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Abstract: An extensive genetic and physiological analysis of the cheetah by O'Brien et al. (1983; 1985; 1987) indicated that the cheetah showed monomorphism at the major histocompatibility complex. This led O'Brien (1985) to propose that the cheetah suffered from an immunodeficiency and was highly susceptible to diseases. It was therefore decided to investigate cell-mediated and humoral immune responses and to apply the limited restriction fragment length analysis (using Pst 1 and Barn H1 enzymes) of the cheetah MHC 1 and MHC II genes. Antibody responses to antigens (feline viruses), as well as mitogen-induced lymphocyte blast transformation responses, were shown to be intact and comparable with that of the domestic cat, indicating a competent immune system in the cheetah. It was also suggested by the results that some polymorphism does exist in the MHC class II genes, but possibly not in the MHC class I genes.

Lymphocyte blast transformation responses and restriction fragment length analysis in the cheetah

J.A SPENCER

Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110 South Africa

ABSTRACT

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An extensive genetic and physiological analysis of the cheetah by O'Brien *et al.* (1983; 1985; 1987) indicated that the cheetah showed monomorphism at the major histocompatability complex. This led O'Brien (1985) to propose that the cheetah suffered from an immunodeficiency and was highly susceptible to diseases. It was therefore decided to investigate cell-mediated and humoral immune responses and to apply the limited restriction fragment length analysis (using Pst 1 and Bam H1 enzymes) of the cheetah MHC I and MHC II genes. Antibody responses to antigens (feline viruses), as well as mitogen-induced lymphocyte blast transformation responses, were shown to be intact and comparable with that of the domestic cat, indicating a competent immune system in the cheetah. It was also suggested by the results that some polymorphism does exist in the MHC class II genes, but possibly not in the MHC class I genes.

INTRODUCTION

The declining numbers of cheetahs found in the wild has led to the implementation of captive breeding programmes for this species. Over the years there have been only sporadic births in captivity, prompting an extensive genetic and physiological analysis of both captive and free-ranging cheetahs (O'Brien, Roelke, Marker, Newman, Winkler, Meltzer, Colly, Evermann, Bush & Wildt 1985). The cheetah appears to be unique among felids and other mammals in its extreme paucity of genetic variation, as estimated by electrophoretic surveys of allozymes and cell proteins resolved by two dimensional gels (O'Brien, Wildt, Goldmann, Merril & Bush 1983). Even more unusual is the observation of acceptance of allogenetic skin grafts among unrelated cheetahs. This reveals genetic monomorphism at the major histocompatibility complex (MHC), an abundantly polymorphic locus in nearly all mammals (O'Brien, *et al.* 1985). A comparative analysis of cheetah ejaculates revealed a sperm count one-tenth of that observed in domestic cats, and an extremely high frequency (71 %) of morphological spermatozoal abnormalities (Wildt, Bush, Howard, O'Brien, Meltzer, Van Dyk, Ebedes & Brand 1983).

In addition to these phenotypic observations, patterns of skeletal variation also show significant asymmetry

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of bilateral characters, a phenomenon generally common in inbred animals (Wayne, Norman, Newman, Martenson & O'Brien 1986). The combined genetic, reproductive and morphological data places the cheetah in a situation reminiscent of deliberately inbred mice. O'Brien *et al.* (1983) were prompted to hypothesize that, in its recent history, the species had probably suffered a demographic contraction or bottleneck associated with inbreeding. However, based on the genetic and physiological similarities of the southern and eastern African subspecies, they concluded that the proposed bottleneck was an ancient one, possibly dating back to the global mammalian extinctions near the end of the Pleistocene epoch, over 10000 years ago (O'Brien, *et al.* 1985; O'Brien, Wildt, Bush, Caro, Fitzgibbon & Leakey 1987).

The theory of natural selection, formulated by Darwin, implies that genetic variation is essential for evolution. It is on genetic heterogeneity that natural selection operates in times of environmental or ecological change. Evolutionary theory predicts that a species with very little genetic variation would be particularly vulnerable in a time of ecological disturbance. Genetic uniformity therefore poses a threat to the survival of a species (Leakey 1979).

The extensive documentation of an epizootic of FIP at Wildlife Safari, Oregon, where 90% of the population showed clinical signs of the virus and 48% of the population died (O'Brien, *et al.* 1985; Pfeifer, Evermann, Roelke, Gallina, Ott & McKeirnan 1983; Evermann, Roelke & Briggs 1986), alerted the wildlife veterinary community to the seriousness of this disease in cheetahs.

O'Brien *et al.* (1985) explained the morbidity of FIP in cheetahs by the monomorphism found in genes within the MHC sublocus.

The MHC contains immune-response (MHC II) genes. A population that becomes monomorphic at the MHC, lacks genetic variation and would be particularly vulnerable to a viral strain able to circumvent the immunological defences. Any virus that evades the immune response system of one animal would subsequently find every other system it encounters in the population to be identical to the first. It would therefore be easy to evade the effects of the immune system of those animals.

On the basis of this premise, it was decided to investigate the immune system of the cheetah to accomplish the following:

- The examination of the *in vitro* functional capabilities of cheetah lymphocytes
- The establishing of a baseline for the evaluation of immune functions
- The determination, by limited restriction fragment length analysis, of whether or not the monomorphism is restricted to the MHC I or MHC II locus, or both loci

These investigations were carried out on the captive cheetahs at the De Wildt Cheetah Breeding and Research Centre, by means of *in vitro* techniques.

MATERIALS AND METHODS

Aseptic techniques were employed in the performance of all the procedures.

Blood collection

Domestic cats

Cats were sedated with "Ketalar" (5 mg/kg I.M.; Ketamine HCl, Parke-Davis, Johannesburg). Blood samples of 5 mℓ were then collected from the cephalic vein and placed in sterile glass tubes containing EDTA. Each tube was inverted several times to ensure mixing and to prevent the blood from clotting.

Cheetahs

Blood samples of 20 mℓ were collected from the medial saphenous vein by venipuncture and placed in tubes containing 1 mℓ 50 U/mℓ preservative-free heparin. The animals were not sedated, but restrained in crush cages.

Lymphocyte separation

The heparinized blood was centrifuged for 10 min at 1800 rpm. The buffy coat was then removed with a pasteur pipette and placed in a clean, sterile 15-mℓ tube. RPM1 medium (Highveld Biologicals, Johannesburg) containing 10% foetal calf serum (FCS) and Gentamycin (0,1 mℓ/100 mℓ medium) were then added to the cell suspension to give a final volume of 10 mℓ. As the addition of RPM1 was seen to cause the clotting cascade to commence, the medium had to be replaced by Mg^{2+}- and Ca^{2+}-free PBS.

This cell suspension was then layered slowly onto 5 mℓ of "Histopaque" (Sigma, St. Louis, United States; d = 1,077) and centrifuged for 40 min at 2200 rpm. The speed of the centrifuge was increased and decreased very slowly to prevent the disruption of the lymphocyte layer.

The lymphocyte layer was removed by gentle pipetting and placed in clean, sterile polypropylene tubes. The lymphocytes were washed three times in Ca^{2+}- and Mg^{2+}-free PBS and then suspended in a final volume of 1 x 10^6 cells/mℓ in RPM1 medium.

Lymphocyte transformation

Samples of 100 µℓ cell suspension were pipetted into round-bottom, 96-well, 300-µℓ, tissue-culture

microtitre plates (Nunc, Roskilde, Denmark). Volumes of 50 µℓ phytohaemagglutinin (PHA) (Grand Island Biological Co., New York) or pokeweed mitogen (PWM) (Grand Island Biological Co.) were added to the cells. Both mitogens (PHA and PWM) were diluted 1:100 in RPM1 medium according to the manufacturers' instructions. Control (unstimulated) cells received only 50 µℓ medium. All tests were performed in triplicate.

Plates were covered with loosely fitting lids and incubated for 48 h at 37 °C, in humidified air containing 5 % CO_2.

Either 50 µℓ or 100 µℓ tritiated thymidine (^{3}H-TdR) (0,5 µCi/mℓ) (Amersham, England) was then added to each well. The plates were covered and incubated for a further 18 h.

The cultures were harvested by means of a multiple semi-automatic cell harvester (Flow Laboratories, Virginia, United States). Lymphocytes were collected on glass-fibre filter paper and left to become air-dry. Once dry, the filter papers were placed in scintillation vials (mini poly-Q vials, Beckman, United States) containing 4 mℓ of toluene-based cocktail (Ready Safe, Beckman).

The incorporation of tritiated deoxynucleoside triphosphates (^{3}H-TdR) into the lymphocytes was determined with a Tri-carb Model-3385 liquid-scintillation spectrometer (Packard, USA). Results were expressed as mean counts per minute (cpm).

Mixed lymphocyte culture (MLC)

Stimulator lymphocytes (B cells) obtained from one cheetah were collected and washed as described above. These cells were treated with Mitomycin C (Sigma, USA), at a concentration of 0,05 mg/2 x 10^6 lymphocytes for 30 min at 37 °C. The stimulator cells were washed three times and resuspended in medium at a final concentration of 1 x 10^6 cells/mℓ.

The stimulator cells were then cultured in 96-well, round-bottom microtitre plates with untreated lymphocytes from other cheetahs.

The plates were set up as follows:

- 75 µℓ treated cells and 75 µℓ untreated cells

or

- 50 µℓ treated cells, 50 µℓ untreated cells and 50 µℓ PHA

Controls consisted of untreated cells and PHA or treated cells and PHA.

The plates were sealed and incubated, as described above, at 37 °C for 96 h. Then 100 µℓ 0,5 µCi ^{3}H-TdR was added to each well. The plates were incubated for a further 18 h before the lymphocytes were harvested and counted as previously described.

The response to the stimulator cells was then compared with the control (normal) response to PHA.

Restriction fragment length analysis

DNA isolation

- Blood samples were collected from the cheetahs and placed in tubes containing heparin, as previously described. The samples were centrifuged at 1 800 rpm for 10 min, after which the buffy coat was harvested. These cells were then washed once in Ca^{2+}- and Mg^{2+}-free PBS and stored frozen at -70 °C in 1-mℓ amounts in Nunc cryotubes until used.
- The thawed samples were placed in sterile polypropylene tubes and 14 mℓ sucrose-triton-X lysis buffer (sucrose 2,19 g 1 M Tris 400 µℓ, 1 M $MgCl_2$ 100 µℓ, Triton X-100 400 µℓ and dH_2O 16 mℓ) was added and gently mixed. The tubes were then placed on ice for 5 min.
- The samples were centrifuged at 3000 rpm at 4 °C for 10 min. The supernatant was removed and the cells were incubated on ice in 14 mℓ lysis buffer for a further 5 min.
- The tubes were centrifuged as described above and the supernatant removed. The following was then added to the cell pellet: 3 mℓ $T_{20}E_5$ (1 M Tris 20 mℓ, 0,5 M EDTA 10 mℓ and dH_2O 970 mℓ), 200 µℓ 10 % sodium dodecyl sulphate (SDS) and 500 µℓ Proteinase K (Boehringer Mannheim, Germany) (2 mg Proteinase K per mℓ 1 % SDS, 2 mM EDTA). The cells were then gently mixed and the tubes incubated overnight in a water bath at 42 °C.
- After an incubation of 16 h, 1 mℓ saturated NaCl was added and the tubes were agitated for 15 s. They were then centrifuged at 2 500 rpm at 4 °C for 15 min to sediment any cell debris.
- The DNA was precipitated with EtOH and left overnight at -20 °C.
- The DNA was sedimented by centrifugation at 6 000 rpm at room temperature for 30 min. The supernatant was removed and the DNA was washed in 70 % ethanol by a gentle shaking of the tubes, and re-centrifuged for 5 min. The DNA sediments were then freeze-dried.
- The DNA sediment was dissolved in sufficient $T_{10}E_1$ (10 mM Tris, 1 mM EDTA) buffer and stored at 4 °C until used.

Restriction enzyme analysis

The analysis was carried out according to the method of Yuhki & O'Brien (1988) using Pst 1 and Bam H1 enzymes only.

Southern blot analysis

The agarose gel was denatured in 0,4 N NaOH by incubating it at room temperature for 1 h. The DNA samples were then transferred to "Hybond" N+ nylon membranes (Amersham, U.K.) by the technique of Southern (1975).

DNA probes

The following two DNA probes, supplied as freeze-dried *E. coli*, containing the plasmids from the American Type Culture Collection (Rockville, Maryland, USA), were used in these experiments:

- HLA-B7, which is a 1,4-kb human-cDNA-probe coding for the α1, α2, α3, transmembrane, cytoplasmic domains and the 3' untranslated region (Yuhki & O'Brien 1988) of the MHC class 1 gene
- DR1ß, which is a 1,3-kb human-cDNA-probe coding for the ß chain peptides

E. coli culture

The cultures were rehydrated with 400 µℓ sterile saline and grown in L-broth according to standard procedures.

Plasmid extraction

Plasmids were extracted by the alkali lysis method of Sambrook, Fritsch & Maniatis (1989).

Isotope labelling of the probes

The plasmid containing the DNA probe was then nick-translated and the unbound ^{32}P was removed by running the solution through a Sephadex G50 column (Sambrook, *et al.* 1989). The probe was boiled for 5 min, snap-cooled on ice and added to the hybridization solution.

Hybridization

The nylon membranes were pre-hybridized at 42 °C for 16 h in a solution consisting of:

20 x SSC	– 6,25 mℓ
Formamide	– 12,50 mℓ
50 x Denhardt's	– 1,00 mℓ
10 % SDS	– 0,25 mℓ
dH_20	– 5,625 mℓ
ss DNA	– 500 µℓ

The volume of the solution was then decreased to 10 mℓ and the DNA probes were added to this solution. Hybridization with the probes was continued at 42 °C for 20 h.

Washing of nylon membrane

After a hybridization of 20 h, the nylon membranes were washed in 2 x SSC (20 x SSC = 175,3 g NaCl, 88,2 g sodium citrate and 800 mℓ dH_2O), 0,1 % SDS at 50 °C to remove the background noise. This represents a 70 % homology stringency. The nylon membranes were autoradiographed at -80 °C for 16 h.

RESULTS

Cheetah lymphocyte transformation

The cpm for each cheetah sample can be seen in Table 1. Two different amounts of tritiated thymidine were added so that it would be possible to compare cheetah results with domestic-cat results, and the present cheetah results with results reported in the literature (Miller-Edge & Worley 1991). Mean cpm with 50 µℓ ^{3}H-TdR for PHA were 8 021 for cheetahs and 12 802 for cats. The responses to PWM were 6 381 and the control values for cheetahs were 1 335. Domestic-cat responses to PWM were not tested and control responses for cats were 167. Mean cpm for cheetahs with 100 µℓ ^{3}H-TdR were higher; 13 529 for PHA and 14 323 for PWM. The control responses were 1 795.

From the results it would initially appear that domestic-cat lymphocytes are more sensitive to PHA than cheetah lymphocytes, but as the range for the domestic cat is much greater than that for the cheetah, this is not a valid comparison.

Double the amount of ^{3}H-TdR had to be added to increase mean cheetah cpm to that of the domestic cat, and also to that of 12 117 cpm, reported in the literature (Miller-Edge & Worley 1991).

Mixed lymphocyte culture (MLC)

There was no response by cheetah responder lymphocytes (mean cpm = 443) to stimulator lymphocytes from other cheetahs. However, there was still a response to PHA (mean cpm = 7 794) (Table 2), indicating that the cheetah responder lymphocytes were still sensitive to mitogens. Furthermore, a student t-test indicated a highly significant difference between the two groups (P = 0,001).

Lymphocyte responses in mixed lymphocyte cultures are caused by the MHC I component of the T-cell receptor. The results showed that cheetah T cells do not respond to cheetah B cells (the stimulator cells).

Restriction fragment length analysis

MHC class 1 genes

A total of 15 samples were digested with Pst 1 and seven were digested with Bam H1. The probe hybridized with animal samples on only two successive attempts; each time a different animal was involved (Fig. 1).

TABLE 1 Lymphocyte blast transformation responses to mitogens expressed as mean counts per minute

Sample	50 µℓ ^{3}H-TdR			Sample	100 µℓ ^{3}H-TdR		
	PHA	PWM	Medium		PHA	PWM	Medium
Cheetah n = 9	8021 ± 2369	6381 ± 2097	1335 ± 536	Cheetah n = 6	13529 ± 2353	14323 ± 9998	1795 ± 1379
Cat n = 6	12802 ± 10969	Not done	167 ± 39	Cat n = 0	Not done		

TABLE 2 Mixed lymphocyte culture responses expressed as mean counts per minute

50 µℓ ^{3}H-TdR		100 µℓ ^{3}H-TdR	
CPM	PHA	CPM	PHA
443 ± 47 n = 8	7794 ± 2810 n = 8	311 ± 54 n = 15*	8839 ± 4511 n = 10*

* Some simulators were cultured with more than one responder

MHC Class II genes

A total of 15 samples were digested with Pst 1 restriction enzyme, and the Southern blotted nylon membranes were hybridized more than once with the DR1ß probe. The results were inconsistent and could not be repeated. For each experiment the probe recognized only a limited number of DNA samples from the animals (Fig. 2). The bands that were detected by these hybridization experiments were not, however, identical—suggesting the existence of polymorphism at the MHC II locus (Fig. 2). Of the seven samples digested with Bam H1, only one confirms hybridization recognition with the DR1ß probe.

DISCUSSION

Until recently, the only way to assess the immune status of felids was by measuring humoral antibody levels or *in vivo* manifestations of delayed-type hypersensitivity. Additional techniques have permitted further studies, and one of the most widely employed assays is the lymphocyte blast transformation test. Although this assay has been used in human research for many years, Cockerell, Hoover, Lobuglio & Yohn (1975) were the first to employ it for the studying of immunocompetence in domestic cats.

The mitogen-induced lymphocyte responses *in vitro* are very suitable as models in the investigation of immune reactions *in vivo* (Janossy & Greaves 1971). *In vitro* techniques can therefore be employed for deducing what happens in the immune system of animals, and may indicate whether or not an animal is immunodeficient.

In this study mean mitogen-stimulated proliferative responses of cheetah and cat lymphocytes were found to be similar. However, the values tended to be lower than those observed in cattle when the same techniques were used (PHA = 72 000 cpm; PWM = 73 000 cpm; Spencer, Jacob & Schultheiss 1991). Considerable variation between individuals occurred within both cheetah and cat species, and could possibly be the result of insufficient Interleukin-2 production in those animals with lower responses (Miller-Edge & Worley 1991).

It has been speculated that cheetahs are more susceptible than domestic cats to coronavirus infection (O'Brien *et al.* 1985). However, in this study lymphocyte blast transformation assays did not provide evidence for species-specific defects in the cheetah's cell-mediated immune functions.

MLC provides a method for studying relatedness between animals of the same species. The technique involves recognition of MHC-gene products at the cellular level. If, as O'Brien *et al.* (1983) postulated, cheetahs are severely restricted at the MHC locus, then there will be limited blast transformation response in an MLC assay. In the present study, MLC from cheetahs led to no transformation, indicating that there is very little genetic variation between the MHC class I genes of individuals of this species.

Although this supports the theory that cheetahs are restricted at the MHC locus because they do not reject skin grafts (O'Brien *et al.* 1985), it also leads to further suggestions. The MHC locus is divided into two groups: MHC I, which is involved in graft rejection and the elimination of tumours and virus-infected cells; and MHC II, which is involved in antibody production (Tizard 1987). Since there was no blast transformation response in cheetah-versus-cheetah mixed-lymphocyte assays, strong antibody responses to vaccine, and an apparent lack of polymorphism as shown by DNA probe analysis, it is possible that the

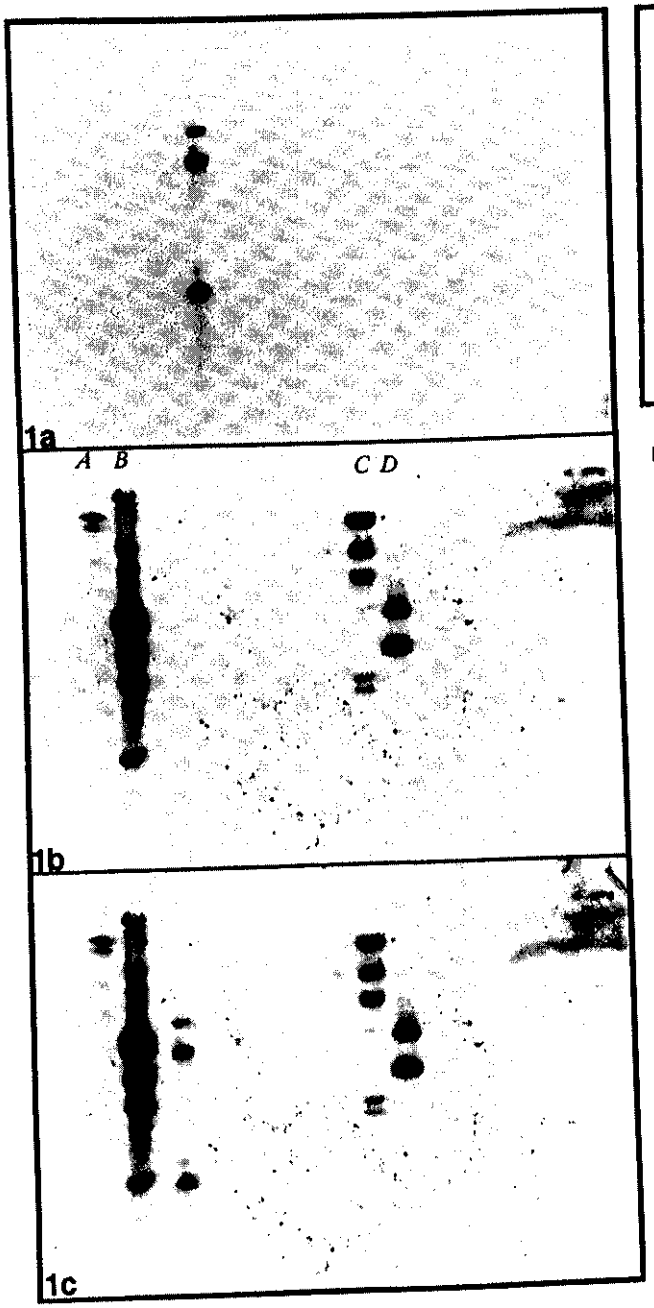

FIG. 1 Hybridization experiments in which the MHC class I gene probe is used:

1a – hybridization with a single animal only, DNA cut with Pst 1

1b – A and B represent DNA cut with Pst 1, C represents the molecular mass marker, and D is the DNA cut with Bam H1

1c – Fig. 1a and 1b are superimposed to facilitate comparison of the results from the two experiments

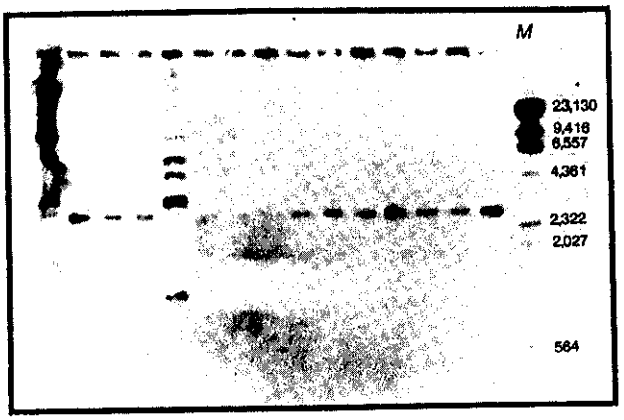

FIG. 2 Hybridization experiments in which the MHC class II gene probe is used. All these samples were cut with Pst 1 and M represents the molecular mass marker

MHC homogeneity within the species is limited to the MHC I locus. This is further supported by the suggestion of polymorphism existing at the MHC II locus as shown by DNA-probe analysis. The apparent lack of polymorphism at the MHC I locus, as shown by RFLP, is supported by Yuhki & O'Brien (1990). They used an in-house feline-pFLA24 probe to detect cheetah MHC I genes.

The results obtained with the feline probe are markedly superior to those obtained with human probes, in that hybridization was seen to occur in all the DNA samples tested. However, as no work has been done in which feline MHC II probes were used, one must rely on the results obtained when the human probe was used. Yuhki & O'Brien (1988), using the human probes employed in this study to examine DNA samples from domestic cats, also experienced inconsistent results. It is therefore hoped that a feline MHC class-II probe will be developed in the near future.

Yuhki & O'Brien (1990) felt that the consequence of genetic uniformity at the MHC would be long-lasting and would increase the chance of extinction due to disease outbreaks. O'Brien *et al.* (1987) speculated that, due to the lack of polymorphism, the cheetah is highly susceptible to diseases such as FIP—as evidenced by previous outbreaks. I would, however, like to dispute this. It is felt that the cheetah is not more susceptible to FIP than any other felid (Pedersen, N.C. 1991. Personal communication. University of California, Davis). The outbreak at Safari Park, Oregon, ran its course in the same way as FIP outbreaks in outbred cats in cattery situations do (Pedersen 1991).

The outbreaks of FIP in cheetahs could be related to stress situations as found in the domestic cat (Pedersen 1988). Stress can lead to a state of immunosuppression, caused by the release of various neurotransmitters and hormones that can modulate the immune response (Khansari, Murgo & Faith, 1990). If the settings of the parks experiencing FIP outbreaks are compared with those of parks such as De Wildt and San Diego Zoo, where no outbreaks of FIP have

occurred, it would seem to suggest that, in disease outbreaks, enclosure design and size are more important than the state of the immune system of the animals. Furthermore, the theory that the animals at De Wildt and the San Diego Zoo are being kept under less stressful situations than the animals in the parks where FIP outbreaks have occurred, is supported by the fact that these two parks have also had the highest captive-birth success rates and the lowest incidence of gastritis—a stress-dependent syndrome.

The cheetah requires spacious enclosures that minimize stress. It would appear that the enclosures in the parks where FIP outbreaks have occurred, are not ideal.

In conclusion, it is proposed that cheetah be described as "immunotolerant" rather than as immunodeficient. They readily accept skin grafts from other cheetahs, indicating a tolerance of these foreign antigens. Their humoral antibody responses and mitogen-induced lymphocyte blast transformation responses are not impaired, indicating an immunocompetent immune system. The greatest threat facing the survival of the species may be man and not disease outbreaks.

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REFERENCES

COCKERELL, G.L., HOOVER, E.A., LOBUGLIO, A.F. & YOHN, D.S. 1975. Phytomitogen and antigen-induced blast transformation of feline lymphocytes. *American Journal of Veterinary Research*, 36:1489–1494.

EVERMANN, J.F., ROELKE, M.E. & BRIGGS, M.B. 1986. Feline coronavirus infections of cheetahs. *Feline Practice*, 16:21–30.

JANOSSY, G. & GREAVES, M.F. 1971. Lymphocyte activation. I. Response of T and B lymphocytes to phytomitogens. *Clinical and Experimental Immunology*, 9:483–498.

KHANSARI, D.N., MURGO, A.J. & FAITH, R.E. 1990. Effects of stress on the immune system. *Immunology Today*, 11:170–175.

LEAKEY, R.G. 1979. *The illustrated origin of species*. London: Faber & Faber.

MILLER-EDGE, M. & WORLEY, M. 1991. *In vitro* mitogen responses and lymphocyte sub-populations in cheetahs. *Veterinary Immunology and Immunopathology*, 28:337–349.

O'BRIEN, S., WILDT, D. E., GOLDMANN, D., MERRIL, C. & BUSH, M. 1983. The cheetah is depauperate in genetic variation. *Science*, 221:459–462.

O'BRIEN, S.J., ROELKE, M.E., MARKER, L., NEWMAN, A., WINKLER, C.A., MELTZER, D., COLLY, L., EVERMAN, J.F., BUSH, M. & WILDT, D.E. 1985. Genetic basis for species vulnerability in the cheetah. *Science*, 227:1428–1434.

O 'BRIEN, S.J., WILDT, D.E., BUSH, M., CARO, T., FITZGIBBON, C. & LEAKEY, R. 1987. East African cheetah: Evidences for two bottlenecks? *Proceedings of the National Academy of Sciences, USA*, 84:508–511.

PFEIFER, M., EVERMANN, J.F., ROELKE, M.E., GALLINA, A., OTT, R. & MCKEIRNAN, A. 1983. Feline infectious peritonitis in a captive cheetah. *Journal of the American Veterinary Medical Association*, 183:1317–1319.

PEDERSEN, N.C. 1988. *Feline infectious diseases*. California: American Veterinary Publications.

PEDERSEN, N.C. 1991. *Feline husbandry. Disease and management in the multiple-cat environment*. California: American Veterinary Publications.

SAMBROOK, J., FRITSCH, E.F. & MANIATIS, T. 1989. *Molecular cloning. A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.

SOUTHERN, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology*, 98:503–517.

SPENCER, J.A., JACOB, B.C. & SCHULTHEISS, W.A. 1991. The effects of a long acting corticosteroid and prostaglandin F2α on lymphocyte blast transformation in the cow. *Proceedings of the 8th Faculty Day, Faculty of Veterinary Science, University of Pretoria.*

TIZARD, I. 1987. *Veterinary immunology. An introduction*. 3rd edition. Philadelphia: W.B. Saunders: 96–98.

WAYNE, R.K., NORMAN, L., NEWMAN, A.K., MARTENSON, J.S. & O'BRIEN, S.J. 1986. Genetic monitors of zoo populations: morphological and electrophoretic assays. *Zoo Biology*, 5: 215–232.

WILDT, D.E., BUSH, M., HOWARD, J.G., O'BRIEN, S.J., MELTZER, D., VAN DYK, A., EBEDES, H. & BRAND, D.J. 1983. Unique seminal quality in South African cheetah and comparative evaluation in the domestic cat. *Biology of Reproduction*, 19:1019–1025.

YUHKI, N. & O'BRIEN, S.J. 1988. Molecular characterization and genetic mapping of class I and class II MHC genes of the domestic cat. *Immunogenetics*, 27:414–425.

YUHKI, N. & O'BRIEN, S.J., 1990. DNA variation of the mammalian major histocompatibility complex reflects genomic diversity and population history. *Proceedings of the National Academy of Sciences, USA*, 87:836–840.