

IMMUNIZATION AGAINST BOVINE BESNOITIOSIS WITH A LIVE VACCINE PREPARED FROM A BLUE WILDEBEEST STRAIN OF *BESNOITIA BESNOITI* GROWN IN CELL CULTURES. 1. STUDIES ON RABBITS

R. D. BIGALKE⁽¹⁾, J. H. SCHOEMAN⁽¹⁾ and R. M. McCULLY⁽²⁾

ABSTRACT

BIGALKE, R. D., SCHOEMAN, J. H. & McCULLY, R. M., 1974. Immunization against bovine besnoitiosis with a live vaccine prepared from a blue wildebeest strain of *Besnoitia besnoiti* grown in cell cultures. 1. Studies on rabbits. *Onderstepoort J. vet. Res.* 41, 1-6 (1974).

The immunizing potency of a live vaccine prepared from a strain of *Besnoitia besnoiti* isolated from blue wildebeest and stored frozen as a low passage stabulate was studied in adult male rabbits. The vaccine consisted of parasites that were grown in primary lamb kidney or an established line of green monkey kidney cell (Vero) cultures and suspended in serum-free Hanks' or Eagle's medium respectively. Freshly prepared doses containing from 1×10^5 to 2×10^6 parasites of the 14th to 34th culture passages were injected subcutaneously. The blue wildebeest strain was pathogenic in rabbits at all the dosage levels tested and 24,6 per cent died as result of vaccination.

The survivors were challenged one to six months after vaccination by subcutaneous inoculation with a bovine strain of *B. besnoiti* grown in lamb or Vero cells from a low passage level frozen stabulate. Immunized rabbits were protected against challenge doses that were 10 to 100 000 times greater than those of the vaccine. Immunized rabbits did not show any evidence of infection, whereas all the controls developed typical reactions (patent parasitaemia, scrotal oedema, orchitis) and 58 per cent died. It was therefore not possible to calculate an ID_{50} for vaccinated and control rabbits in this investigation, but one experiment revealed that more than 5 logs of protection had been induced by the vaccine.

Rabbits injected with Vero cells only were not protected.

INTRODUCTION

Bovine besnoitiosis is a disease that is widespread in the cattle ranching areas of South Africa (Pols, 1960; Bigalke, 1968). Although it does not have a high mortality rate (Pols, 1960), it is nevertheless of considerable economic importance as it has a high morbidity (Bigalke, 1968) and affected bulls often become sterile (Pols, 1960).

Investigations aimed at finding a method of controlling the disease have failed to provide a satisfactory answer. Hitherto no drugs have been found which will either cure a clinical case or prevent a reaction from occurring if administered during the incubation period of the disease (Pols, 1960). The only prophylactic method which offers any hope of success, namely the elimination of chronically infected carrier cattle (Bigalke, 1968), would probably have to be applied on a country-wide scale to be effective and would therefore be very costly. The development of an effective vaccine therefore seems to be the only way to protect cattle against this disease.

An opportunity to develop a live vaccine arose after the discovery of *Besnoitia* cysts in blue wildebeest and impala (Basson, Van Niekerk, McCully & Bigalke, 1965). Contrary to expectations it was found that these strains of *Besnoitia besnoiti* were not only of low virulence to cattle, but also induced immunity to challenge with bovine strains in cattle and rabbits (Bigalke, Van Niekerk, Basson & McCully, 1967). Moreover the antelope strains could be grown in cell culture.

The blue wildebeest strain of *B. besnoiti* was selected for vaccination purposes. An extensive investigation was launched which involved laboratory trials in rabbits and cattle, as well as field investigations in cattle.

Rabbits have been found to be excellent models for studies on *B. besnoiti* of bovine (Pols, 1960; Bigalke, 1967) and antelope origin (Bigalke *et al.*, 1967). Their

use in this study served two purposes: to furnish preliminary information on the immunizing potency of the blue wildebeest strain of *B. besnoiti*, and to provide results for later comparison with those obtained in cattle inoculated with vaccine prepared from the same batch of parasites. The results of the experiments conducted on rabbits are reported here; those in cattle will be reported separately.

MATERIALS AND METHODS

Rabbits

Mature male rabbits bred at this institute were utilized in six experiments. They were caged singly and fed a balanced concentrate ration.

Cell cultures

Two cell types were used, namely primary lamb foetal kidney cells and green monkey kidney (Vero) cells. They were grown either in 500 ml McCartney roller bottles or in Roux flasks. Hanks' solution containing 0,5 per cent lactalbumin hydrolysate, 10 per cent bovine serum and 100 units penicillin and 100 micrograms streptomycin per ml was used as nutrient medium in all experiments involving the use of lamb kidney cells, whereas Eagle's medium containing serum and antibiotics as above was employed with Vero cells.

Blue wildebeest strain of *B. besnoiti*

The strain was isolated in rabbits from blue wildebeest in 1965 (Bigalke *et al.*, 1967) and serially passaged 77 times in this laboratory host by subinoculation of blood taken at the height of the clinical reaction. It was isolated in lamb kidney cultures by seeding them with a suspension prepared from the spleen collected aseptically from a moribund rabbit. The parasites were multiplied by serial passage in lamb kidney cultures (Bigalke, 1962) and stored frozen at -76°C as a glycerinated stabulate at the seventh passage.

Counts of parasites

Haemocytometer counts were conducted as described by Bigalke (1968). Only refractile organisms with a typical merozoite form were counted.

⁽¹⁾ Veterinary Research Institute, Onderstepoort
⁽²⁾ Colonel, USAF, VC, Geographic Zoonoses Branch, Armed Forces Institute of Pathology (AFIP), Washington, D.C. 20306

Preparation of vaccines

Two of the batches of vaccine used in this investigation were produced from parasites grown in confluent monolayers of lamb kidney cells and the other three from organisms grown in Vero cells.

The glycerinated stabilate was used to infect cell cultures on five occasions and the parasites multiplied by several passages in fresh cultures (Bigalke, 1962) until sufficient living parasites were present. They were then harvested, together with the associated cells, and suspended in an appropriate volume of ice cold serum-free Hanks' (lamb kidney cells) or Eagle's medium (Vero cells), to give the required number of parasites per vaccine dose of 1.0 ml. The vaccine was administered immediately.

For control purpose a sixth batch of vaccine, containing Vero cells only, was prepared in Eagle's medium and injected in 1.0 ml doses.

Immunization

With parasites grown in lamb kidney cultures. The two batches of vaccine for Experiments 1 and 2 contained 1×10^4 and 2×10^5 parasites per dose and were administered subcutaneously over the abdominal wall and in the neck respectively.

With parasites grown in Vero cells. Two of the batches of vaccine respectively contained 1×10^4 (Experiment 3) and 1×10^5 (Experiment 4) parasites per dose, whereas serial tenfold dilutions ranging from 1×10^5 to 1×10^1 were prepared from the third batch (Experiment 5). The vaccine was injected subcutaneously over the abdominal wall in all cases.

With Vero cells only. This batch of control vaccine contained 1×10^5 cells and was also injected over the abdominal wall (Experiment 6).

Challenge of immunity

Rabbits were challenged 1 to 6 months after vaccination with a bovine strain of *B. besnoiti* stored frozen at -76°C as a stabilate. It was originally isolated from a naturally infected bull by subcutaneous inoculation of a saline suspension of cyst-bearing skin into two rabbits. When the latter developed an acute orchitis 17 days later they were sacrificed and a suspension prepared aseptically from their heavily parasitized testes was seeded onto lamb kidney cultures. The parasites were multiplied as described by Bigalke (1962) and stored frozen as a glycerinated suspension at the fifth passage on 26 June 1967.

For challenge purposes this stabilate was multiplied in lamb kidney cells for Experiments 1 to 4 and in Vero cells for Experiments 5 and 6. When sufficient parasites were present they were harvested together with the associated cells and suspended in an appropriate volume of ice cold serum-free medium to give the required number of organisms per challenge dose. This was immediately administered subcutaneously in the neck to both vaccinated and control rabbits.

In Experiment 2 an attempt was made to demonstrate differences between the ID_{50} of the parasitic challenge for immunized and control rabbits (Table 1). Rabbits in the other experiments received a constant challenge dose (Tables 1, 2 and 3).

Assessment of immunity

The following criteria were used to assess susceptibility to challenge:

1. The development of typical "bovine" besnoitiosis reactions characterized by microscopically detectable parasitaemia and/or symptoms of scrotal oedema and orchitis.

2. Death from besnoitiosis 1 to 4 weeks after infection following development of one or more of the abovementioned signs.

Titration of infectivity in culture

In all the experiments the infectivity of the vaccine and the challenging inoculum were determined by titration in roller tube cultures containing the homologous type of cell. The tubes were examined for signs of infection 18 days later by direct microscopic examination for parasites and cytopathic changes, and the cells scraped off the flask into the medium, centrifuged and the sediment examined after staining with Giemsa. The TCID_{50} was calculated by the method of Reed & Muench (1937).

RESULTS

Vaccination with parasites grown in lamb kidney cells

The results of these experiments are summarized in Table 1.

Experiment 1. The 17th passage of the blue wildebeest strain of *B. besnoiti* was used to prepare the vaccine. It contained 1×10^4 parasites per dose and its infectivity in cell culture was $10^{4.5}$ $\text{TCID}_{50}/\text{ml}$. Four of the 10 rabbits died 15 to 23 days after vaccination during or following pronounced febrile episodes which are typical of infection with this strain in rabbits (Bigalke *et al.*, 1967). A fifth rabbit died later from an undetermined cause.

The five survivors and control rabbits were challenged two months after vaccination with 1×10^6 parasites ($10^{4.5}$ $\text{TCID}_{50}/\text{ml}$) of the 13th passage of the bovine strain. The vaccinated rabbits showed no sign of a reaction to the challenging infection. The four controls all developed a patent parasitaemia which was accompanied by typical symptoms in three of the animals. All the controls died from 9 to 15 days after challenge. Protection against clinical besnoitiosis had therefore been afforded by the vaccine.

Experiment 2. Only one of the 10 rabbits inoculated with 2×10^5 parasites (10^4 $\text{TCID}_{50}/\text{ml}$) of the 14th passage of the vaccine strain died 29 days after vaccination following a prolonged febrile reaction.

The nine survivors were challenged 1 month after vaccination with a logarithmic series of doses varying from 2×10^4 to 2×10^8 parasites ($10^{2.9}$ to $10^{6.9}$ $\text{TCID}_{50}/\text{ml}$) of the 16th passage of the challenge strain. The maximum challenge dose, as determined by a haemocytometer count, was therefore a thousand times as high as that of the vaccine. The 10 control rabbits received 2×10^3 to 2×10^7 parasites ($10^{1.9}$ to $10^{5.9}$ $\text{TCID}_{50}/\text{ml}$).

Vaccinated rabbits failed to show any indications of a reaction to the challenge. They all survived and there was no evidence of testicular involvement or a patent parasitaemia. As their immunity did not break down it was not possible to calculate the ID_{50} , which was obviously in excess of 2×10^8 ($=10^{8.3}$) parasites.

The controls were all fully susceptible. One died within 24 hours of being challenged, apparently from the toxic effects of the inoculum (Bigalke, 1967). The remaining nine animals developed typical symptoms and patent parasitaemia; seven died from 11 to 25 days after infection. Again it was not possible to calculate the ID_{50} , which must have been less than 2×10^3 ($=10^{3.3}$) parasites. It can therefore be deduced that more than 5 logs protection against challenge had been induced by the vaccine.

TABLE 1. Vaccination of rabbits with parasites grown in lamb kidney cells

		Vaccination			Challenge				
		No. of rabbits	Dose	No. of rabbits died	No. of rabbits	Time (months)	Dose	No. of rabbits	
								Reacted	Died
Exp. 1.....	Vaccinated rabbits	10	1×10^4	5	5	2	1×10^6	0	0
	Controls.....				4	—	1×10^6	4	4
Exp. 2.....	Vaccinated rabbits	10	2×10^5	1	2	1	2×10^8	0	0
					2	1	2×10^7	0	0
					2	1	2×10^6	0	0
					2	1	2×10^5	0	0
					1	1	2×10^4	0	0
	Controls.....				2	—	2×10^7	1*	0
					2	—	2×10^6	2	2
					2	—	2×10^5	2	1
					2	—	2×10^4	2	2
					2	—	2×10^3	2	2

* One of controls died from toxic effects of challenging inoculum

Vaccination with parasites grown in Vero cells

The results of these experiments are summarized in Table 2.

Experiment 3. Inoculation with 1×10^4 ($10^{4.5}$ TCID₅₀/ml) parasites of the 34th passage of the vaccine strain caused the death of two rabbits: one died within 24 hours, from the toxic effects of the inoculum (Bigalke *et al.*, 1967), and the other on Day 11 during a pronounced febrile reaction. When the eight survivors were challenged 3 months later with 1×10^6 parasites ($10^{4.8}$ TCID₅₀/ml) of the 13th passage of the challenge strain no reactions could be detected.

The eight controls, however, all developed patent parasitaemia; seven showed typical symptoms and four succumbed to the disease from 12 to 14 days after inoculation. Vaccination with parasites grown

in Vero cells had therefore also induced protection to clinical infection with the challenge strain of *B. besnoiti*.

Experiment 4. Two of the 10 rabbits inoculated with 1×10^5 parasites ($10^{4.3}$ TCID₅₀/ml) of the 26th passage of the vaccine strain died from the infection 14 days later during pronounced febrile episodes. A third rabbit died 35 days after infection from an undetermined cause. The seven survivors showed no evidence of disease when they were challenged 6 months later with 1×10^7 parasites ($10^{6.5}$ TCID₅₀/ml) of the 14th passage of the challenge strain.

In contrast to this the controls all developed typical symptoms and patent parasitaemias, although only one died 10 days after infection. The vaccinated rabbits were therefore still fully immune 6 months after vaccination.

TABLE 2. Vaccination of rabbits with parasites grown in Vero cells

		Vaccination			Challenge				
		No. of rabbits	Dose	No. of rabbits died	No. of rabbits	Time (months)	Dose	No. of rabbits	
								Reacted	Died
Exp. 3.....	Vaccinated rabbits	10	1×10^4	2*	8	3	1×10^6	0	0
	Controls.....				8	—	1×10^6	8	4
Exp. 4.....	Vaccinated rabbits	10	1×10^5	3	7	6	1×10^7	0	0
	Controls.....				7	—	1×10^7	7	1
Exp. 5.....	Vaccinated rabbits	5	1×10^5	2	3	2	1×10^4	0	0
		5	1×10^4	0	5	2	1×10^6	0	0
		5	1×10^3	2	3	2	1×10^6	0	0
		5	1×10^2	1	4	2	1×10^6	0	0
		5	1×10	3	2	2	1×10^6	0	0
	Controls.....				5	—	1×10^6	5	3

* One rabbit died from toxic effects of vaccine

IMMUNIZATION AGAINST BOVINE BESNOITIOSIS WITH A LIVE VACCINE

Experiment 5. Twenty-five rabbits, divided into five equal groups, were inoculated with a logarithmic series of parasite doses varying from 1×10^5 (Group 1) to 1×10 (Group 5) that were harvested from the 15th passage of the vaccine strain. The expected correlation between the size of the infective inoculum and the mortality rate did not materialize (Table 2). Three rabbits, for example, died in the group given 1×10 parasites whereas 1×10^5 parasites killed only two rabbits. Deaths occurred from 21 to 30 days after injection and were associated with pronounced febrile reactions. Parasites were found in spleen smears of the six rabbits that were examined: both rabbits in Group 1, one of the two in Group 3, the sole representative of Group 4 and two of the three rabbits of Group 5. It was therefore clear that the vaccine had been infective to rabbits at as low a dosage level as 1×10 parasites. The infectivity in culture at the 1×10^5 level was $10^{1.75}$ TCID₅₀/ml.

The 17 survivors were challenged with 1×10^6

with bovine parasites (Bigalke *et al.*, 1967), was selected for trial as an experimental vaccine for three reasons. The strain from blue wildebeest could be passaged serially in rabbits by subinoculation of blood drawn during acute infection whereas the impala strain was invariably lost after a few passages (Bigalke *et al.*, 1967). The blue wildebeest strain was in fact passaged 77 times before it was isolated in cell culture for vaccination purposes. Secondly, since the parasitic strain originated from a free-living antelope of the family Bovidae, it was possible that the original isolate could have been contaminated with adventitious infectious agents that were pathogenic for cattle: To attempt to eliminate such infectious agents the parasites were serially passaged in an unrelated host. A third consideration was that the efficacy of cryopreservation was still being tested at the time, and the fact that the blue wildebeest strain could be maintained by serial passage was a further safeguard against its loss.

TABLE 3 Control vaccination of rabbits with Vero cells only

		Vaccination		Challenge			
		No. of rabbits	Dose	Time (months)	Dose	No. of rabbits	
						Reacted	Died
Exp. 6.....	Vaccinated rabbits.....	5	1×10^5	2	1×10^6	5	3
	Controls.....	5		—	1×10^6	5	4

parasites ($10^{3.25}$ TCID₅₀/ml) of the 15th passage of the bovine strain. No evidence of disease was seen in any of the vaccinated rabbits. The rabbits that received only 1×10 parasites were also protected against the massive challenge that they experienced. The vaccine had therefore been 100 per cent infective and protective at all dosage levels.

The five controls, on the other hand, developed patent parasitaemia, accompanied by typical symptoms; three died from the disease 13 to 15 days post-challenge.

In terms of the criteria used to assess the immunogenicity of the blue wildebeest strain for rabbits, the data obtained in Experiments 1 to 5 can be summarized as follows: the 46 rabbits that survived vaccination showed no evidence of a reaction to challenge and were therefore all resistant to clinical infection. The 33 unvaccinated controls all developed typical "bovine" besnoitiosis after challenge and 19 (58%) died from the disease.

Control vaccination with Vero cells only

Experiment 6. The rabbits injected with 1×10^5 Vero cells and the uninoculated controls were fully susceptible when they were challenged with 1×10^6 parasites ($10^{3.25}$ TCID₅₀/ml) of the 14th passage of the challenge strain (Table 3). Parasites could be demonstrated in all the animals and only two that were vaccinated with Vero cells failed to show symptoms of besnoitiosis. Four controls and three vaccinated rabbits died from the disease. There was therefore no evidence of protection.

DISCUSSION

A blue wildebeest rather than an impala strain of *B. besnoiti*, which is apparently also of low virulence for cattle and also induced immunity to challenge

It was decided to use an established line of cells (Vero) rather than primary cells as foetal lamb kidney cells are not always available. Moreover there is less likelihood of extraneous infectious agents being introduced into the vaccine with an established line of cells.

From the results of these experiments it can be concluded that inoculation of rabbits by the subcutaneous route with the blue wildebeest strain of *B. besnoiti*, grown in either lamb or Vero cells, produced an excellent immunity. The number of organisms used in the vaccine varied from 1×10 to 2×10^5 and evoked complete protection to clinical infection with from 10 to 100 000 fold greater challenge doses. There was no evidence that the cells used for culture contributed to the induction of immunity.

The blue wildebeest strain of *B. besnoiti* was pathogenic for rabbits. Sixteen (24.6%) of the 65 rabbits succumbed to the disease induced by vaccination and one apparently died from the toxic effects of the inoculum. An increase in the pathogenicity was noticed with serial passage and was thought to be due to adaptation of the parasite to its rabbit host (Bigalke *et al.*, 1967). The results obtained in Experiment 5, where a dose of 1×10 parasites killed more rabbits than one of 1×10^5 parasites, suggest that the pathogenicity is not related to the number of parasites in the inoculum. The phenomenon of death within 24 hours of administration of large numbers of organisms has been ascribed to toxicity (Bigalke *et al.*, 1967; Bigalke, 1967), but requires further investigation.

The discrepancy between direct parasite counts and the titre of infectivity in culture was unexpected. Although the differences were usually not very great, they applied to both blue wildebeest and bovine strains of *B. besnoiti*. Experiment 5, where infectivity

of the vaccine was titrated in rabbits, revealed closer relationship between actual counts and infectivity in rabbits than between infectivity in culture and counts. In fact, a distinct possibility exists that the infectivity of the inoculum was higher than that reflected by the parasite counts. This curious phenomenon could possibly be explained by the presence of intracellular parasites which are difficult to detect microscopically but which are probably in a privileged position with regard to viability and infectivity. These parameters are clearly in need of further investigation to standardise titration methods that will provide reproducible results.

The potential value of the rabbit as a model for testing the potency of a vaccine used for cattle, and hence for standardization of the vaccine, is self-evident. The results of laboratory and field trials on cattle inoculated with vaccine prepared from some of the batches used in this investigation will be correlated with those obtained in rabbits in further publications.

REFERENCES

- BASSON, P. A., VAN NIEKERK, J. W., McCULLY, R. M. & BIGALKE, R. D., 1965. Besnoitiosis in South African antelopes: A preliminary note on the occurrence of *Besnoitia* cysts in the cardiovascular system. *Jl S. Afr. vet. med. Ass.*, 36, 578.
- BIGALKE, R. D., 1962. Preliminary communication on the cultivation of *Besnoitia besnoiti* (Marotel, 1912) in tissue culture and embryonated eggs. *Jl S. Afr. vet. med. Ass.*, 33, 523-532.
- BIGALKE, R. D., 1967. The artificial transmission of *Besnoitia besnoiti* (Marotel, 1912) from chronically infected to susceptible cattle and rabbits. *Onderstepoort J. vet. Res.*, 34, 303-316.
- BIGALKE, R. D., 1968. New concepts on the epidemiological features of bovine besnoitiosis as determined by laboratory and field investigations. *Onderstepoort J. vet. Res.*, 35, 3-138.
- BIGALKE, R. D., VAN NIEKERK, J. W., BASSON, P. A. & McCULLY, R. M., 1967. Studies on the relationship between *Besnoitia* of blue wildebeest and impala, and *Besnoitia besnoiti* of cattle. *Onderstepoort J. vet. Res.*, 34, 7-28.
- POLS, J. W., 1960. Studies on bovine besnoitiosis with special references to the aetiology. *Onderstepoort J. vet. Res.*, 28, 265-356.
- REED, I. J. & MUENCH, M., 1937. A simple method of estimating 50 per cent end points. *Am. J. Hyg.*, 27, 493-497.