CORTICOSTEROID-INDUCED SUPPRESSION OF IN VITRO LYMPHOCYTE PROLIFERATION IN FOUR CAPTIVE RHINOCEROS SPECIES

Terri L. Roth, M.S., Ph.D., and Carrie K. Vance, M.S., Ph.D.

Abstract: Captive African black rhinoceroses (*Diceros bicornis*) are unusually susceptible to several diseases not commonly observed in any of the other three rhinoceros species maintained in captivity. The potential role of corticosteroids (either endogenously produced or exogenously administered) in the development of these sometimes fatal diseases has been questioned. In this study, the suppressive effects of two therapeutic corticosteroids (dexamethasone and hydrocortisone) on in vitro lymphocyte proliferation was examined in four rhinoceros species, including the Sumatran rhinoceros (*Dicerorhinus sumatrensis*, $n = 3$), Indian rhinoceros (*Rhinoceros unicornis*, $n = 4$), African black rhinoceros ($n = 10$), and African white rhinoceros (*Ceratotherium simum*, $n = 5$). Three blood samples collected from each rhinoceros 1 mo to 1 yr apart provided replicates for the study. Both dexamethasone and hydrocortisone suppressed ($P < 0.05$) lymphocyte proliferation stimulated by B-cell mitogens (pokeweed and lipopolysaccharide) and T-cell mitogens (phytohemagglutinin and concanavalin A). Suppressive effects of the glucocorticoids differed ($P < 0.05$) depending on the mitogen used to stimulate the lymphocytes. Overall, dexamethasone was a more potent suppressor of cell proliferation when compared with hydrocortisone ($P < 0.05$). However, black rhinoceros cell proliferation in response to any of the four mitogens was never completely suppressed, even in cultures containing the highest steroid concentration tested (10^{-3} M). The effect of the two corticosteroids differed slightly among the rhinoceros species and subspecies tested, but there was no evidence that eastern or southern black rhinoceros lymphocytes were more sensitive to the suppressive effects of corticosteroids than the other rhinoceros species.
Key words: Black rhinoceros, white rhinoceros, Indian rhinoceros, Sumatran rhinoceros, immunology, disease, dexamethasone, hydrocortisone.

INTRODUCTION

The African black rhinoceros (*Diceros bicornis*) has been one of the more difficult rhinoceros species to maintain in captivity because of its unusual susceptibility to several diseases that are absent or much less prevalent in the other three captive rhinoceros species. Hemolytic anemia was the leading cause of death in the 1980s, and approximately half the cases were associated with leptospirosis.[11,15] Most black rhinoceroses now are vaccinated for leptospirosis, and the incidence of hemolytic anemia has decreased in recent years.[15] The most common disease, superficial necrolytic dermatitis, has affected approximately 50% of the captive population[16,17] and can occur as either a primary or secondary condition. A more recently identified disease, idiopathic hemorrhagic vasculopathy syndrome, plagued seven rhinoceroses in the late 1990s[18] but has not been reported much in the past few years. In a few cases, individuals have survived this syndrome when treated aggressively with corticosteroid and fluid therapy.[4] Fungal pneumonia

From the Center for Conservation and Research of Endangered Wildlife, Cincinnati Zoo and Botanical Garden, 3400 Vine Street, Cincinnati, Ohio 45220, USA. Correspondence should be directed to Dr. Terri Roth (terri.roth@cincinnatizoo.org).

was associated with 25% of black rhinoceros deaths from 1988 to 1994, but all of these rhinoceroses had concurrent diseases, suggesting the pneumonia was secondary.[25] In several cases, fungal pneumonia was diagnosed after corticosteroid treatment for the primary illness.[25]

Several hypotheses currently are being tested regarding heightened disease susceptibility in black rhinoceroses. One hypothesis is that increases in corticosteroid concentrations in black rhinoceroses, either through exogenous therapeutic administration or endogenous release in response to environmental stimuli, predispose individuals to disease. In support of this hypothesis are recent reports correlating elevated fecal corticoid production with specific captive conditions, mortality, and disease.[6,7]

The underlying mechanism of stress-induced disease susceptibility is suppression of the immune system by chronic elevated circulating steroids. Fatal fungal infections are not uncommon in humans with high levels of endogenously produced corticosteroids or on corticosteroid therapy,[9,24] and the same likely holds true for the rhinoceros. Results from one recent study of immune cell function in the four captive rhinoceros species suggest that black rhinoceros T-cell proliferative responses are less vigorous than those of the other rhinoceros species when exposed to mitogens in an in vitro sys-

tem.[23] In the same study, serum corticoid levels were higher in the black rhinoceroses compared with corticoid levels measured in serum of the other rhinoceros species. Although one must be very conservative about extrapolating from in vitro immune cell studies to in vivo conditions, the in vitro system does provide a controlled environment ideal for a number of studies that would not be possible in vivo.

To further investigate the potential relationship between corticosteroids and immune cell suppression in the rhinoceros, in vitro lymphocyte proliferation studies were conducted with white blood cells collected from African black, African white (*Ceratotherium simum*), Indian (*Rhinoceros unicornis*), and Sumatran rhinoceroses (*Dicerorhinus sumatrensis*). The first study objective was to evaluate the suppressive effects of two commonly used therapeutic corticosteroids, dexamethasone and hydrocortisone, on mitogen-induced immune cell proliferation in vitro. The second objective was to determine whether the effects of the two glucocorticoids on B- and T-lymphocyte proliferation were predominantly attributed to just one cell subset. Finally, differences in the suppressive effects of these glucocorticoids on in vitro lymphocyte proliferation in the black rhinoceros compared with the other three rhinoceros species were examined.

MATERIALS AND METHODS

Animals and sample collection

Captive rhinoceroses maintained in U.S. zoos and wildlife parks were used in this study. Individuals were chosen on the basis of their current healthy status, the ability to obtain multiple blood samples from them, and the institution's willingness to participate in the study. A total of 22 rhinoceroses were included in the study, ranging in age from 2 to 33 yr and consisting of 10 males and 12 females. The breakdown by species and subspecies was: eastern black rhinoceros (*Diceros bicornis michaeli*, $n = 4$), southern black rhinoceros (*D. b. minor*, $n = 6$), white rhinoceros ($n = 5$), Indian rhinoceros ($n = 4$), and Sumatran rhinoceros ($n = 3$). Three blood samples were collected from each rhinoceros over a 2-yr period with a minimum of 1 mo and a maximum of 1 yr between successive collections. The blood collection technique was left up to each contributing institution. Typically, blood was collected from the medial tarsal vein of white, black, and Indian rhinoceroses and from the ear or tail vein of the Sumatran rhinoceroses with 25-gauge butterfly catheters and syringes. The blood (2–20 ml) was transferred into ethylenediamine tet-raacetic acid tubes and sent overnight, lightly insulated on ice packs, to the Cincinnati Zoo's Center for Conservation and Research of Endangered Wildlife (CREW), where it was immediately processed.

Lymphocyte proliferation assays

Peripheral blood lymphocyte proliferation was quantified by cell proliferation enzyme-linked immunosorbent assay BrdU kits (Roche Diagnostics, Indianapolis, Indiana 46250, USA) as described in detail previously.[23] Briefly, buffy layers were obtained from whole blood samples by density gradient centrifugation through Ficoll-paque PLUS (Amersham Biosciences, Piscataway, New Jersey 08855, USA). The buffy layer cells were washed twice with 10 ml of Hank's Balanced Salt Solution (Sigma Chemical Company, St. Louis, Missouri 63178, USA) and then suspended in 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 cultures of each blood sample were prepared in triplicate wells of 96-well flat-bottom tissue culture plates (Becton-Dickinson). Each well contained 1×10^5 cells delivered in 50 μl, plus 100 μl of RPMI media containing the appropriate dosage of mitogen and steroid. Lymphocytes were cultured for 72 hr at 38°C in a humidified atmosphere of air containing 5% CO_2, before labeling with BrdU for 22 hr and harvesting per kit instructions. The plates were read on an ELISA reader at $\lambda = 490$ nm.

Mitogens and corticosteroids

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 mitogen (PWM, Sigma). These mitogens were added to the cultures at the dosages previously determined to illicit optimal responses in rhinoceroses (0.3, 5.0, 5.0, and 0.1 μg/ml, respectively).[23]

Water soluble forms of two commonly used therapeutic corticosteroids, hydrocortisone (Sigma, H0396) and dexamethasone (Sigma, D2915), were tested for their ability to suppress lymphocyte proliferation. The corticosteroids were serially diluted in culture media, and aliquots were added to triplicate well sets to achieve final concentrations of 10^{-3}, 5×10^{-4}, 10^{-4}, 10^{-6}, 10^{-8}, and 10^{-10} M per well.

For each blood sample and each plate, several

controls were run in triplicate wells. One set of blank wells into which nothing was added provided a mean background value that the plate reader automatically subtracted from each well's value. A second set of triplicate wells contained cell cultures with no mitogen or steroid added. The mean value of this set of wells was used in confirming that the cells were not already stimulated by an active infection in the animal or contamination during processing before they were added to the cultures. Finally, a set of triplicate wells for each mitogen served as the stimulated controls. These wells contained the cells and mitogens but no steroid, and the mean values of these triplicates were used in calculating the level of suppression caused by the steroids.

Quantitative and statistical analysis

The suppressive effects of the steroids were quantitatively calculated as the ratio of the optical density (OD) in the colorimetric assay produced by the steroid-treated cell cultures to that of the OD for the stimulated control cells (with no steroid added) cultured on that plate. Hence, a response of 1.0 is equal to cell proliferation in the stimulated control sample and would indicate no suppression (or 100% of control proliferation), whereas a value of 0.5 would indicate that proliferation was reduced by half. Data were analyzed with the statistics program Statview (Statview 5.0.1 Macintosh statistical software package), in a repeated measures analysis of variance with the wells containing serially diluted steroid serving as the repeated measure. Main effects included species, steroid, and mitogen. Pairwise comparisons of significant main effects were conducted by Fisher's protected least significant difference test. Data are reported as $\bar{x} \pm$ SEM, and statistical significance was determined at $P < 0.05$.

RESULTS

Both dexamethasone and hydrocortisone suppressed in vitro lymphocyte proliferation across all rhinoceros species and mitogens tested (Fig. 1). Lymphocyte proliferation was suppressed most ($P < 0.05$) at the highest concentrations (10^{-3} M) of steroids, and proliferation increased ($P < 0.05$) with each subsequent dilution.

Dexamethasone was more powerful in suppressing lymphocyte proliferation compared with hydrocortisone ($P < 0.05$). Even at the highest hydrocortisone concentration tested (10^{-3} M), lymphocyte proliferation was approximately 24% of the stimulated control compared with just 7% in the presence of the same concentration of dexamethasone. Additionally, lymphocyte proliferation was
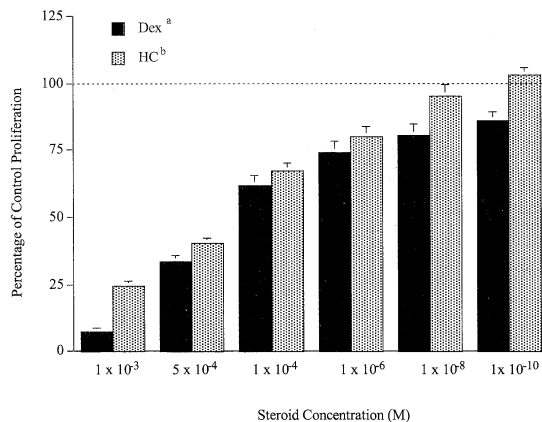

Figure 1. Mitogen-stimulated lymphocyte proliferation in cultures containing serial dilutions of dexamethasone (Dex) or hydrocortisone (HC). Data represent mean responses ($\bar{x} \pm$ SEM) across all rhinoceros species and all mitogens tested. Different superscripts denote differences in suppression between glucocorticoids ($P < 0.05$). Lymphocyte proliferation is expressed as the ratio of OD values for mitogen-stimulated, corticosteroid-treated cell cultures vs. mitogen-stimulated control cell cultures.

not suppressed ($P > 0.10$) in cultures containing 10^{-8} and 10^{-10} M hydrocortisone, whereas proliferation was suppressed ($P < 0.05$) in cultures containing those same concentrations of dexamethasone.

Steroid-induced suppression of lymphocyte proliferation was affected by the mitogen used to stimulate the cells, but there was no significant interaction between steroid treatment and mitogen, so dexamethasone and hydrocortisone data were pooled in Figures 2 and 3. When cells were stimulated with ConA, proliferation was suppressed only in cultures containing steroid concentrations of 10^{-4} M or higher. Similarly, when cells were stimulated with PWM, the level of proliferation was more than 80% of that for stimulated control cultures until steroid concentrations reached 5×10^{-4} M or greater. In contrast, when cells were stimulated with PHA or LPS, proliferation was suppressed, even at the most dilute concentrations of steroids (10^{-8} M and 10^{-10} M) compared with stimulated controls ($P < 0.05$), and proliferation decreased linearly with increasing concentrations of steroids. However, cell proliferation in response to any of the four mitogens was never completely suppressed, even in cultures containing the highest steroid concentration tested (10^{-3} M).

Because there were no significant species by steroid interactions, data for dexamethasone and hydrocortisone were pooled to examine interspecific
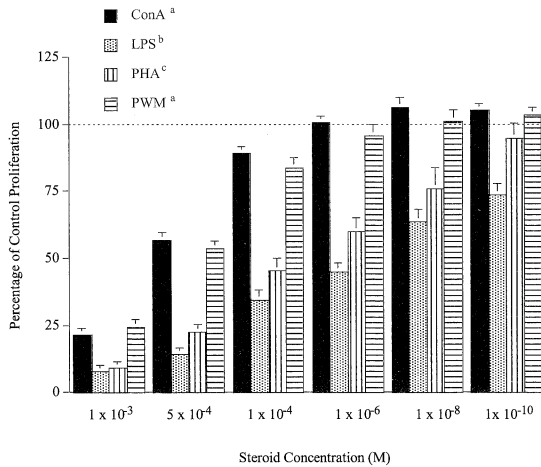
Figure 2. Cortocosteroid-induced suppression of rhinoceros lymphocyte proliferation stimulated by the four mitogens: concanavalin A (ConA), lipopolysaccharide (LPS), phytohemagglutinin (PHA), and pokeweed mitogen (PWM). Different superscripts denote differences in corticosteroid-induced suppression among mitogen treatments ($P < 0.05$). Lymphocyte proliferation is expressed as the ratio of OD values for mitogen-stimulated, corticosteroid-treated cell cultures and mitogen-stimulated control cell cultures.

differences within each mitogen (Fig. 3). Eastern black rhinoceros T-lymphocyte proliferative responses to PHA and Con A were greater than or equivalent to those in the other rhino species (Fig. 3a, b). Although the southern black rhinoceros cell response was not as robust as that exhibited by the eastern black rhinoceros ($P < 0.05$), it was similar ($P > 0.05$) to that of the Indian rhinoceros and white rhinoceros. In Con A–stimulated cultures containing the most dilute concentrations of glucocorticoids, Sumatran rhinoceros and eastern black rhinoceros lymphocyte proliferation appeared to increase above stimulated control levels. However, there was significant variation in the data that was at least partially responsible for this unanticipated result. Similarly, B-lymphocyte proliferation in response to PWM was greater than that for stimulated controls at the lower concentrations of glucocorticoids for the Sumatran rhinoceros and eastern black rhinoceros (Fig. 3d). Again, significant between-replicate and between-animal variation was observed in these data. Similar to their response to T-cell mitogens, lymphocytes of eastern black rhinoceros and southern black rhinoceros mounted a proliferative response to B-cell mitogens that was greater than or equivalent to that of the other rhino species (Fig. 3c, d). Only in the Indian rhinoceros cell cultures stimulated with LPS was

lymphocyte proliferation completely suppressed to control, nonstimulated levels at the highest concentrations of glucocorticoids.

DISCUSSION

The underlying cause of captive black rhinoceros disease susceptibility is unknown, but research is underway on several fronts in an effort to discover a cause-and-effect relationship between suspected predisposing factors. One working hypothesis receiving significant attention since 1992 is that captive black rhinoceroses suffer from iron overload, and this condition predisposes them to disease.[12,20] Clearly, high serum ferritin concentrations and iron deposition observed in most major organs at necropsy are conditions that appear to be specific for captive rhinoceroses in that studies of wild animals have not revealed the same.[12] However, the elevation in serum ferritin appears to occur very quickly in all black rhinoceroses brought into captivity, and many of these animals live relatively healthy, long lives, suggesting that iron storage disease alone might not be the culprit. Another evolving theory under investigation is a possible antiphospholipid syndrome. Preliminary data suggest that antibodies of phospholipids are elevated in captive versus wild black rhinoceroses.[2] Finally, several publications have reported higher corticosteroid concentrations in black rhinoceroses compared with the other rhinoceros species in captivity.[6,7,23] Therefore, a hypothesis gaining momentum is that stress-related increases in glucocorticoid production predispose black rhinoceroses to disease.

The immunosuppressive effects of glucocorticoids are numerous. They affect virtually every cell involved with immune and inflammatory responses, often by several mechanisms.[14] Perhaps one of the most rapid and pronounced physiological responses after glucocorticoid treatment is the redistribution of immune cells out of circulation to the organs, resulting in lymphopenia and monocytopenia.[3] However, glucocorticoids also act directly at the cellular level impairing effector cell function and thereby suppressing immune responses. By collecting blood samples from healthy rhinoceroses, isolating the white blood cells and then exposing the cells to glucocorticoids in the presence of stimulatory reagents in vitro, the effects of glucocorticoids on lymphocyte proliferation could be measured without interference from the confounding factors that occur in vivo when animals are treated with glucocorticoids.

Although both dexamethasone and hydrocortisone suppressed rhinoceros lymphocyte proliferation in vitro, there were some differences worth
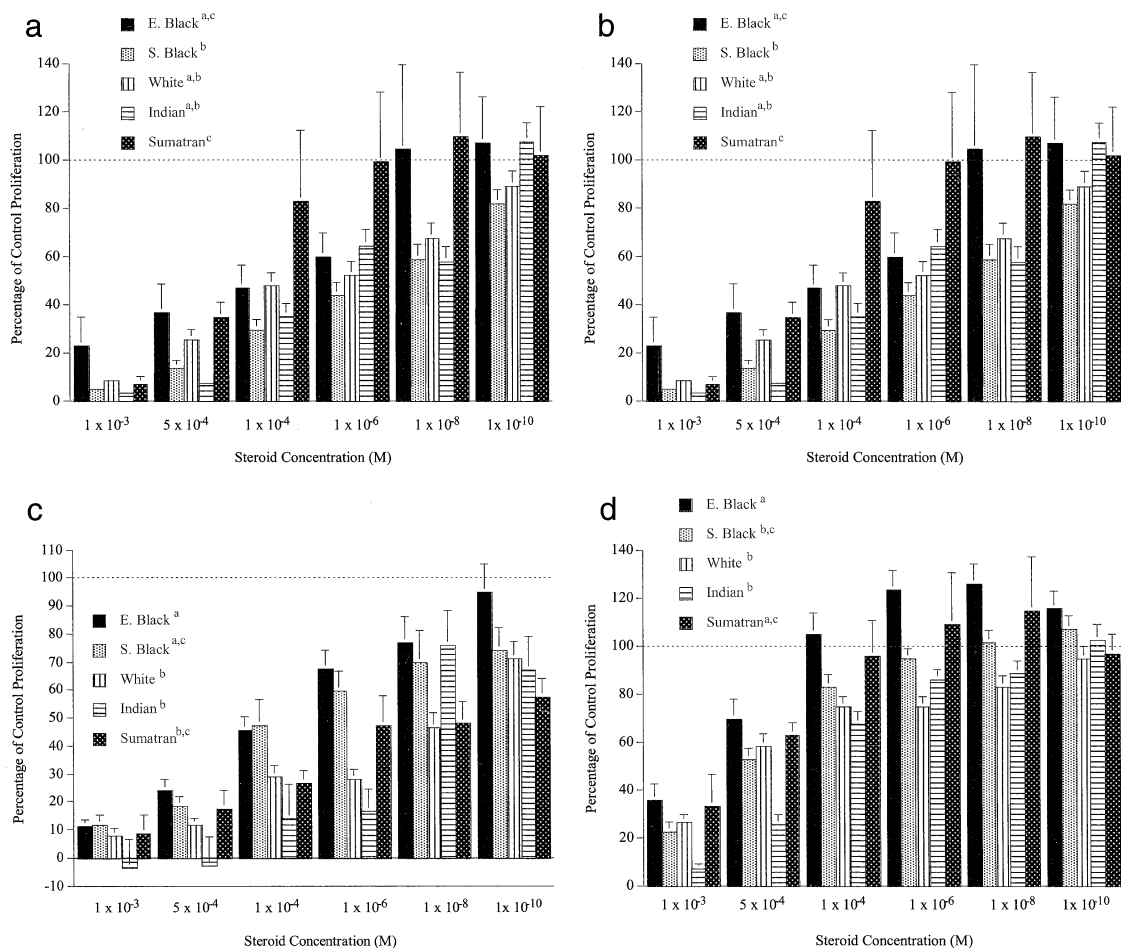
Figure 3. Glucocorticoid-induced suppression of rhinoceros lymphocytes stimulated by the mitogens **a.** phytohemagglutinin, **b.** concanavalin A, **c.** lipopolysaccharide, and **d.** pokeweed mitogen. Within each mitogen, different superscripts denote interspecific or subspecific differences in lymphocyte proliferation over the range of glucocorticoid concentrations tested ($P < 0.05$). Lymphocyte proliferation is expressed as the ratio of OD values for mitogen-stimulated, corticosteroid-treated cell cultures and mitogen-stimulated control cell cultures.

noting. In addition to being a more potent suppressor of lymphocyte proliferation overall, dexamethasone significantly suppressed lymphocytes even at the lowest concentration tested (10^{-10} M), whereas hydrocortisone was only suppressive at concentrations greater than 10^{-8} M. Therefore, treatment of rhinoceroses with low dosages of hydrocortisone might not affect lymphocyte proliferation significantly, whereas even very low dosages of dexamethasone could compromise this cell function. Unfortunately, the effect of prednisone, another commonly used corticosteroid, could not be tested because the compound was insoluble in an aqueous solution and the addition of organic solvents to the culture would likely have affected the cells, thereby confounding the results.

The suppressive effects of glucocorticoids on cell proliferation were not consistent across all mitogens tested. Studies on other species have demonstrated that T-lymphocytes are potently inhibited by glucocorticoids.[3,13,14] However, in this study, rhinoceros cell cultures stimulated with Con A (a T-cell mitogen) were the least inhibited. Only the highest concentrations of glucocorticoids reduced proliferation. In contrast, PHA-stimulated cultures were affected at much lower concentrations, and PHA also targets T-cells. Clearly, the type of stimulant used affects cell sensitivity to glucocorticoids. Similar findings have been reported in cows. In one study,[22] Con-A–stimulated lymphocytes from dexamethasone-treated cows proliferated at a level similar to that of controls. In another study,[1] PHA-stimulated

lymphocytes from dexamethasone-treated cows were suppressed compared with controls. Therefore, it seems like good practice to incorporate more than just one mitogen in studies designed to measure the suppressive effects of glucocorticoids on lymphocyte proliferation.

Although most reports on the effects of glucocorticoids focus on T-lymphocytes, B-lymphocytes are also affected. In this study, B-cell proliferation was similarly suppressed by glucocorticoids, and the response likewise varied depending on the mitogen used to stimulate the cells. Much like the T-cell response when stimulated with ConA, the B-cell response to PWM was significantly suppressed only at higher concentrations, whereas those cultures treated with LPS were suppressed even at the lowest concentration of glucocorticoid.

Regardless of the variations in glucocorticoid-induced suppression associated with the different mitogens tested, study results indicate that glucocorticoids suppress rhinoceros B- and T-lymphocyte proliferation in vitro. However, even at the highest concentrations of dexamethasone and hydrocortisone tested, cell proliferation was not completely suppressed all the way down to control levels (except possibly in the Indian rhinoceros), and such high concentrations of glucocorticoids should never be attained physiologically by a rhinoceros undergoing treatment because clinicians tend to use the prescribed dosage for horses as a guideline (1–4 mg/kg hydrocortisone,[21] 0.05–0.2 mg/kg dexamethasone; Package insert; Azium, Schering, Berlin, Germany).

Even within the human race, racial differences in T-lymphocyte responses to glucocorticoids has been documented. For example, 50% more dexamethasone is required to suppress PHA-stimulated lymphocytes in asthmatics of African descent compared with asthmatics of European descent.[8] Additionally, after renal transplants, larger concentrations of immunosuppressive reagents are required to treat individuals of African descent compared with individuals of European descent.[10] Therefore, it seemed logical to hypothesize that there would be interspecific differences in rhinoceros lymphocyte responses to glucocorticoids. Results from several studies have already shown that black rhinoceroses have higher concentrations of endogenous corticoids when compared with white rhinoceroses.[5,7,23] If black rhinoceros lymphocytes also express an increased sensitivity to glucocorticoids, the effect of steroids on immune function could be exacerbated and more readily lead to disease.

Although interspecific differences were revealed in this study, black rhinoceros lymphocytes did not appear to be more sensitive to the suppressive effects of glucocorticoids than those of the other rhinoceros species. In fact, proliferation of eastern black rhinoceros lymphocytes in response to all four mitogens was often greater than that for the southern black rhinoceros, Indian rhinoceros, and white rhinoceros, suggesting greater resilience to the suppressive effects of these two glucocorticoids. Although proliferation of glucocorticoid-treated lymphocytes from the southern black rhinoceros was not always as robust as that for the eastern black rhinoceros, glucocorticoid-induced suppression of the southern black rhinoceros lymphocytes consistently was equivalent to that of the white rhinoceros and Indian rhinoceros across all mitogens tested. The increase in proliferation exhibited by lymphocytes from eastern black rhinoceroses and Sumatran rhinoceroses in cultures containing PWM or Con A and the lower concentrations of glucocorticoids were at least partially the result of significant variation in the data. Data were examined closely in an effort to determine whether this variation was due to a particular animal or particular day or month the assays were run, but nothing consistent could be identified that might justify omitting a particular data set from the results.

In a previous study, mitogen-induced proliferation of T-lymphocytes from black rhinoceroses was reduced compared with that of the other rhinoceros species.[23] This study was designed to determine whether, in addition to being less responsive, black rhinoceros lymphocytes were also more sensitive to suppressive effects of glucocorticoids that might compound immune system effects during steroid treatments. Data herein suggest that black rhinoceros lymphocytes are not more susceptible to glucocorticoid-induced suppression than those of other rhinoceros species. Whereas these results represent another important step forward in studying immune function and its relevance to black rhinoceros diseases, interpretation of these data needs to be conservative. Although multiple blood samples from each rhinoceros were used, the numbers of Sumatran rhinoceroses and Indian rhinoceroses in the study were low because of their limited availability. Therefore, the interspecies comparisons with these two species should be considered preliminary only. Furthermore, although studying cell function in a controlled in vitro system has merit, one cannot assume the cells will act similarly in vivo when many other physiological processes are affected simultaneously. Therefore, it would not yet be appropriate to conclude that under glucocorticoid treatment, black rhinoceros lymphocyte proliferation is similar to that of other rhinoceroses. However, these data

do demonstrate that the inherent sensitivity of black rhinoceros lymphocytes to direct suppressive effects of glucocorticoids is not greater than those of other rhinoceroses.

A final point from a more applied perspective is to note that in this in vitro system, lymphocyte proliferation was significantly reduced with even the lowest concentration of dexamethasone tested (10^{-10} M), and suppression increased with higher concentrations. Rhinoceroses treated with a typical 10- or 20-mg dosage of dexamethasone[4,19] are almost sure to reach systemic concentrations well above the lowest concentration tested in this study. Therefore, one must carefully consider the benefits and potential drawbacks of reduced immunity and secondary disease susceptibility when treating rhinoceroses with glucocorticoids.

LITERATURE CITED

1. Anderson, B. H., D. L. Watson, and I. G. Colditz. 1999. The effect of dexamethasone on some immunological parameters in cattle. Vet. Res. Commun. 23(7): 399–413.

2. Ball, R. L., M. Morrow, M. Hofmeyr, P. Buss, and D. Cooper. 2006. Comparison of anti-phospholipid antibodies between wild and captive black rhinoceros (*Diceros bicornis*): implications for health and repatriation. Proc. Am. Assoc. Zoo Vet. 2006: 322–324.

3. Balow, J. E., D. L. Hurley, and A. S. Fauci. 1975. Immunosuppressive effects of glucocorticosteroids: differential effects of acute vs chronic administration on cell-mediated immunity. J. Immunol. 114: 1072–1076.

4. Bobko, C. M. 1996. Detection and treatment of a possible new disease syndrome in a captive black rhinoceros (*Diceros bicornis*). Proc. Am. Assoc. Zoos Aquar. Reg. Conf. 1996: 452–455.

5. Brown, J. L., A. C. Bellem, M. Fouraker, D. E. Wildt, and T. L. Roth. 2001. Comparative analysis of gonadal and adrenal activity in the black and white rhinoceros in North America by noninvasive endocrine monitoring. Zoo Biol. 20: 463–486.

6. Carlstead, K., and J. L. Brown. 2005. Relationships between patterns of fecal corticoid excretion and behavior, reproduction and environmental factors in captive black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses. Zoo Biol. 24: 215–232.

7. Carlstead, K., J. Fraser, C. Bennett, and D. G. Keiman. 1999. Black rhinoceros (*Diceros bicornis*) in U.S. Zoos: II. Behavior, breeding success, and mortality in relation to housing facilities. Zoo Biol. 18: 35–52.

8. Federico, M. J., R. A. Covar, E. E. Brown, D. Y. M. Leung, and J. D. Spahn. 2005. Racial differences in T-lymphocyte response to glucocorticoids. Chest 127(2): 571–578.

9. Graham, B. S., and W. S. Tucker. 1984. Opportunistic infections in endogenous Cushing's syndrome. Ann. Intern. Med. 101: 334–338.

10. Hricik, D. E., H. A. Anton, T. C. Knauss, V. Rodriguez, D. Seaman, C. Siegel, J. Valente, and J. A. Schulak. 2002. Outcomes of African American kidney transplant recipients treated with sirolimus, tacrolimus and corticosteroieds. Transplant. 74: 189–193.

11. Jessup, D. A., R. E. Miller, C. A. Bolin, M. D. Kock, and P. Morkel. 1992. Retrospective evaluation of leptospirosis in free-ranging and captive black rhinoceroses (*Diceros bicornis*) by microscopic agglutination titers and fluorescent antibody testing. J. Zoo Wildl. Med. 23: 401–408.

12. Kock, N., C. Foggin, M. Kock, P. Trembath, and D. Jessup. 1992. Hemosiderosis in the black rhinoceros (*Diceros bicornis*): a comparison of free-ranging and recently captured translocated and captive animals. J. Zoo Wildl. Med. 23: 230–234.

13. Langhoff, E., K. Olgaard, and J. Ladefoged. 1987. The immunosuppressive potency in vitro of physiological and synthetic steroids on lymphocyte cultures. Int. J. Immunopharmacol. 9: 469–473.

14. Lionakis, M. S., and D. P. Kontoyiannis. 2003. Glucocorticoids and invasive fungal infections. Lancet 362: 1828–1838.

15. Miller, R. E. 1993. Hemolytic anemia in black rhinoceroses. *In:* Fowler, M. E. (ed.). Zoo and Wild Animal Medicine, 3rd ed. W. B. Saunders Co., Philadelphia, Pennsylvania. Pp. 455–458.

16. Munson, L., J. W. Koeler, J. E. Wilkinson, and R. E. Miller. 1998. Vesicular and ulcerative dermatopathy resembling superficial necrolytic dermatitis in captive black rhinoceroses (*Diceros bicornis*). Vet. Pathol. 35: 31–42.

17. Munson, L., and R. E. Miller. 1999. Skin diseases of black rhinoceroses. *In:* Fowler, M. E., and R. E. Miller (eds.). Zoo and Wild Animal Medicine, 4th ed. W. B. Saunders Co., Philadelphia, Pennsylvania. Pp. 551–555.

18. Murray, S., N. P. Lung, T. P. Alvarado, K. C. Gamble, M. A. Miller, D. E. Paglia, and R. J. Montali. 1998. Idiopathic hemorrhagic vasculopathy syndrome in seven black rhinoceroses. J. Am. Vet. Med. Assoc. 216: 230–233.

19. Ott, J. E., S. E. McDonald, P. T. Robinson, and F. H. Wright. 1982. Ulcerative stomatitis in a black rhinoceros (*Diceros bicornis*). Proc. Am. Assoc. Zoo Vet. Annu. Meet. 1982: 68–71.

20. Paglia, D. E., and P. Dennis. 1999. Role of chronic iron overload in multiple disorders of captive black rhinoceroses (*Diceros bicornis*). Proc. Am. Assoc. Zoo Vet. Annu. Meet. 1999: 163–171.

21. Robinson, N. E. 1987. Table of common drugs: approximate doses. *In:* Robinson, N. E. (ed.). Current Therapy in Equine Medicine, 2. W. B. Saunders Co., Philadelphia, Pennsylvania. Pp. 761.

22. Thanasak, J., R. Jorritsma, A. Hoek, J. P. T. M. Noordhuizen, V. P. M. G. Rutten, and K. E. Muller. 2004. The effects of a single injection of dexamethasone-21-isonicotinate on the lymphocyte functions of dairy cows at two weeks post partum. Vet. Res. 35: 103–112.

23. Vance, C. K., S. Kennedy-Stoskopf, A. R. Obringer, and T. L. Roth. 2004. Comparative studies of mitogen- and antigen-induced lymphocyte proliferation in four captive rhinoceros species. J. Zoo Wildl. Med. 35(4): 435–446.

24. Wajszczuk, C. P., J. S. Dummer, M. Ho, D. H. Van Thiel, T. E. Starzl, S. Iwatsuki, and B. Shaw. 1985. Fungal infections in liver transplant recipients. Transplant. 40: 347–353.

25. Weber, M., and R. E. Miller. 1996. Fungal pneumonia in black rhinoceros (*Diceros bicornis*). Proc. Am. Assoc. Zoo Vet. Annu. Meet. 1996: 34–46.