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COMPARATIVE STUDIES OF MITOGEN- AND ANTIGEN-INDUCED LYMPHOCYTE PROLIFERATION IN FOUR CAPTIVE RHINOCEROS SPECIES

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Abstract: Cellular immune function in four rhinoceros species was evaluated by way of in vitro lymphocyte proliferation responses to mitogenic and antigenic stimuli to establish normative data on white blood cell activity for each species and to identify species-specific differences that might help explain the predisposition of black rhinoceroses (*Diceros bicornis*) to disease. A cross section of the U.S. rhinoceros population encompassing all four captive species was sampled, including the Sumatran rhinoceros (*Dicerorhinus sumatrensis*) ($n = 3$); Indian rhinoceros (*Rhinoceros unicornis*) ($n = 4$); African black rhinoceros ($n = 16$); and African white rhinoceros (*Ceratotherium simum*) ($n = 10$). Of the four species evaluated, African black rhinoceroses exhibited the weakest ($P < 0.05$) lymphocyte proliferative responses to the mitogens: pokeweed (0.1 μg/ml), phytohemagglutinin (0.3 μg/ml), and concanavalin A (5.0 μg/ml). Total cell density at the end of culture was only 70% of that achieved with lymphocytes isolated from African white rhinoceroses, Indian rhinoceroses, and Sumatran rhinoceroses. However, lymphocyte response to bacterial endotoxin lipopolysaccharide was similar ($P > 0.05$) across species. Antigenic stimulation produced much weaker responses than mitogenic stimulation. No differences ($P > 0.05$) were observed among rhinoceros species in response to 1 and 10 μg/ml of *Leptospira icterohemorrhagiae* or *Leptospira gryppotyphosa*. Lymphocytes from African white rhinoceroses proliferated weakly in the presence of *Aspergillus fumigatus* filtrate, whereas lymphocytes from the southern black rhinoceros subspecies appeared slightly suppressed in the presence of increasing doses (0.1, 1, and 10 μg/ml) of *Aspergillus* filtrate. This comparative data set characterizing lymphocyte proliferation in the rhinoceros reveals several differences in immune cell responses among rhinoceros species and provides some evidence that lymphocytes of captive African black rhinoceroses are less vigorous than those of the other rhinoceros species.

Key words: Black rhinoceros, white rhinoceros, Indian rhinoceros, Sumatran rhinoceros, immunology, disease, stress, corticoids.

INTRODUCTION

Although the imminent extinction of the African black rhinoceros (*Diceros bicornis*) has been averted by intensive in situ conservation efforts, continued management and propagation of captive populations are still considered important for ensuring its long-term survival. The black rhinoceros has been maintained in zoologic environments for decades, and reproduction has been more successful in this species than in the other three rhinoceros species in captivity.[8] However, the sustainability of captive black rhinoceroses is jeopardized by the frequent occurrence of ill-fated diseases and syndromes.[20,21,23–25,38] Typical disease manifestations, which are often fatal in captive African black rhinoceroses, have been notably absent from populations of free-ranging black rhinoceroses and the captive populations of African white rhinoceroses (*Ceratotherium simum*), Indian rhinoceroses (*Rhinoceros unicornis*), and Sumatran rhinoceroses (*Dicerorhinus sumatrensis*).

Fungal pneumonia is a rare infection in mammals and usually is associated with immunosuppression. Alarmingly, it has been associated with 25% of the black rhinoceros deaths between 1988 and 1994.[38] Hemolytic anemia has been implicated in 40% of all black rhinoceros deaths.[19,22] In approximately 50% of all cases involving red blood cell hemolysis, there have been concurrent *Leptospira interrogans* infections.[20] At least four captive eastern black rhinoceroses (*D. bicornis micheali*) have died in recent years from leukoencephalomalacia.[29] The genealogies of these particular animals reveal eight other family members with documented episodes of primary hemolytic anemia. In 1998, it was reported that approximately 50% of the black rhinoceroses in the United States had been affected by ulcerative dermatitis.[23] Finally, seven black rhinoceroses have

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exhibited idiopathic hemorrhagic vasculopathy syndrome (IHVS), a more recently described disease in rhinoceroses. Six of these seven were of the southern subspecies (*D. bicornis minor*), suggesting a subspecies or regional predisposition to it.[25]

Investigators have proposed several potential predisposing factors to the assortment of clinical disorders appearing in the North American captive black rhinoceros population. Early research efforts focused largely on red cell physiology and function[27,30,32,33] and the relationship to a nutritionally based chronic iron overload syndrome.[15,28] Specifically, it has been suggested that iron overload in black rhinoceroses exacerbates an already inherent sensitivity to oxidant-induced injury.[31] Chronic stress and subsequent adverse effects on the immune system should also be considered as predisposing factors to unusual disease manifestations in captive black rhinoceroses.

The immunocompetence of captive African black rhinoceroses has been an area of increasing interest; yet, very little is known about white blood cell (WBC) physiology and function in the Rhinocerotidae. Establishing general characteristics of immune cell function in rhinoceros species would lay the foundation for identifying potential breakdowns in the immune response and cell-mediated defense mechanisms that lead to disease in black rhinoceroses. Lymphocyte proliferation assays measure the ability of lymphocytes to rapidly divide before differentiation into functional effector cells.[13,39] These classical in vitro studies are well accepted as valid and reliable methods for evaluating immunocompetence. They are especially valuable when studying rare and endangered species because the assays do not require species-specific reagents and in vivo studies would be difficult and unlikely to be approved, even if risks to animal health were minimal. Furthermore, the application of in vitro lymphocyte proliferation assays offers several advantages over in vivo studies. First, when comparing cell function between populations, the environment is defined and consistent for cells from each individual. Second, specific mitogens or antigens that preferentially stimulate T or B cells can be chosen so that differential responsiveness between cell subsets can be determined. Finally, cells from a single blood sample can be divided into several treatments so that multiple studies can be conducted concurrently with minimal requirements for hands-on manipulations.

The general goal of this study was to compare in vitro lymphocyte proliferation responses to stimulation by several mitogens and specific antigens among four rhinoceros species. This study was designed to lay the foundation for testing the hypothesis that species-specific immune dysfunction in captive African black rhinoceroses is linked to disease susceptibility. Lymphocyte proliferation was compared first between the African black rhinoceroses and African white rhinoceroses. The study was then expanded to include the Sumatran and Indian rhinoceroses. Specific objectives were to 1) characterize and compare lymphocyte proliferative responses in representative populations of each rhinoceros species through stimulation with well-known mitogens, 2) measure lymphocyte proliferation in response to *Aspergillus* and *Leptospira* antigens in each rhinoceros species, and 3) evaluate responses of two black rhinoceros subspecies.

MATERIALS AND METHODS

Animals and sample collection

Captive rhinoceroses of all four species held in U.S. zoos and wildlife parks were studied. Animals ranged in age from 9 mo to 45 yr and were represented by 15 males and 17 females. The species and numbers of animals used are described in the experimental design of each study. Whole blood (2–20 ml) was obtained from each individual by the host institution staff and placed in ethylenediaminetetraacetic acid tubes to prevent coagulation. Blood was typically collected from the medial tarsal vein of white rhinoceroses, black rhinoceroses, and Indian rhinoceroses and from the ear or tail vein of the Sumatran rhinoceroses, using 25-ga butterfly catheters and syringes (Terumo Medical Corporation, Elkton, Maryland 21921, USA). Samples from collaborating institutions were sent overnight, lightly insulated on ice packs, to the Cincinnati Zoo's Center for Conservation and Research of Endangered Wildlife (CREW), where they were immediately processed.

Study 1: The mitogenic and antigenic responses of lymphocytes obtained from 10 southern black and 10 southern white rhinoceroses were compared to determine whether differences existed in cell-mediated immune function between these two African species. Blood samples were collected by nonstressful methods that included operant conditioning; thus, animals were selected based on the institution's ability to obtain samples. All samples were collected and processed during a 3-mo interval. Except for one young, unvaccinated calf, all black rhinoceroses in study 1 had been vaccinated against leptospirosis with Leptoferm-5 (Norden Laboratories, Lincoln, Nebraska 68502, USA) or Brativac-6 (Pfizer Animal Health, Cambridge, Massachusetts 02139, USA) annually or every 2–3 yr.

Blood was collected from these animals no less than 10 wk and not more than 26 mo since their last vaccination. There also were three unvaccinated adult white rhinoceroses in study 1; the other seven had been vaccinated at least once within a year of the study. All animals were considered healthy at the time of blood collection. However, one white rhinoceros suffered from chronic arthritis and one black rhinoceros had an extensive medical history that included two episodes of IHVS, chronic diarrhea, and upper respiratory infections.

Fecal samples were collected from each animal on the day of blood collection. Fecal samples were stored at −20°C until analyzed for corticosteroid metabolites by the Endocrine Research Laboratory at the Smithsonian Institute's Center for Conservation and Research.

Study 2: Study 2 expanded upon the work in study 1 by including all four rhinoceros species in captivity. Three samples were obtained from each study animal to control for the high variation that can occur in single-sample immune cell function measurements. The animals sampled were African black rhinoceros (n = 11 animals; 33 blood samples), African white rhinoceros (n = 6 animals; 18 blood samples), Indian/Nepali rhinoceros (n = 4 animals; 12 blood samples), and Sumatran rhinoceros (n = 3 animals; 9 blood samples). Six southern black rhinoceroses and six southern white rhinoceroses from study 1 were randomly chosen for extended participation in study 2. In addition, five eastern black rhinoceroses were included to provide both better species representation for black rhinoceroses and an opportunity for subspecies comparison between black rhinoceroses in study 3. Unfortunately, a limited number of Indian rhinoceroses and Sumatran rhinoceroses were available for the study because either few were trained for blood collection (Indian rhinoceros) or few exist in the United States (Sumatran rhinoceros). During a 2-yr period, blood (2–20 ml) was collected from each animal three times, with a minimum of 1 mo and a maximum of 1 yr between successive collections. All individuals in study 2 had been previously vaccinated against leptospirosis with Leptoferm-5 or Brativac-6. The only case in which blood was routinely obtained from an anesthetized animal was for an Indian rhinoceros undergoing bimonthly footwork, and lymphocyte responses of this animal were similar to those of unanesthetized Indian rhinoceroses. All other samples were obtained from unanesthetized animals that were trained to allow blood collection, and all animals were considered healthy when samples were collected. Single samples from a Sumatran rhinoceros and a black rhinoceros were split into two aliquots and used to establish matched cultures the day of collection and the day after collection after overnight storage in a refrigerator. Proliferation data for each culture at 72 hr were similar for both the fresh cultures and those established with cells stored overnight.

Study 3: This study was designed to compare lymphocyte responses of individuals representing different subpopulations of captive African black rhinoceroses. Lymphocyte responses of the two black rhinoceros subspecies, *D. b. micheali* (eastern black rhinoceros, n = 5) and *D. b. minor* (southern black rhinoceros, n = 6), were compared. These 11 black rhinoceroses were the same animals used in study 2. Although neither subspecies represents an exclusively healthy or unhealthy population, four of the five eastern black rhinoceroses were related to animals with documented episodes of primary hemolytic anemia and calves affected by fatal leukoencephalomalacia. However, all animals were considered in good health when blood was collected for the study. The six southern black rhinoceroses were those from study 2 and had no history of serious illness.

Cell counts and differentials

All blood samples were evaluated for total WBC and differential cell counts immediately upon arrival at CREW. For WBC counts, 20 μl of blood was diluted into a 2-ml Unopette (Becton-Dickinson, Rutherford, New Jersey 07417, USA) in which the red blood cells were lysed. The WBCs were counted using a hemocytometer, and all samples were counted in duplicate. For the differential counts, duplicate slides were spread with 2 μl of undiluted blood and allowed to dry before staining with Quick II Stain Diff (VWR Scientific, Atlanta, Georgia 30024, USA). After staining, slides were allowed to air dry and were examined under oil at ×100 magnification. A total of 100 or 200 WBCs were identified per slide.

Lymphocyte proliferation assays

Peripheral blood lymphocyte proliferation was quantified using Cell Proliferation ELISA BrdU kits (Roche Diagnostics, Indianapolis, Indiana 46250, USA). Each whole-blood sample was separately diluted 1:1 with Hanks' balanced salt solution (HBSS, Sigma Chemical Co., St. Louis, Missouri 63103, USA). Lymphocytes were isolated by density-gradient centrifugation in 15-ml conical tubes by layering 5 ml diluted blood over 3 ml of Ficoll-Paque PLUS (Amersham Biosciences, Piscataway, New Jersey 08855, USA) and centrifuging 35 min at 400 *g*. The buffy layer, suspended above the Fi-

coll gradient, was collected and washed twice with 10 ml of HBSS by pelleting the lymphocytes (600 *g*, 10 min). Finally, the lymphocytes were suspended in Rosewell Park Memorial Institute (RPMI) media (GIBCO BRL, Grand Island, New York 14072, USA) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, Utah 84321, USA), 0.3 mg/ml L-glutamine, and 1 U/ml penicillin–streptomycin. Cell concentration was determined using a Mackler chamber, and the lymphocytes were subsequently diluted to 2.0×10^6 cells/ml. Cell cultures of each blood sample were prepared in triplicate wells using 96-well flat-bottom tissue culture plates (Becton-Dickinson). Each well contained 1×10^5 cells (50 μl) plus 100 μl of RPMI media containing the required dose of mitogen or antigen. Lymphocytes were cultured with mitogens or *Leptospira* antigens for 72 hr and *Aspergillus* antigens for 96 hr, at 38°C in a humidified atmosphere of air containing 5% CO_2, before labeling with 5′-bromo-2′-deoxyuridine (BrdU) as per kit instructions. The plates were incubated an additional 22 hr, harvested by plate centrifugation as described in the Cell Proliferation ELISA BrdU kit, and read using an enzyme-linked immunosorbent assay reader at $\lambda = 490$ nm.

Mitogens and antigens

The four mitogens used to stimulate lymphocyte proliferation were phytohemagglutinin (PHA, Sigma), concanavalin A (Con A, Sigma), lipopolysaccharide (LPS, Sigma #L2654, *Escherichia coli* 026: B6), and pokeweed (Sigma). Mitogen concentrations required to optimize the lymphocyte proliferation response were determined by testing blood samples from Sumatran rhinoceroses ($n = 2$) and African black rhinoceroses ($n = 2$) against a titration of each mitogen, ranging from 0.1 to 5.0 μg/ml. Maximum lymphocyte proliferation occurred when PHA, Con A, LPS, and pokeweed mitogen (PWM) were at concentrations of 0.3, 5.0, 5.0, and 0.1 μg/ml, respectively, and optimal doses did not differ between species. Proliferation was greater at 72 hr than 96 hr, so all mitogen assays in all studies were incubated for 72 hr.

Immunologic memory is reflected by lymphocyte proliferation in response to specific antigens to which individuals have previously been exposed. Vaccination history is the most reliable way to record exposure of the study population to a particular antigen, and most rhinoceroses have been vaccinated with a leptospiral bacterin containing *Leptospira gryppotyphosa* and *Leptospira icterohemorrhagiae*, two serovars previously isolated from animals with acute hemolytic anemia. For this reason, crude protein preparations of *L. gryppotyphosa* and *L. icterohemorrhagiae* were evaluated for their ability to induce lymphocyte proliferation in the black rhinoceros compared with other rhinoceros species.

Bulk cultures of formalin-inactivated *L. interrogans* serovar *gryppotyphosa* and serovar *icterohemorrhagiae* were provided by Rollins Animal Disease Diagnostic Laboratory (Raleigh, North Carolina 27607, USA). Leptospires were pelleted by centrifugation at 20,000 *g* for 15 min at 4°C and washed twice with 10 ml of bovine serum albumin-polysorbate medium without albumin (National Veterinary Services Labs, Ames, Iowa 50010, USA). The organisms were suspended in 5 ml of HBSS and sonicated four times at 30-sec intervals. The suspension was passed through a 0.45-μm syringe filter (Corning, Corning, New York 14831, USA) and protein concentration determined by the Bradford method using a commercially available kit (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, California 94547, USA). Optimal protein concentrations were determined by titrating each antigen from 0.01 to 50 μg/ml and incubating lymphocytes for 3–5 days. Maximal lymphocyte proliferation occurred after 3 days in culture in response to concentrations of 50 and 30 μg/ml of *L. icterohemorrhagiae* and *L. gryppotyphosa*, respectively. However, because of the limited available quantities of these antigens and the minor reduction in proliferation induced by lower doses, 1 and 10 μg/ml were actually used in the study.

An *Aspergillus fumigatus* antigen–specific lymphoproliferative assay was tested because of the high incidence of *Aspergillus* spp. pneumonia associated with black rhinoceros mortalities. An incubation period of 5 days generated the best (albeit weak) proliferative response of lymphocytes in the presence of *A. fumigatus* cellular filtrate (Greer Laboratories, Inc., Lenoir, North Carolina 28645, USA). The concentrations tested were 0.1, 1.0, and 10 μg/ml, but a dose-dependent increase in cell proliferation was not readily apparent in the titration study.

Quantitative and statistical analysis

Lymphocyte proliferation requires the replication of cellular DNA. Incorporation of the labeled pyrimidine analog BrdU into the DNA of proliferating cells permits quantification of cell replication by spectrophotometric analysis. The response is quantitatively calculated as the ratio of the optical density (OD) in the colorimetric assay produced by the stimulated cells to the OD for control (nonstimulated) cells cultured in parallel. Hence, a response

of 1.0 is equal to cell proliferation in the nonstimulated sample. Mitogen-stimulated cell cultures for each blood sample were set up in triplicate wells on each of three separate plates, and the mean values of each set of triplicate were used in the database. Therefore, for each blood sample tested with mitogens, three values representing nine total wells were used for statistical analysis. For the antigen studies, individual blood samples were only tested on a single plate, also using triplicate wells that produced one mean value for statistical analysis. Data were analyzed using the statistics program Statview (version 5.0.1, MacIntosh statistical software package). Outlier data points for individual wells were identified and eliminated from the analysis if they were >2.5 SD from the population mean. Data are reported as species' means (±SEM), and statistical significance was determined at $P < 0.05$. Unpaired *t*-tests were used to determine differences in cell counts as well as mitogen and antigen responses between rhinoceros species or subspecies, and repeated-measures analysis of variance was used in study 2 to determine dose-dependent responses to concentrations of antigens. Fecal corticoid data were analyzed using the statistics program JMP (version 4, SAS Institute, Cary, North Carolina 27513, USA). The Kruskal–Wallis test was used to determine differences in corticosteroid means between species. A *P* value of <0.05 was considered significant.

RESULTS

Total cell counts and differentials

Complete WBC counts and hematocrits were performed on every sample both after shipment by the investigating laboratory as well as before shipment by the host institution. The mean WBC values obtained for each rhinoceros species in this study were within the range reported in the International Species Inventory System;[7] however, the variation was reduced, likely because of the consistency of methodology and technician. The mean WBC count for black rhinoceroses was lower ($P < 0.05$) than that for white rhinoceroses in samples shipped overnight. Whole blood obtained from black rhinoceroses contained <70% of the number of WBC found in white rhinoceroses, with a mean of only $6{,}470 \pm 430 \times 10^6$ cells/L compared with $9{,}320 \pm 450 \times 10^6$ cells/L for white rhinoceroses. A similar trend was noted when WBC counts were performed by the host institutions before overnight shipment. Although the interspecific difference was greater after shipping the blood overnight, even fresh samples from black rhinoceroses contained fewer ($P < 0.05$) WBCs than those from white rhinoceroses ($7{,}990 \pm 430 \times 10^6$ cells/L vs. $9{,}660 \pm 660 \times 10^6$ cells/L, respectively). Whereas storage and shipping can affect WBC stability in any species, the average decrease in the number of WBCs was three times greater ($P < 0.05$) in samples obtained from black rhinoceroses (23.1% decrease) than from white rhinoceroses (7.8% decrease). Blood samples obtained from three Indian rhinoceroses and all Sumatran rhinoceroses were evaluated for WBC count on the day of blood collection only. The mean WBC concentrations for Indian rhinoceroses and Sumatran rhinoceroses were $8{,}410 \pm 500 \times 10^6$ cells/L and $8{,}540 \pm 440 \times 10^6$ cells/L, respectively. In contrast, no differences ($P > 0.05$) were found between any of the rhinoceros species in the distribution of WBC types (percent neutrophils, lymphocytes, monocytes, eosinophils, and basophils).

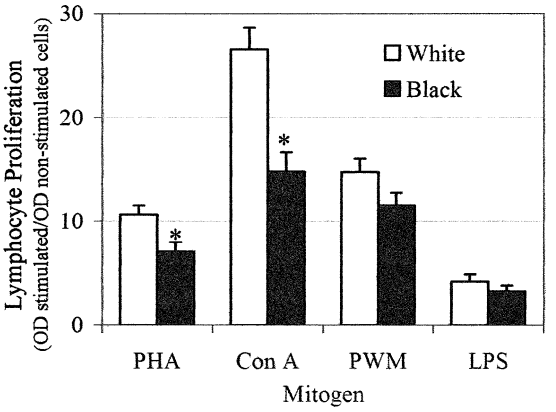

Figure 1. Mitogen-stimulated lymphocyte proliferation in captive southern black rhinoceroses ($n = 10$) and southern white rhinoceroses ($n = 10$). Mean responses (+SEM) for each species are shown, and the asterisk denotes differences ($P < 0.05$) between species. Lymphocyte proliferation is expressed as the ratio of OD values for stimulated vs. nonstimulated control cell cultures.

Study 1: African black rhinoceros and white rhinoceros lymphocyte responses to mitogen stimulation revealed significant differences between species (Fig. 1). Proliferation of lymphocytes from black rhinoceroses was inferior ($P < 0.05$) to that of lymphocytes from white rhinoceroses when stimulated with PHA or Con A. In the presence of either mitogen, lymphocyte proliferation was at least 50% greater for white rhinoceroses than for black rhinoceroses. In contrast, PWM-induced cell proliferation remained comparable ($P > 0.05$), although the mean response of lymphocytes from black rhinoceroses is only 75% of that for white rhinoceroses. Likewise, LPS affected lymphocytes

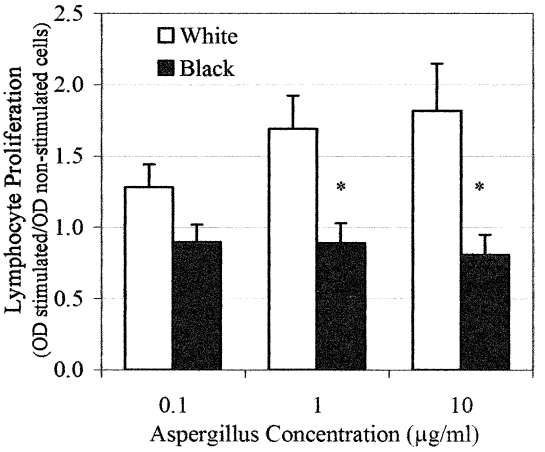

Figure 2. Lymphocyte proliferation in the captive southern black rhinoceros and southern white rhinoceros after exposure to three concentrations of *Aspergillus fumigatus* antigen. Species' means (+SEM) are shown, and the asterisk denotes differences ($P < 0.05$) between species. Lymphocyte proliferation is expressed as the ratio of OD values for stimulated vs. nonstimulated control cell cultures.

from both rhinoceros populations similarly ($P > 0.05$), with cells proliferating at 3.2–4.2 times that of the nonstimulated control.

A similarly weak ($P > 0.05$) proliferative response was induced by *Leptospira* antigens (*L. gryppotyphosa* and *L. icterohemorrhagiae*) in both rhinoceros species. Although the response was greater ($P < 0.05$) overall with higher antigen doses, within each antigen and species, there was no significant difference between doses ($P > 0.05$). At the lower dose (1.0 μg/ml) of *L. gryppotyphosa* and *L. icterohemorrhagiae*, the mean proliferation ratios were 1.2 ± 0.2 and 1.4 ± 0.3 for black rhinoceroses and 1.6 ± 0.2 and 1.4 ± 0.2 for white rhinoceroses, respectively. Mean proliferation ratios reached 2.8 ± 0.5 and 2.6 ± 0.5 for black rhinoceroses and 2.3 ± 0.5 and 2.2 ± 0.3 for white rhinoceroses in the presence of 10 μg/ml of *L. gryppotyphosa* and *L. icterohemorrhagiae*, respectively. Of the unvaccinated rhinoceroses, two (one white rhinoceros and one black rhinoceros) were less than 1 yr of age and exhibited lymphocyte responses near unity to both antigens. The remaining two unvaccinated animals were the oldest white rhinoceroses, and they also exhibited poor responses to *L. icterohemorrhagiae* (=1.0), yet relatively strong lymphocyte recognition of *L. gryppotyphosa* (>3.0), for both concentrations tested.

Higher concentrations of *A. fumigatus* antigen appeared to stimulate proliferation of lymphocytes from white rhinoceroses (albeit minimally) but not black rhinoceroses (Fig. 2). At the lowest concentration tested (0.1 μg/ml *A. fumigatus* filtrate), neither species appeared to respond. However, at higher concentrations of *A. fumigatus* filtrate, very low levels of cell proliferation were induced in the white rhinoceroses, but the black rhinoceros cells failed to respond. In fact, exposure to 1 and 10 μg/ml *A. fumigatus* appeared to suppress black rhinoceros mononuclear cells because proliferation never exceeded 90% of the nonstimulated control. Overall, the proliferation responses to *A. fumigatus* were negligible.

Corticoid metabolite levels were significantly higher in the feces from black rhinoceroses than white rhinoceroses ($P < 0.005$). Means for black rhinoceroses and white rhinoceroses were 92.08 ± 30.19 ng/g dry weight and 55.80 ± 17.73 ng/g dry weight, respectively.

Study 2: Of the four rhinoceros species studied, Indian rhinoceroses typically exhibited the greatest lymphocyte proliferative responses to all four mitogens tested, especially compared with those of the two African rhinoceros species (Table 1). African white rhinoceroses and Sumatran rhinoceroses exhibited intermediate levels of cell density after mitogen stimulation. In general, with the exception of LPS, African black rhinoceroses exhibited the weakest ($P < 0.05$) responses to mitogen stimulation, with cell density levels typically reaching only

Table 1. Comparison of mitogen-induced lymphocyte proliferation among four rhinoceros species.[a,b]

Species	PHA[c]	Con A	PWM	LPS
African black rhinoceros ($n = 11$)	$5.6^{a} \pm 0.4$	$10.5^{a} \pm 0.7$	$7.3^{a} \pm 0.5$	$3.6^{a} \pm 0.3$
African white rhinoceros ($n = 6$)	$7.9^{b} \pm 0.7$	$17.1^{b} \pm 1.3$	$9.7^{b} \pm 0.8$	$3.0^{a} \pm 0.3$
Sumatran rhinoceros ($n = 3$)	$12.4^{c} \pm 1.3$	$16.3^{b} \pm 1.9$	$10.8^{b} \pm 1.3$	$3.1^{a} \pm 0.3$
Indian rhinoceros ($n = 4$)	$13.5^{c} \pm 1.0$	$27.0^{c} \pm 2.5$	$17.8^{c} \pm 1.4$	$5.6^{b} \pm 0.6$

[a] Values are species' means (±SEM) calculated from triplicate blood samples from each individual and represent the ratio of the OD of stimulated cells to the OD of nonstimulated cells.

[b] Different superscripts within columns denote differences in mean values between rhinoceros species ($P < 0.05$).

[c] PHA, phytohemagglutinin; Con A, concanavalin A; PWM, pokeweed mitogen; LPS, lipopolysaccharide.

Table 2. Lymphocyte proliferation in four rhinoceros species in response to *Leptospira* antigens.[a]

Species	*L. gryppotyphosa*		*L. icterohemorrhagiae*	
	1.0 μg/ml	10 μg/ml	1.0 μg/ml	10 μg/ml
African black rhinoceros ($n = 11$)	1.3 ± 0.2	1.8 ± 0.3	1.2 ± 0.2	1.8 ± 0.2
African white rhinoceros ($n = 6$)	1.2 ± 0.2	2.1 ± 0.4	1.2 ± 0.1	2.4 ± 0.5
Indian rhinoceros ($n = 4$)	1.0 ± 0.3	1.7 ± 0.3	1.3 ± 0.2	2.6 ± 0.5
Sumatran rhinoceros ($n = 3$)	1.8 ± 0.4	2.3 ± 0.6	1.8 ± 0.2	2.2 ± 0.5

[a] Values are species' means (±SEM) calculated from duplicate blood samples from each individual and represent the ratio of the OD of stimulated cells to the OD of nonstimulated cells. All rhinoceroses in the study were previously vaccinated against leptospirosis.

75% of the levels achieved by the other three rhinoceros species.

Both Asian rhinoceros species exhibited greater ($P < 0.05$) PHA-induced lymphocyte proliferation than the two African species. For the other three mitogens (Con A, PWM, and LPS), the Sumatran rhinoceros and African white rhinoceros responses were similar ($P > 0.05$), whereas the Indian rhinoceros responses were consistently higher ($P < 0.05$), with proliferation at least 60% better than that measured for the other species. Lymphocytes from African black rhinoceroses exhibited the weakest response ($P < 0.05$) to PHA, Con A and PWM. The differences in African black rhinoceros and white rhinoceros lymphocyte proliferation after PHA and Con A stimulation were consistent with those noted in study 1. Moreover, study 2 revealed that black rhinoceros lymphocytes exhibit the weakest response to PWM ($P < 0.05$), whereas in study 1, this was not clearly evident ($P = 0.07$).

Despite the fact that all animals in study 2 were vaccinated against leptospirosis, lymphocyte proliferation responses to *L. icterohemorrhagiae* and *L. gryppotyphosa* antigens were again very weak. There were no differences among species, although the dose effect for both antigens appeared to persist (Table 2).

In contrast to study 1 results, cellular responses to three concentrations of *A. fumigatus* filtrate did not differ between African species ($P > 0.05$; Table 3). In fact, the only species that appeared to exhibit a weak response to the antigen at all three doses was the Sumatran rhinoceros ($P < 0.05$). There was no *A. fumigatus* dose-dependent increase in lymphocyte proliferation for any of the four rhinoceros species tested.

Study 3: A subspecies comparison of black rhinoceroses in study 3 revealed a notably weaker proliferative response to PHA by cells of the eastern vs. southern black rhinoceros ($P < 0.05$; Fig. 3). Although Con A–induced cell proliferation was similar between the two black rhinoceros subspecies, both responses were weaker ($P < 0.05$) than those for the other three rhinoceros species in study 2. Cell proliferation also was similar ($P > 0.05$) in the eastern and southern black rhinoceroses after PWM exposure. In contrast, proliferation induced by LPS was greater ($P < 0.05$) in the eastern black rhinoceros than in the southern black rhinoceros.

The apparent suppressive effect of *A. fumigatus* on southern black rhinoceros lymphocytes observed in study 1 was again noted in study 3 (Table 4) but still was not statistically significant. In contrast, the eastern black rhinoceros was not inhibited below nonstimulated control levels in the presence of *A. fumigatus*, and data for this subspecies more closely resembled that recorded in study 2 for African black rhinoceroses. Regardless, subspecies

Table 3. Comparison of lymphocyte proliferation in four rhinoceros species in response to three doses of *Aspergillus* antigen.[a]

Species	*Aspergillus*[b]		
	0.1 μg/ml	1.0 μg/ml	10 μg/ml
African black rhinoceros ($n = 11$)	1.1^{a} ± 0.1	1.2^{a} ± 0.1	1.0^{a} ± 0.1
African white rhinoceros ($n = 6$)	1.5^{ab} ± 0.2	1.5^{ab} ± 0.2	1.4^{a} ± 0.2
Indian rhinoceros ($n = 4$)	1.3^{ab} ± 0.2	1.4^{ab} ± 0.2	1.0^{a} ± 0.2
Sumatran rhinoceros ($n = 3$)	1.9^{b} ± 0.4	1.9^{b} ± 0.4	2.3^{b} ± 0.6

[a] Values are species means (±SEM) calculated from triplicate blood samples from each individual and represent the ratio of the OD of stimulated cells to the OD of nonstimulated cells.

[b] Different superscripts within columns denote differences in mean values between rhinoceros species ($P < 0.05$).

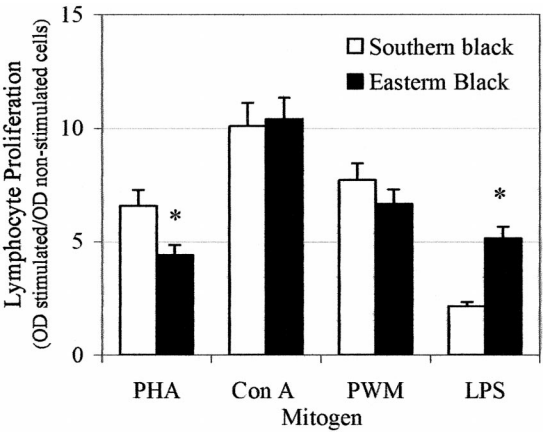

Figure 3. Mitogen-induced lymphocyte proliferation in eastern ($n = 5$) and southern ($n = 6$) African black rhinoceroses. Subspecies' means (+SEM) are shown, and the asterisk denotes differences ($P < 0.05$) between subspecies. Lymphocyte proliferation is expressed as the ratio of OD values for stimulated vs. nonstimulated control cell cultures.

did not differ ($P > 0.05$) in their responses. Statistically, the southern black rhinoceros subspecies' response to *A. fumigatus* was weaker than responses recorded for Sumatran rhinoceroses, white rhinoceroses, and Indian rhinoceroses in study 2; the eastern black rhinoceros response was similar ($P > 0.05$) to that of the white rhinoceros, but no rhinoceros species exhibited a strong response to *A. fumigatus*. Finally, no differences ($P > 0.05$) were detected between previously vaccinated eastern and southern black rhinoceros lymphocyte responses to *Leptospira* antigens (data not shown). The responses were similar ($P > 0.05$) to those reported for African black rhinoceroses in study 2.

DISCUSSION

This study provides the first comparative data set on rhinoceros lymphocyte proliferation, an important step in the immune system's response to disease. Information was generated from a cross section of the U.S. captive African black rhinoceros, African white rhinoceros, Indian rhinoceros, and Sumatran rhinoceros populations. These data are the first to demonstrate decreased T-cell responsiveness in the black rhinoceros compared with that of other rhinoceros species, a finding that might help explain this species' unusual predisposition to certain diseases.

Both T and B lymphocytes proliferate in response to antigen recognition. Specific antigens stimulate a very small, select subset of lymphocytes. In contrast, mitogens that originate from certain plants and bacteria induce mitosis and proliferation in large fractions (5–30%) of specific (B or T) lymphocyte subpopulations.[12,13,39] Con A and PHA are two plant-derived lectins that directly stimulate unprimed peripheral T lymphocytes to proliferate by binding nonspecifically to numerous plasma membrane surface glycoproteins.[39] Pokeweed mitogen is a plant lectin that preferentially stimulates B cells but also induces some T-cell reactivity, whereas LPS stimulates only B cells to proliferate.[12] Mitogen stimulation of lymphocytes in vitro, therefore, is used to evaluate general lymphocyte activity and detect differential proliferative responses in lymphocyte subpopulations.

Because the mitogen dose that effectively induces maximum stimulation can vary among species, initial trials focused on mitogen titration assays with cells collected from several rhinoceroses of different species. Mitogen concentrations required for eliciting the greatest proliferative response from rhinoceros lymphocytes (Con A, 5.0 μg/ml; PHA, 0.3 μg/ml; PWM, 0.1 μg/ml; LPS, 5.0 μg/ml) were approximately 30-fold lower for PHA and 10-fold lower for PWM but 10-fold higher for LPS than those required in the horse.[36] Surprisingly, results in these titration trials revealed little variation in dose response within or between individual rhinoceroses or species tested, so the doses eliciting optimal responses were held constant and used for all rhinoceroses across all species in these three studies.

Table 4. Comparison of lymphocyte proliferation between two subspecies of African black rhinoceroses in response to *Aspergillus* antigen.[a]

	Aspergillus		
Species	0.1 μg/ml	1.0 μg/ml	10 μg/ml
Southern black ($n = 6$)	0.9 ± 0.1	0.9 ± 0.1	0.7 ± 0.1
Eastern black ($n = 5$)	1.1 ± 0.1	1.4 ± 0.1	1.2 ± 0.2

[a] Values are species' means (±SEM) calculated from triplicate blood samples from each individual and represent the ratio of the OD of stimulated cells to the OD of nonstimulated cells.

When black rhinoceros and white rhinoceros lymphocytes were exposed to PHA and Con A in study 1, black rhinoceros T-cell proliferation was 30–50% lower than that achieved by white rhinoceroses. To rule out the possibility that white rhinoceros lymphocytes are unique in exhibiting higher responses to these mitogens, the work was expanded in study 2 to include all four rhinoceros species in captivity, and triplicate blood samples were tested from each animal to control for single-sample variation. Study 2 results were consistent with those in study 1 and provided further evidence that black rhinoceros T-cell responsiveness to mitogen stimulation is substantially less vigorous than that exhibited by the other three rhinoceros species. There are many factors that could cause decreased T-cell proliferation including impaired intracellular signaling mechanisms, insufficient energy transduction, or premature cell death. Support for the last factor exists in the cell count data that indicate a notable reduction in cell number during transit to the laboratories.

The black rhinoceros and white rhinoceros lymphocyte responses to LPS did not differ in study 1 or 2, indicating no species differences in B-cell proliferation. With the addition of more animals and repetitive sampling in study 2, black rhinoceroses had a decreased response to PWM compared with white rhinoceroses that were not statistically significant in study 1. Considering that PWM stimulates both B and T cells, the response to PWM in black rhinoceroses may reflect their diminished T cell reactivity rather than decreased B-cell proliferation. Among all four species, the Indian rhinoceros exhibited the strongest response to the mitogens, but with a sample size of just four animals, it would be premature to conclude that this is a species-specific trait. It is possible that some of the difference could be attributed to the fact that for three of the Indian rhinoceroses, cell cultures were set up the day of blood collection and not after a day in cold storage.

In addition to nonspecific mitogenic responses, antigen-specific immune function to *A. fumigatus* and *L. gryppotyphosa* and *L. icterohemorrhagiae* antigens was studied. Although fungal pneumonia from *Aspergillus* spp. infection has been reported in numerous black rhinoceroses, exposure of individuals and therefore the potential for acquired immunity to *Aspergillus* spp. are uncertain. In contrast, exposure to *Leptospira* is well documented because of vaccination histories, and memory responses would be expected when lymphocytes are cultured in vitro with leptospiral antigens.

Antigen-specific, proliferative responses typically are much weaker than mitogen-stimulated responses because only a small subset of cells contain specific receptors that recognize and respond to the antigen. However, the low responses in both black rhinoceroses and white rhinoceroses to *L. gryppotyphosa* and *L. icterohemorrhagiae* were somewhat disappointing. There are several possible explanations for this poor proliferative response. Antigens can vary in immunogenicity, and the specific antigens generated for these assays may not have been broadly recognized by specific receptors on the memory cells. In addition, because of the limited volume of antigen available, concentrations lower than what produced maximum proliferative responses were used. Although the doses tested provided a means to evaluate species differences, they may not have provided ideal conditions for measuring optimal responses. Finally, the efficacy of *Leptospira* vaccines has not been thoroughly tested in rhinoceroses. Although measurable increases in antibody titers have been detected after vaccination,[10] the extent to which the vaccine generates a cell-mediated response is not fully understood. However, since vaccination of black rhinoceroses against leptospirosis began in the early 1990s, hemolytic anemia, a presenting clinical feature of infection, has become very rare in the captive North American population.

Lymphocyte responses to *A. fumigatus* filtrate were even weaker than those to *Leptospira* antigens. This failure of cells to respond could be explained, at least in part, by the unknown history of *Aspergillus* spp. exposure among rhinoceroses. However, it is worth noting that *A. fumigatus* appeared somewhat suppressive to black rhinoceros cells in vitro in study 1, whereas white rhinoceros cells did proliferate in response to the higher *A. fumigatus* doses, suggesting a difference between species. When the two subspecies of black rhinoceros were compared in study 3, it was clear that the suppressive effect of *A. fumigatus* was only evident in the southern black rhinoceros subspecies. When southern and eastern black rhinoceros cell responses were combined in study 2, however, there were no differences between black rhinoceroses and white rhinoceroses or Indian rhinoceroses in response to *A. fumigatus* antigens in vitro.

Although an interesting observation, the subspecies variation in cellular response to *A. fumigatus* antigen is probably not significant. *Aspergillus* spp. pneumonia was associated with 25% of black rhinoceros deaths from 1988 to 1994,[38] but all these were eastern black rhinoceroses, the subspecies not apparently suppressed by *A. fumigatus* antigen. The eastern subspecies also exhibits a higher incidence

of leukoencephalomalacia and hemolytic anemia[28,29] than the southern subspecies, so the reduced response to *A. fumigatus* does not appear to be an indicator of increased, overall disease susceptibility in southern vs. eastern black rhinoceroses. In fact, data from the mitogen stimulation trials do not support the hypothesis that one subspecies is more compromised than the other. Both subspecies demonstrated similar lymphocyte proliferative responses to Con A and PWM, whereas southern black rhinoceroses exhibited a stronger T-cell response to PHA and eastern black rhinoceroses exhibited a stronger B-cell response to LPS.

Fecal corticosteroid levels were 65% higher in black rhinoceroses than white rhinoceroses in study 1. Although these represent single samples collected the day that blood was drawn, the data are consistent with previously published data showing a 30% increase in fecal corticoid concentrations in black rhinoceroses compared with white rhinoceroses when samples were collected weekly for an entire year.[1] Whether the corticoid concentrations of captive black rhinoceroses reflect a chronic stress response or normal physiologic values is not known because there are no comparative data from free-ranging animals. However, chronic stress has been shown to decrease leukocyte redistribution and to depress immune function.[3,4] Furthermore, corticosteroids are known to suppress T-lymphocyte responses to mitogens in certain species and individuals within a species.[2,14,40] Therefore, both the lower WBC count and reduced T-cell proliferation observed in black rhinoceroses compared with other rhinoceros species in this study are observations consistent with expected chronic, stress-induced effects on the immune system and also could facilitate the development of clinical aspergillosis.

The relationship between *Aspergillus* spp. pneumonia and immunocompromised individuals has long been recognized, but defining all elements involved and describing their roles have been difficult. Based on studies conducted in vitro and in vivo in the mouse, it appears that T cells play a critical role in the immune system's response to aspergillosis through both increased killer cell activity and interleukin production.[16,34,35] Although a depressed T-cell proliferative response was observed in this study, additional functional assays will need to be performed in multiple rhinoceros species before any general statement can be made regarding compromised immune function in black rhinoceroses.

The apparent iron overload syndrome occurring in black rhinoceroses continues to concern conservationists participating in the captive breeding program. Data suggest that eastern black rhinoceroses may exhibit higher serum iron and ferritin than the southern black rhinoceroses but that both groups are clearly exhibiting higher levels of iron than their wild counterparts, white rhinoceroses, Indian rhinoceroses, or equine species.[15,29] Iron and ferritin data from black rhinoceroses and white rhinoceroses in this study are in agreement with these earlier findings (data not shown). However, a direct cause and effect mechanism between high body iron and disease susceptibility has not been delineated. Although excess iron has been associated with diminished phagocytic and bactericidal activities of mononuclear and polymorphonuclear leukocytes in humans,[11,37] it is not clear whether high iron loads necessarily impair lymphoproliferative responses.[5,26] Of relevance to this study, one proposed mechanism for cellular iron toxicity is apoptosis of cells mediated by iron-catalyzed oxidative destabilization of lysosomes and subsequent release of destructive enzymes.[6] The unexpected substantial reduction in WBC in 24-hr-old vs. fresh black rhinoceros blood samples suggest that cell death may be occurring at an unusually rapid rate in this rhinoceros species, which would be consistent with oxidative injury. However, no data were collected during the study to support this speculative cause and effect theory.

A common link between excess iron, stress, and immune cell function could be the hypothalamic–pituitary–adrenal (HPA) axis. The immune system is integrally connected to the HPA axis through a series of complex biochemical and physiologic mechanisms. Whereas dysfunction of the HPA axis and resulting clinical disease has been documented in humans with hereditary hemochromatosis,[9,18] more relevant to the rhinoceros is the recent report of elevated cortisol concentrations in Africans with dietary iron overload.[17] Although unproven, the rhinoceros diet is the presumed source of iron contributing to high levels of iron in black rhinoceroses. The similar finding of elevated cortisol in populations of two species experiencing dietary iron overload is intriguing, but interpretation of corticosteroid levels is problematic in any species because both concentration and duration of a response are important in determining whether immune function may be enhanced or suppressed.[3] Reconciling the pathophysiology of excessive iron accumulation in black rhinoceroses with their perceived compromised immune function in captivity is daunting and so far has proven elusive. Despite a common link to the HPA axis, the pathogenesis of iron accumulation and stress-mediated immunosuppression is more likely temporally related rather than strictly

cause and effect. More work will be necessary to fully evaluate immune function in rhinoceroses before any definitive statements can be made regarding immune function deficiencies in the captive black rhinoceroses. The information generated from this study indicating that black rhinoceroses have depressed proliferative responses to T-cell mitogens and declining WBC numbers with increased storage time provides an important foundation for future investigations.

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