpesviruses (Rouse and Babiuk, 1974; Lopez and O'Reilly, 1977). Although all the cheetahs used in this study had been previously immunized with a commercial feline herpesvirus vaccine, it was not possible to control for other environmental conditions that could affect immune response, such as nutrition, stress, and other subclinical disease conditions. However, individuals from which serial samples were tested showed consistent responses.

Virus-induced suppression has been observed in several species (Lopez and O'Reilly, 1977; Filion et al., 1983; Del Gobbo et al., 1990). Studies have shown that herpesvirus-infected cells can suppress T cell proliferation in vitro (Carter et al., 1989). This effect was due to viable virus since UV-inactivated virus did not mediate inhibition. Because UV-inactivated noninfectious virus was used as antigen in our experiments, suppression due to infection or cytotoxicity in vitro could not account for low responses.

Initial studies investigating mechanisms resulting in low responsiveness suggested that decreased IL-2 production may be one possible factor involved. IL-2 is an antigen-nonspecific lymphokine which is necessary for stimulated T cell expansion (Smith, 1988). Supplementation of virus-stimulated

cultures with exogenous IL-2 resulted in significant enhancement of certain individual cheetahs' responses. As a group, the mean response of FHV-1-stimulated cheetah PBM supplemented with IL-2 was comparable to domestic cat PBM responses, with or without IL-2. Therefore, cheetah PBM appear to have similar potential immunologic competence as that of domestic cats. Interestingly, the individuals with the poorest responses were those that tended to respond most favorably with IL-2 supplementation. This trend has been repeated in other *in vitro* systems (Miller-Edge and Splitter, 1986). Our results appear to rule out an inability of cheetah PBM to respond to antigen by lack of IL-2 receptor expression, as has been shown to occur in some low responder conditions (Pink and Vainio, 1983; Kierszenbaum et al., 1989). However, further studies of IL-2 production by stimulated cheetah PBM are required to determine whether insufficient endogenous IL-2 is the underlying cause of poor response. If IL-2 supplementation can augment *in vivo* responses as well, incorporation of this lymphokine into prophylactic regimens with killed or subunit vaccines may be beneficial, especially in exotic species where data are lacking regarding safety and efficacy of current vaccines. Preliminary investigations using IL-2 *in vivo* have shown that it may provide an immunological boost in vaccination programs (Weinberg and Merigan, 1988; Kawashima and Platt, 1989; Reddy et al., 1989).

The observed wide individual variation in responses to mitogen and antigen has special significance with regard to the cheetah since it has been proposed that apparent increased susceptibility to certain viral infections is due to a uniform species-specific unresponsiveness linked to genetic monomorphism (O'Brien et al., 1985). In order to assess the effect of similar genetic background on immune responses, mitogen- and antigen-stimulated proliferation were compared among cheetah littermates. Differences were observed even between related individuals (Fig. 5). Although T cell responses appear to be restricted to epitopes that can bind particular MHC molecules, there is variation in responses even among syngeneic individuals (Gammon et al., 1990). Recent results (Yuhki and O'Brien, 1990; Miller-Edge and Worley, manuscript in preparation) have demonstrated some polymorphism in cheetah MHC class I-like genes. However, attempts to correlate level of mitogen- or antigen-stimulated proliferation with specific RFLP were unsuccessful. Other studies attempting to correlate expression of specific MHC antigens with ability to respond to antigens have given equivocal results (Clerici et al., 1989). Therefore, mechanisms, in addition to genetic factors, influence the final outcome of the host's response. Selection and proliferation of specific T cell clones depend on *in vivo* factors such as route, dose, form, and number of exposures to antigen, as well as host factors (Hammerberg et al., 1989).

In summary, *in vitro* responses of antigen-stimulated cheetah PBM show wide individual variation, even among littermates, and could not be correlated with specific RFLP patterns in MHC class I Southern blots. Therefore,

a direct effect of genetic variation on level of immune response could not be demonstrated. FHV-stimulated cheetah PBM proliferated significantly less than PBM from domestic cats. Further analyses suggested that this species difference was probably mediated by the cellular immune response. Further characterization of the mechanisms resulting in low responses may lead to the use of immunomodulators to supplement *in vivo* responses.

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