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Vaccine-induced protection against anthrax in cheetah (*Acinonyx jubatus*) and black rhinoceros (*Diceros bicornis*)

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Abstract

Institution of a policy of vaccination in endangered species with a vaccine not previously administered to it cannot be undertaken lightly. This applies even more in the case of cheetah (*Acinonyx jubatus*) with their unusually monomorphic gene pool and the potential restrictions this places on their immune responses. However, the recently observed mortalities from anthrax in these animals in the Etosha National Park, Namibia, made it imperative to evaluate vaccination. Black rhinoceros (*Diceros bicornis*), another endangered species in the park, have been vaccinated for over three decades but the effectiveness of this has never been evaluated. Passive protection tests in A/J mice using sera from 12 cheetahs together with enzyme immunoassay indicated that cheetah are able to mount seemingly normal primary and secondary humoral immune responses to the Sterne 34F2 live spore livestock vaccine. Overall protection rates in mice injected with the sera rose and fell in concert with rises and declines in antibody titres, although fine analysis showed that the correlation between titre and protection was complex. Once a high level of protection (96% of mice 1 month after a second booster in the cheetahs) had been achieved, the duration of substantial protection appeared good (60% of the mice 5 months after the second booster). Protection conferred on mice by sera from three of four vaccinated rhino was almost complete, but, obscurely, none of the mice receiving serum from the fourth rhino were protected. Sera from three park lions with naturally acquired high antibody titres, included as controls, also conferred high levels of protection. For the purposes of wildlife management, the conclusions were that vaccination of cheetah with the standard animal anthrax vaccine causes no observable ill effect in the animals and does appear to confer protective immunity. At least one well-separated booster does appear to be desirable. Vaccination of rhino also appears to be justified from the limited data obtained.

Keywords: Cheetah; Rhinoceros; Anthrax

1. Introduction

The susceptibility of cheetah (*Acinonyx jubatus*) to anthrax was recently noticed in the Etosha National Park, Namibia [1]. While a policy of hands-off management is generally in place in national parks, being an endangered species, cheetah qualify for directed control measures such as, in this case, vaccination.

The lack of genetic diversity in cheetah is well recognised [2,3]. It has been proposed, albeit with some divergence of opinion [4–6], that this is the result of a bottleneck in their recent evolutionary history. Corresponding to this monomorphism is a singular lack of variation in the major histocompatability complex (MHC) genes in the cheetah as a species, reflected in failure to reject allografts [2]. MHC gene products play a key role in how an animal mounts an immune response to an infectious disease agent and, although the evidence from serology for a number of infectious agents, microparasites and viral vaccines points to individual cheetahs mounting differing responses [4], institution of a policy of vaccination of these animals with a vaccine not previously administered to them cannot be undertaken lightly. It was felt, therefore, that, in the case of anthrax, the value of administering the existing animal vaccine needed to be assessed scientifically.

Black rhinoceros (*Diceros bicornis*) are also an endangered species; the additional danger anthrax poses to these

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animals has long been recognised in the Etosha National Park [7] and vaccination campaigns have been carried out since the 1970s. However, the effectiveness of vaccination has never been monitored and, furthermore, vaccination is done by means of drop-out darts leaving it uncertain whether a dose, or complete dose has been delivered.

This paper describes work primarily aimed at evaluating the effect of vaccinating cheetah against anthrax but with reference also to assessing the merits of the existing vaccination policy for black rhinoceros in the park.

2. Materials and methods

2.1. Locations of the work

A total of 12 cheetahs were involved in the study (Table 1). These were located at the AfriCat Foundation, Otjiwarongo, Namibia. Vaccinations and test bleeding were carried out there.

Lion sera were obtained from the serum bank in the Etosha Ecological Institute, Etosha National Park, Namibia. The black rhinoceros are free-roaming in the Etosha National Park. Serology and passive protection studies were carried out in the Central Veterinary Laboratory, Windhoek, Namibia.

2.2. Cheetah and vaccinations

Of the 12 cheetahs included in the study, 9 received a single dose (1 ml containing 10^7 cfu of spores) of live spore livestock (Sterne strain 34F2) vaccine (Onderstepoort Biological Products, South Africa) on 9 September 2000. Five of these were re-vaccinated 11 and 12 months later. Serum samples were collected at zero time and 1 and 2 months after dose 1 and then again at the times of doses 2 and 3 and 1, 2 and 5 months after dose 3 (Fig. 1). Three new cheetah were added to the study at the 11-month point so that their first and second vaccinations were administered at the same time as the second and third doses of the five previously vaccinated animals.

2.3. Black rhinoceros and vaccinations

Four rhinos with an uncertain overall vaccination history, but with definite records in 1998 and 1999 of vaccination by drop-out darts delivering 2 ml of the Onderstepoort vaccine, were immobilised in May 2000 for blood collection. One unvaccinated animal was also bled.

2.4. Serology

Following the first vaccination of the study on 9 September 2000, the sera collected from the initial group of nine cheetahs at zero, 1 and 2 months were examined by a conventional ELISA procedure for antibodies to protective antigen (PA) and lethal factor (LF). Coating concentrations (75 μl per well) were 5 μg/ml in PBS and, for duplicate tests, high pH carbonate coating buffer. The rhino sera were similarly examined by conventional ELISA.

Following the vaccinations of the second group of eight cheetahs, an inhibition ELISA procedure [8] was used for the greater confidence in specificity it afforded under field conditions. Those sera still available from the initial group of nine animals were re-tested. Antigen coating concentrations were 5 μg/ml PA or 7.5 μg/ml LF in carbonate coating

Table 1
Histories of the cheetah included in the study

Cheetah ID	Sex	Age at 09/01 (years)	Antibody titre before vaccination		Captivity at AfriCat (years)	History before arrival at AfriCat
			Anti-PA	Anti-LF		
AJ2/01	Male	2	128	128	0.5	Siblings; wild caught as 7-month cubs. Spent 13 months in captivity in Windhoek area with unrelated cheetahs
AJ48/00	Female	2	Negative	32	1	
AJ47/00	Male	2	32	64	1	
AJ70[a]	Male	6.5	8	32	6	Wild caught as 4-month cub. Spent 2 months in veterinary clinic in Otjiwarongo
AJ79	Male	6.5	na	4	6.5	Siblings; wild caught as 2-month cubs. Came straight to AfriCat
AJ80	Male	6.5	16	16	6.5	
AJ81	Female	6.5	na	na	6.5	
AJ82[b]	Female	6.5	32	16	6.5	
AJ302	Male	5.5	256	64	3	Origins unknown; spent 16 months at game dealer's in Okahandja area
AJ303	Male	12.5	32	16	3	
AJ12/99	Male	3.5	256	64	2.5	Wild caught as 1-year cub in Gobabis area
AJ279	Female	4	64	32	3	Wild caught as 8-month cub in Steinhausen area. Spent 10 days at place of capture in cage in farm garden

na: not available, insufficient serum for test.
[a] Euthanised June 2002 (bone cancer).
[b] Euthanised February 2002 (broken leg that failed to heal).

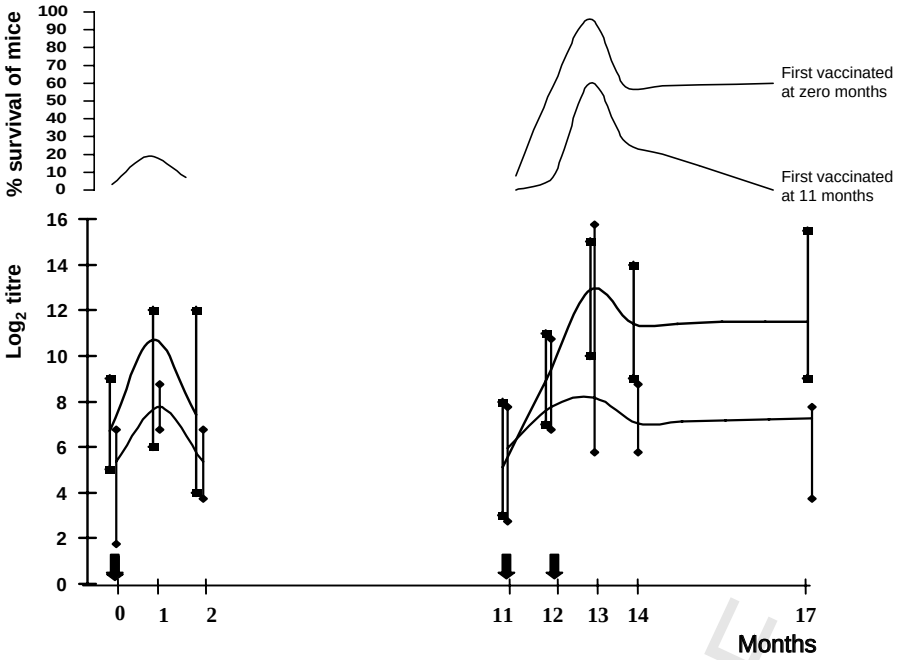

Fig. 1. Titres of antibodies to the protective antigen (PA, ■) and lethal factor (LF, ◆) components of the anthrax toxin in sera from cheetahs vaccinated with the Sterne 34F2 vaccine (lower curves) and protection conferred by these sera on A/J mice (upper curves). Arrows indicate vaccination dates. Bars indicate ranges of titres in the cheetah sera at each time point.

buffer (pH 9.4), 50 μl per well. The plates were held in a refrigerator overnight and washed with phosphate buffered saline containing 0.5 ml/l Tween-20 (PBST); 150 μl PBST containing 10% (w/v) dehydrated skim milk (Difco) (PBSTM) were then added to each well and the plates left at room temperature for approximately 1 h. After washing with PBST, two rows of wells were used for each test. In the first row (test line of wells), 50 μl PBSTM were dispensed into each well with an extra 25 μl in the first well. The wells in the second row (inhibition line of wells) each received 50 μl of PBSTM containing the antigen at 7.5 μl/ml for PA and 10 μg/ml in the case of LF. Again an extra 25 μl was added to the first well. Twenty-five microlitres of the serum being tested, pre-diluted where necessary, were added to the first wells of each row followed by serial doubling dilutions to the ends of the rows. The plates were incubated (37 °C) for approximately 1 h before washing, addition of conjugate (1:2000 in PBSTM) and, after further incubation (30 min) and washing, subsequent addition of substrate ABTS (Kirkegaard and Perry Laboratories, MD, USA). The reactions were read after a 40 min incubation period at 37 °C.

Antibodies to cheetah and lion immunoglobulins being unavailable, the ELISAs were performed using conjugated feline antibodies (goat anti-cat IgG-Fc, Bethyl Laboratories, Inc). In the case of the rhino, conjugated horse antibodies were used as the relation alternative.

2.5. Passive protection tests

(The housing and handling of test animals was done in accordance with the National Code for the Handling and Use of Animals in Research, Education, Diagnosis and Testing of Drugs and Related Substances in South Africa, Public Services Department of the National Zoological Gardens of South Africa, Pretoria, South Africa, 1990).

After an initial check with two mice to confirm tolerance to the foreign sera, and following the procedure described previously [9], 0.5 ml volumes of the sera were injected intraperitoneally into A/J mice (Harlan UK Ltd., Oxfordshire). The aim was to use five mice per serum sample, but in a few instances with the initial group of nine cheetahs, four or three mice were used because of shortage of serum.

As near as possible to 24 h later, each mouse received a subcutaneous injection of Sterne 34F2 vaccine strain *B. anthracis* spores prepared by washing past-expiry date vaccine batches 42 and 318 (Onderstepoort Biological Products, South Africa) with sterile deionised water. As assessed by viable spore counts, mice passively immunised with sera from the initial group of nine cheetahs following a single dose of vaccine at the outset of the study received 1.75×10^6 spores. This was higher than had been intended and, in the later set of challenge tests after the 18-month point, the mice received 3×10^5 spores. The same spore preparation was used for both sets of tests and had shown no significant loss of viability in the intervening 18 months in the refrigerator. The rhino sera were tested at the same time as the initial group of cheetahs and the recipient mice also received 1.75×10^6 spores.

Positive controls took the form of sera from a horse that had been repeatedly vaccinated (13 times) in 1960s and 1970s with the Sterne 34F2 livestock vaccine (from the former Burroughs-Wellcome, Beckenham, Kent or the then

Central Veterinary Laboratory, Weybridge, UK) over a period of several years and a goat that had received purified PA together with the Ribi Adjuvant System (Corixa Corp., Seattle, WA, USA) at 0, 1 and 6 months. Sera from three Etosha lions were also included. These were expected from previous experience [10] to have high titres of naturally acquired antibody to PA and LF and subsequently this proved to be the case.

Serum from an unvaccinated goat constituted a negative control in addition to the zero-time sera from the cheetah and the serum from the unvaccinated rhino.

Over a 12-day observation period, deaths in the mice were confirmed by culture with diagnostic 'gamma' phage and penicillin sensitivity testing as being due to the infecting *B. anthracis.*

2.6. Statistics

Differences in protection of A/J mice by sera from cheetah at different time points were analysed by Chi-squared tests using 2×2 contingency tables, as were differences in protection conferred by sera from the five cheetah which had had been vaccinated three times when compared with sera from the three animals that had been vaccinated twice. Significances of differences in anti-PA and anti-LF titres were analysed by Student's two-tailed *t*-test for means of small samples. Regression analysis to assess correlation between protection conferred on the mice and anti-PA and anti-LF titres in the cheetah sera was done using the Statlets package on http://www.statlets.com.

3. Results

3.1. Reactogenicity to the vaccine

The cheetah tolerated the vaccine well and showed no signs of adverse reactions to the immunisations. There were similarly no records of adverse reactions in the rhinos.

3.2. Antibody titres in the cheetahs

While anti-PA ranges of several titration units were seen among the cheetahs at every sampling time, including zero time serum samples, post-vaccination trends were apparent from a comparison of the means at each sampling time (Fig. 1, lower curves). Following the single dose of vaccine at the commencement of the study, a rise in mean titer was apparent after 1 month followed by a fall back to zero time levels at 2 months. Subsequent boosters 11 and 12 months later resulted in a rise in titres to higher levels which then fell to a steady and apparently persisting level. These trends are quite similar to the pattern reported in vaccinated humans [8].

Mean anti-LF titres followed a path parallel to the anti-PA titres but at lower titration values (Fig. 1). Again, however, ranges among individual animals were quite wide at all sampling times.

3.3. Protection conferred on A/J mice by the cheetah sera

The overall protection rates in the mice rose and fell in concert with the rises and falls of the mean anti-PA and anti-LF antibody titres in the cheetah sera (Fig. 1). Survival rates in the mice receiving sera from the first group of nine cheetahs 0, 1 and 2 months after the single dose of vaccine at the beginning of the study were 2, 19 and 7%, respectively (Fig. 1, top left). In the five cheetahs from this group still available a year later, overall protection rates following doses 2 and 3, administered 11 and 12 months after dose 1 respectively, rose to a high of 96% at 1 month after dose 3, falling to 58% a month later. Five months after dose 3, the last test point in the study, the proportion of protected mice was still 60% (Fig. 1, top right, Table 2).

With the three cheetahs brought into the study at the time of administration of dose 2 to the initial five animals, the overall mouse survival rate of 7% at 1 month compared with 19% at the equivalent time point for the nine cheetahs the year before. Following their second dose 1 month later, protection conferred by the sera from these three cheetahs had risen to 60% at the end of another month but then fell to 27% over the month after that and to zero by the end of the study 3 months later (Fig. 1, top right, Table 2).

All the mice receiving the negative control goat serum died within 48 h of challenge. All the mice that had received the positive control horse and goat sera survived the 12-day observation period.

3.4. Cheetah antibody titre versus conferred protection

Although Fig. 1 gives the impression of a good correlation between anti-PA and anti-LF titres and the degrees of protection, finer analysis revealed that the correlations were less clear-cut. This is apparent in Table 2 where it can be seen that, from the time of the last dose, the protection conferred by the initial five cheetah sera was significantly greater than that conferred by the sera from the three animals added to the study 11 months later, while mean anti-PA and anti-LF titres in the two groups did not show correspondingly significant differences. On the other hand, regression analyses on the numbers of mice surviving in relation to titre (Fig. 2) showed an 80% correlation coefficient between protection of the mice and anti-PA titre in the cheetah sera (but only a 50% correlation coefficient between protection and anti-LF titre). As assessed on the basis of mouse groups showing total protection (no deaths in the group), it was not possible to identify anti-PA or anti-LF titres in the cheetah sera that were predictive of certain survival in the mice.

The three lions which were included had naturally acquired anti-PA titres of >1:16,400, 1:32,800 and 1:65,600 (the first being a conversion estimate from standard ELISA to inhibition ELISA titre) conferring protection on,

Table 2
Comparison of anti-PA and anti-LF titres in sera from the two groups of cheetahs with differing vaccination histories and of the passive protection conferred by these sera in A/J mice

Vaccination 11 months before	Zero time (time of dose 1)			1 month (time of dose 2)			2 months			3 months			7 months		
	Mean $\log_2$ titer		Mice which lived (%)	Mean $\log_2$ titer		Mice which lived (%)	Mean $\log_2$ titer		Mice which lived (%)	Mean $\log_2$ titer		Mice which lived (%)	Mean $\log_2$ titer		Mice which lived (%)
	PA	LF		PA	LF		PA	LF		PA	LF		PA	LF	
Yes	5.2	5.2	8	9.5	5.5	58	12.3	8.6	96	11	6.5[a]	58	12.5	6.5	60
No	5	7	0	8.3	6.5	7	13.7	5.7	60	12	8.3[a]	27	10.3	6.6	0
Significance	NS	NS	NS	NS	NS	$P < 0.01$	NS	NS	$P < 0.01$	NS	$P < 0.05$	$P = 0.05$	NS	NS	$P < 0.001$

[a] Although there is a significant difference, the difference is inverse to what would be anticipated; the 'yes' group would be expected to have a higher mean titre than the 'no' group. This is probably an artifact but conceivably could result from a neutralisation effect in the more highly immunised group.

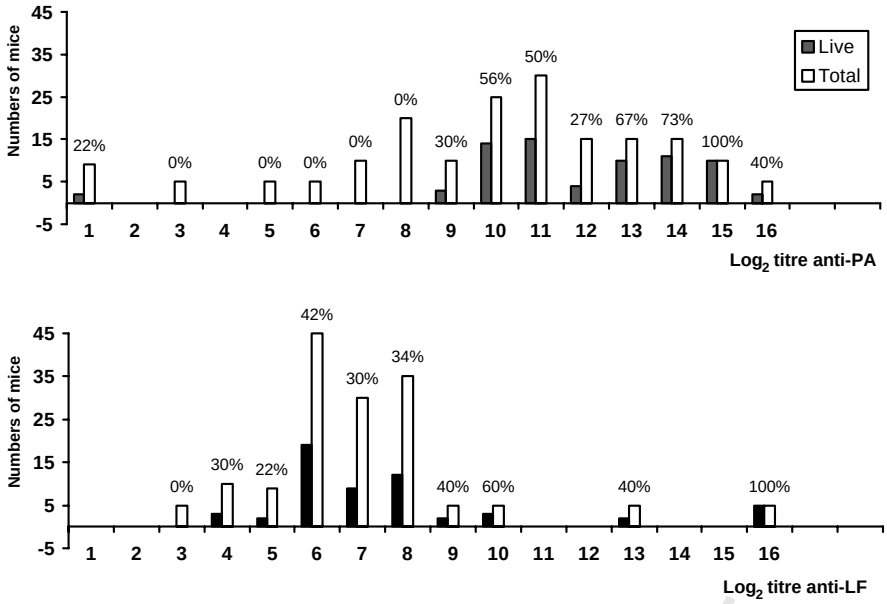

Fig. 2. Anti-PA (upper histogram) and anti-LF (lower histogram) titre in the cheetah sera vs. overall survival in the recipient A/J mice. Each bar represents the number of mice receiving serum with that particular titre.

Table 3
Immunisation histories and test results for the black rhinoceros

Black rhinoceros ID	Vaccination(s)			Titre (reciprocal)		Surviving A/J mice
	May 1998	September 1999	Months since last dose	Anti-PA	Anti-LF	
DB4			7	200	100	8/10
DB30F			7	400	200	5/5
DB30M			7	800	400	5/5
DB42			7	400	200	0/3
DB11			na	<50	50	0/4

na: not applicable.

respectively, 60, 60 and 100% of passively protected mice. The titre in the positive control goat serum was not known and could not be tested as anti-goat conjugate was not available. The titre in the positive control horse serum was ≫1:32,800. In that they utilised different reagents, it would have been difficult to relate the goat and horse titres to those of the cheetahs in any precise manner.

3.5. Antibody titres and passive protection with the rhino sera

Sera from three of the vaccinated rhino conferred protection on 80–100% of the mice (Table 3). No protection was conferred by serum from the fourth rhino despite having antibody profiles in line with those of the other vaccinated animals.

4. Discussion

Recent concerns over human anthrax vaccines [11] have led to an intense search for markers of protection. The need for a reliable passive protection model was a consequence of this. It has been known from the first half of the twentieth century that protective immunity to anthrax can be transferred with serum from immune animals [9,12–15] suggesting that antibodies are the fundamental elements of immunity to anthrax. Although mice have been used frequently in the study of vaccine-induced immunity in anthrax, it is a common experience that they are unsatisfactory in protection studies. Anthrax vaccines induce immunity to the toxin complex of *B. anthracis*, particularly the PA component, and anomalous results frequently obtained in protection studies have been attributed to interference by the bacterium's capsule [9,16]. The dose-dependent susceptibility of A/J mice to tox^+/cap^- strains, such as the Sterne 34F2 and Russian STI vaccine strains [9,17] overcomes this and has supplied a valuable system for passive protection studies [9]. It has the added advantage of not requiring fully virulent *B. anthracis* for the challenge.

The protective effect of a single dose of strain 34F2 vaccine is said to last about 1 year [18] and annual boosters are recommended for livestock in endemic areas. In a study on antibody levels to PA in vaccinated zebra in the Etosha

National Park [19] it was evident that two initial doses approximately 8 weeks apart were necessary for development of dependably measurable antibody titres and the decline in titre by 1 year after the second booster indicated that would be the time to administer a booster. However, the duration of actual protection induced by the livestock vaccine has never been systematically studied in laboratory animals or livestock either directly or by means of a passive protection study. Thus, the sensitivity of the adoptive immunity test used in the present study has not been determined and no algorithm exists yet for converting degree of protection in the mice into degree of protection in the donor animal. Altogether, therefore, apart from the limited data emerging from the simultaneous tests done on the rhino (see below), there is nothing at present with which to compare the performance of the vaccine in the cheetahs, or the cheetahs' response to it, to the performance and response in "normal" polymorphic species.

The choice of 0.5 ml as the volume of passively transferred serum with challenge 24 h later, although based on a previous study [9] was empirical. The extent to which altering the volume or delivering it as purified immunoglobulin could enhance sensitivity is undetermined. Similarly, how the sensitivity of the test might be enhanced by altering the time and size of the challenge dose administered to the mice is also not known. However, there was no obvious difference in the performance of the test with the two challenges doses used (1.75×10^6 spores after the initial vaccination and 3×10^5 spores after the boosters). In that the innate defence system of the recipient mice will destroy the foreign serum as rapidly as it can, it seems reasonable to infer that 100% protection in the mice probably indicates substantial protection in the donor animal(s). Protection levels significantly less than 100% in the mice may still indicate that the donor animal would survive the type of challenge that it is likely to encounter in the field, but this will remain speculative until further information is available.

It has been frequently noted that titres of antibodies to the toxin components, anti-PA in particular, are not, per se, predictors of protection levels even though there is a strong association between the presence of anti-PA antibodies and protection (reviewed in [9]) and though also, for a given immunogen/host combination, it may be possible to establish titres which will predict protection [20]. The anomaly was again apparent here when the five cheetahs vaccinated three times were compared with the three animals brought into the study at 11 months and only vaccinated twice (Table 2). A significant difference was found between the protection conferred by the former as compared with the latter while there were no significant differences in anti-PA and anti-LF titres. On the other hand, correlation coefficients of 80 and 50% between protection of the mice and, respectively, anti-PA and anti-LF titres in the cheetah sera indicated a positive correlation between protection and at least anti-PA titre. Anti-PA or anti-LF titres, or combinations thereof, that were predictive of certain protection were not found.

In in vitro cultures of *B. anthracis* PA and LF are produced simultaneously but in the ratio of approximately 1:5 [21]. This may reflect the in vivo situation (although this has not been established) and perhaps explain why the anti-LF titres were so much lower than the anti-PA titres. There are few data on the antibody response in animals to the live spore vaccine. In one study [8], the mean anti-LF titre in guinea pigs vaccinated with Sterne strain spores was two titration units lower than the anti-PA titre, but, perversely, in those immunised with spores of the analogous live Russian STI vaccine strain, mean anti-LF titres were two titration units higher than anti-PA titres. The assumption is made both in that paper and this one that, in using the same coating concentrations of the two antigens and otherwise identical test conditions, anti-PA and anti-LF titres are directly comparable. This may, or may not be valid, or may be only partially valid. Also PA and LF have similar molecular weights; purification of one completely free of the other was always difficult and is now done by using mutant strains lacking one or other of the relevant genes. However, the antigens used here and in the 1986 study were derived from the unmutated Sterne strain, although purification procedures will have been refined in the period between the two studies. Overall, interpretation of the anti-PA/anti-LF differences seen here awaits information from better laboratory models.

The rise and fall of antibody titres in line with what would be expected in any vaccination programme indicate that the use of anti-cat conjugate for the cheetah sera was valid. The titres obtained with the lion sera using anti-cat conjugate were comparable with those obtained using anti-lion Igs previously [10]. It is probably legitimate to compare the titres in the cheetah and lions directly. Similarly, the anti-horse conjugate apparently worked well with the rhino sera.

The natural acquisition of anthrax-specific antibodies in lions and other carnivores in the Etosha National Park has been detailed elsewhere [10]. In the light of that, the positive, if generally low antibody titres (Table 1) in the cheetahs at the times of their first vaccinations may be significant. The unreliability of ELISA at low titres is well-known, although, in theory, the inhibition ELISA should be reliable from the lowest titre at which the criteria for a positive—three consecutive dilutions in which the ODs differ by $\geq$20%—become apparent. A comparison of titre and histories of the animals (Table 1) does not rule out the possibility of past exposure to the disease in these animals. In terms of protection conferred on the mice, there was no obvious difference in protection induced by naturally acquired antibodies in the lions and that induced by the livestock vaccine in the cheetah, rhino and horse or by the purified PA vaccine in the goat.

In livestock, the recommended route of administration of the animal vaccine is subcutaneous [22]. However, in wildlife, vaccination is frequently done by dart gun, and therefore is intramuscular. With this realization, although the initial doses in the cheetahs were given subcutaneously, the three cheetahs being vaccinated for the first time at the 11-month point received the vaccine intramuscularly and

then all doses at the 12-month point were administered intramuscularly. No obvious divergences on the rising titres or levels of transferred protection resulted from this change in procedure.

Although not a major part of this study, the results obtained with sera from the black rhinoceros (*D. bicornis*) are included for the extra data they supply. Had all the mice receiving the serum from DB42 lived (Table 3), the conclusion might have been that vaccine appeared to perform better in the rhino than in the cheetah. This then might have been discussed in the light of the immune system of the cheetah as related to its special genetic characteristics as referred to in the introduction. As it is, it can only be concluded that the cheetahs did mount an apparently normal immune response to the vaccine, although more than one dose of vaccine was required to induce a substantial protective immunity.

In terms of recommendations for wildlife management, vaccination of cheetah with the standard animal anthrax vaccine causes no observable ill effect in the animal and does appear to confer protective immunity. The manner in which the vaccinations were given in this study do not permit the recommendation of a precise schedule, but they do show that at least one booster is desirable. The most logical time for this would be 2 months or more after the first dose when the protection from the primary dose has fallen to baseline levels and then probably annually after that.

With a black rhinoceros population estimated to exceed 700 animals spread throughout the park, regular vaccination of these animals in Etosha would be impractical and prohibitively expensive. However, the limited data from this study appear to justify the implementation of dart vaccination when the need arises, as when there is the threat of an impending epidemic.

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