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Infectivity to cattle of metacyclic forms of *Trypanosoma* (*Nannomonas*) *congolense* propagated in vitro

I. Development of localized skin reactions following intradermal inoculation

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Summary

Skin reactions similar to those induced by tsetse infected with *Trypanosoma congolense* were elicited in cattle at sites of intradermal inoculation of in vitro propagated parasites which morphologically resembled metacyclic trypanosomes. The time to detection of the reaction, the time to maximal size and the maximal size attained were dependent on the number of parasites inoculated, although it was possible to induce a skin reaction with as few as 20 trypanosomes. All cattle became infected with the initial detection of the skin reaction preceding parasitaemia by 3 to 7 days.

Key words: *Trypanosoma congolense;* in vitro culture; metacyclic trypanosomes; cattle; local skin reactions; parasitaemia.

Introduction

Following the successful feeds of tsetse infected with *Trypanosoma congolense,* cattle develop local skin reactions of delayed onset that persist for several days and can measure up to 10 centimetres in diameter (Bolton, 1965; Roberts et al., 1969; Luckins and Gray, 1978; Gray and Luckins, 1979; 1980; Emery et al., 1980; Akol and Murray, 1982). This reaction is commonly called a chancre and it acts as an extravascular focus for proliferation of the parasite prior to passage into the bloodstream via the draining lymphatics (Akol, 1983). This initial interaction between *T. congolense* and the host at the level of the skin plays an important role in the induction of immunity as it is only after regression

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of the chancre that cattle are immune to tsetse-transmitted homologous challenge (Akol and Murray, 1985).

Attempts to induce skin reactions by the intradermal inoculation of large numbers of bloodstream forms of *T. congolense* (10^7) have been unsuccessful, although the cattle became infected (Bolton, 1965; Akol and Murray, 1982). On the other hand, small numbers of tsetse-derived metacyclic forms of *T. congolense* (10^2–10^3) induced skin reactions in cattle when inoculated intradermally (Akol and Murray, 1982). Metacyclic forms of *T. congolense* have now been successfully propagated in vitro (Gray et al., 1981; Hirumi et al., 1982) and were found to induce local skin reactions in rabbits (Luckins et al., 1981).

In this study, varying numbers of metacyclic trypanosomes, propagated in vitro at 28 °C in the presence of purified bovine dermal collagen or at 35 °C in the presence of bovine endothelial cell feeder layers, were inoculated intradermally to determine their capacity to induce local skin reactions similar to those produced by tsetse-transmitted parasites, and to infect cattle.

Materials and Methods

Cattle

Four Boran, one Hereford and two Guernsey cattle, aged 6 to 8 months, were used. Hereford and Guernsey cattle were purchased from an area of Kenya which was free from trypanosomiasis and were serologically negative for antibodies to *Trypanosoma* and *Theileria* species by immunofluorescence and to *Anaplasma* species by the card agglutination test. Boran cattle were reared in ILRAD and had not been exposed to haemoparasitic infections. Animals were housed in insect-proof accomodation and managed as described by Akol and Murray (1982).

Trypanosomes

T. congolense IL 687 was an uncloned derivative of STIB 212 (Swiss Tropical Institute Basel 212) which was a stabilate prepared after a single passage in rats of an isolate made from a lion in Serengeti National Park by Geigy and Kauffmann (1973). STIB 212 was passaged twice in normal mice and made into stabilate material as IL 20E-8. IL 687 was derived from IL 20E-8 by a single transmission through tsetse and three passages in normal mice.

In vitro cultivation of trypanosomes

Animal infective forms of *T. congolense* IL 687 were cultivated in vitro as described by Gray et al. (1981) and modified by Hirumi et al. (1982). Briefly, trypanosomes from infected tsetse probosces were cultured in the presence of purified bovine dermal collagen (Vitrogen, Collagen Company, Palo Alto, California, USA) using HEPES-buffered (25 mM) Eagle's Minimum Essential Medium (MEM) with Earle's balanced salts (GIBCO, Paisley, Scotland, UK) supplemented with 20% (v/v) heat-inactivated foetal bovine serum (Flow Lab., Irvine, Scotland, UK) at 28 °C. The cultures were comprised of long slender procyclic forms, epimastigotes and metacyclic-like forms which became infective for mice after 53 days in culture and were referred to TC 28 °C (*T. congolense*, 28 °C) in this study. When these parasites were passed through columns of diethylaminoethyl cellulose (DE52, Whatman Chemical Separation Limited, England), using phosphate-buffered saline containing 1% (w/v) glucose (PSG), pH 8.0, 2–4% metacyclic-like forms were recovered from the original mixed population. These were designated as TC 28 °C DE52 (*T. congolense*, 28 °C, DE52). When the mixed population was transferred to bovine endothelial cell feeder layer cultures (Hirumi and Hirumi, 1984) and incubated at 35 °C, the majority (over 95%) of the parasites which were actively alive retained morphological characteristics of metacyclic trypanosomes while some (less than 5%) of them trans-

formed within 4 days into forms that morphologically resembled bloodstream forms. Procyclic forms and epimastigotes in the mixed populations died off 24 h after they were transferred to the 35 °C culture system. When the cultures were maintained at 35 °C by changing ⅓ or ½ volume of the culture medium with the same volume of fresh medium at 48 h intervals, only morphologically metacyclic-like forms with some bloodstream form-like trypanosomes remained in the cultures by day 4. These trypanosome populations were used to inoculate cattle and were designated as TC 35 °C (*T. congolense*, 35 °C). No procyclic forms and epimastigotes, including their debris, were seen in such populations.

Prior to inoculation into cattle, all trypanosomes were washed three times with sterile PSG, pH 8.0, to remove culture medium, containing possibly remaining debris of dead trypanosomes.

Experimental design

Intradermal inoculation of cattle with T. congolense IL 687 propagated in vitro

Cattle were sedated with 15–30 mg of xylazine hydrochloride (Rompun, Bayer, Leverkusen, Germany). Individual animals were inoculated intradermally with varying doses of TC 28 °C, TC 28 °C DE52 or TC 35 °C trypanosomes as outlined in Table 1. Each animal received duplicate inocula, on one or both flanks, of titrated trypanosome concentrations, given in 200 µl volume of sterile PSG, pH 8.0 (Akol and Murray, 1982).

Control sites on the same cattle were inoculated intradermally with sterile PSG alone.

Table 1. Experimental design for intradermal inoculation of cattle with *Trypanosoma congolense* IL 687 propagated in vitro

Cattle		Sex	Trypanosome populations inoculated[b]	Number of trypanosomes in each inoculum of 200 µl[a]				
Case No.	Breed							
B 348	Boran	M	TC 28 °C	4×10^5	4×10^4	4×10^3	4×10^2	ND
B 349	Boran	F	TC 28 °C DE52	2×10^5	2×10^4	2×10^3	ND	ND
B 350	Boran	M	TC 35 °C	2×10^5	2×10^4	2×10^3	ND	ND
B 347	Boran	F	TC 35 °C	ND	4×10^4	4×10^3	4×10^2	ND
B 429	Hereford	M	TC 28 °C DE52	2×10^5	2×10^4	2×10^3	2×10^2	2×10
B 696	Guernsey	M	TC 28 °C DE52	2×10^5	2×10^4	2×10^3	2×10^2	2×10
B 697	Guernsey	F	TC 28 °C DE52	2×10^5	2×10^4	2×10^3	2×10^2	2×10

[a] For each concentration of trypanosomes, the inoculations were made in duplicate.

[b] TC 28 °C:
Mixed trypanosome population comprised of procyclic forms, epimastigotes and metacyclic-like trypanosomes, which was propagated at 28 °C over 53 days.
TC 28 °C DE 52:
Trypanosome population comprised of metacyclic-like trypanosomes which were derived from the mixed population (TC 28 °C) by passing through a DE52 column.
TC 35 °C:
Trypanosome population comprised of forms with morphological appearance of metacyclic trypanosomes after transferring the mixed population (TC 28 °C) from 28 °C to 35 °C and incubating in the presence of bovine endothelial cell layers for 4 days.

ND = Not done

Sampling techniques

Skin thickness at the site of trypanosome inoculation was measured every other day using vernier calipers. Skin biopsies were obtained and histological processing was carried out as described by Akol and Murray (1982). Jugular blood samples were collected into 10 ml EDTA-treated vacutainers daily from day 0 to 20 and twice weekly up to day 60. The buffy coat was examined for trypanosomes by phase-contrast microscopy as described by Paris et al. (1982).

Results

Local skin reactions induced by in vitro propagated T. congolense

Intradermal inoculation of the trypanosome population of TC 28 °C DE52, TC 35 °C or TC 28 °C, induced skin reactions at 46 out of 48 sites injected (Table 2). The time to detection of the skin reactions, the subsequent maximal thickness and the time to attainment of maximal thickness were found to be dependent on the number of trypanosomes inoculated into the skin (Table 2).

The reactions induced by metacyclic-like trypanosomes purified from a mixed population on a DE52 column (TC 28 °C DE 52), were tender, raised, indurated and oedematous plaques that by day 8 measured up to 10 cm in diameter at sites inoculated with 2×10^5 parasites. As few as 20 parasites were able to induce reactions that measured up to 4 cm in diameter by day 10. Parasitaemia was detected on day 11 in two cattle and day 12 in the other two animals infected with these parasites.

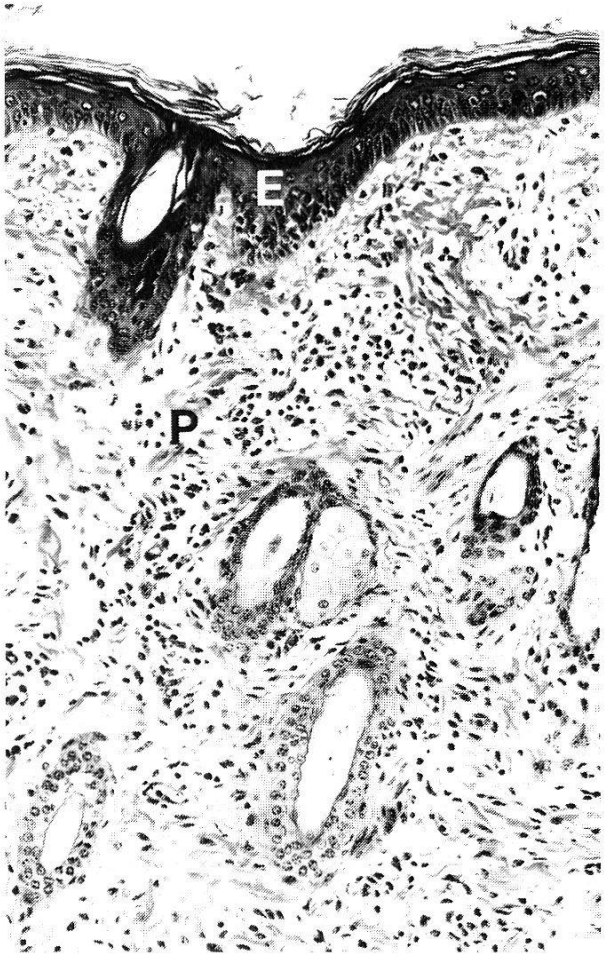
Table 2. Skin reactions of three representative cattle to intradermal inoculation with *Trypanosoma congolense* IL 687 propagated in vitro

Cattle		Trypanosome populations inoculated	No. of trypanosomes in 200 µl PSG	Number[a]	Chancres		
Case No.	Breed				Days to detection	Maximum thickness	
						Percent increase[b]	Day attained
B 429	Hereford	TC 28 °C DE52	2×10^5	4/4	5	100.0	8
			2×10^4	4/4	6	101.6	8
			2×10^3	4/4	6	63.7	8
			2×10^2	4/4	7	63.6	10
B 347	Boran	TC 35 °C	4×10^4	4/4	5	138.0	7
			4×10^3	4/4	5	89.0	7
			4×10^2	2/4	7	78.0	8
B 348	Boran	TC 28 °C	4×10^5	4/4	4	89.0	9
			4×10^4	4/4	4	87.0	9
			4×10^3	4/4	5	50.0	9
			4×10^2	4/4	5	39.0	9

[a] The number of chancres that developed out of the total sites inoculated.
[b] The percentage increase to maximum skin thickness in relation to day 0 thickness.

Skin reactions induced by trypanosomes propagated in the presence of bovine endothelial cell feeder layers (TC 35 °C) occurred at all sites inoculated with 4×10^4 and 4×10^3 trypanosomes, but only two reactions developed out of a possible four at sites inoculated with 4×10^2 trypanosomes. These reactions were similar in size and appearance to those induced by purified metacyclic-like trypanosomes propagated at 28 °C. The two animals inoculated with these parasites developed microscopically detectable parasitaemia 8 and 12 days after infection.

Following inoculation of the preparation that consisted of a mixture of procyclic forms, epimastigote, and metacyclic-like forms (T 28 °C), the reactions first appeared as red inflamed maculae on days 4 to 5. The maximum increase in skin thickness was reached on day 9 at all trypanosome concentrations. The



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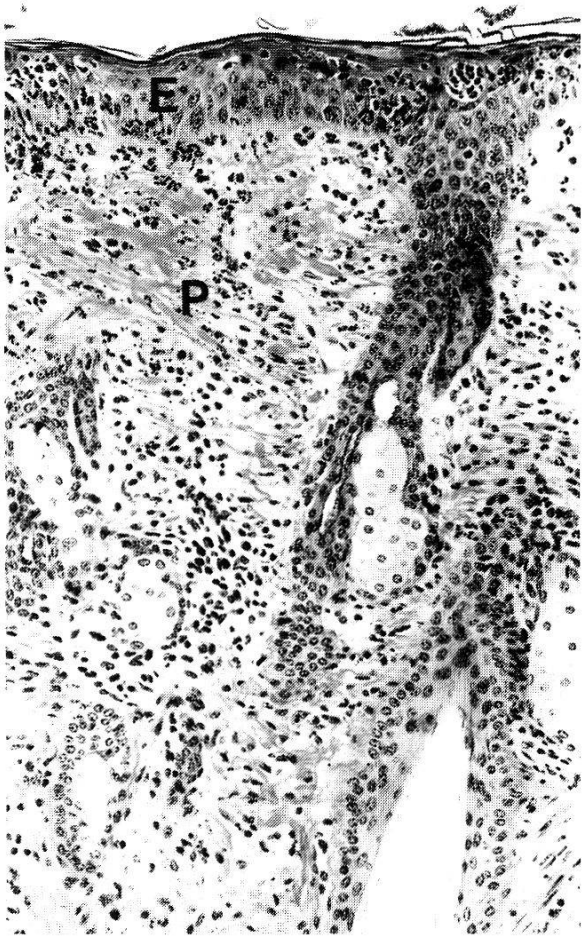

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Fig. 1. Bovine skin (B350) 7 days after intradermal inoculation of metacyclic-like *Trypanosoma congolense* parasites propagated for 4 days at 35 °C in the presence of bovine endothelial cell feeder layers after being cultivated over 53 days at 28 °C in the presence of purified bovine dermal collagen. Note the intact epidermal layer (E) and the infiltration of the papillary dermis (P) mainly by small lymphocytes: there is also marked congestion and oedema. ×170. Haematoxylin and Eosin.

Fig. 2. Bovine skin (B348) 7 days after intradermal inoculation of the mixed metacyclic-like, epimastigotes, and procyclic *Trypanosoma congolense* parasites propagated at 28 °C in the presence of purified bovine dermal collagen. Note the massive numbers of viable and dead neutrophils in the epidermis (E) and papillary dermis (P). ×170. Haematoxylin and Eosin.

maximum diameter of the reactions was approximately 5 to 7 cm, but, in contrast to the other preparations (TC 28 °C DE52 and TC 35 °C), no induration was seen. At sites inoculated with 4×10^5 trypanosomes, the chancres developed dark central necrotic areas on day 9. By day 10, all other sites also became soft, dark and necrotic. Dry crusts had formed at all sites by day 11 and they persisted up to day 14. Parasitaemia was detected microscopically on day 10 in the animal inoculated with these parasites.

Reactions did not develop at control sites inoculated with PSG alone.

Histopathology

Skin reactions induced in cattle by the metacyclic-like trypanosomes purified on DE52 columns (TC 28 °C DE52) from the mixed populations and the populations propagated in the presence of bovine endothelial cell feeder layers (TC 35 °C) were different from those produced by the unpurified mixed population (TC 28 °C). The reactions induced by parasites from the two former culture systems were dominated by numerous small and medium lymphocytes (Fig. 1) and later by plasma cells; a few neutrophils were also present but only in the initial stages of development between days 5 and 7. On the other hand, the reactions caused by the unpurified mixed population (TC 28 °C) were characterized by an intense inflammatory response, dominated by neutrophils many of which were undergoing karyorrhexis and karyolysis (Fig. 2); severe necrotizing vasculitis was also present. At the height of the reaction, superficial necrosis and desquamation were extensive in the epidermis. With all trypanosome preparations as the height of the reaction, parasites were readily identified and there was intense congestion and oedema, with disruption of collagen fibres and distension of the dermal lymphatics.

Sites inoculated with PSG alone did not exhibit any histological changes.

Discussion

T. congolense organisms prepared with the three different culture systems used in this study all induced local skin reactions when inoculated intradermally into cattle. Similar results have also been achieved in rabbits (Luckins et al., 1981). Previous studies with *T. congolense* have shown that following intradermal inoculation it was only metacyclic trypanosomes derived from tsetse flies that induce typical chancres (Akol and Murray, 1982), whereas, procyclic (R. H. Dwinger, personal communication) and bloodstream forms (Bolton, 1965; Akol and Murray, 1982) did not. Thus based on the ability to induce skin reactions, as well as on morphological appearance (Hirumi et al., 1982), it was concluded that the metacyclic-like parasites propagated in this study were metacyclic forms.

The reactions induced were dose-dependent as assessed by the time to detection, time to peak reactivity and the percentage increase in skin thickness, with as few as 20 parasites capable of causing skin reactions. While, in most

cases, peak skin reactivity was attained 1 to 3 days earlier than has been demonstrated for tsetse-transmitted *T. congolense,* 20 to 200 purified *T. congolense* metacyclics propagated at 28 °C, induced skin lesions that reached peak size on day 10. This timing was the same as that observed in tsetse-transmitted *T. congolense* (Akol and Murray, 1982) confirming the estimation that tsetse probably do not inoculate more than 200 metacyclic trypanosomes during each feed (Harley and Wilson, 1968; Otieno and Darji, 1979; Nantulya et al., 1980). Also, reactions produced by the purified *T. congolense* metacyclics propagated at 28 °C (TC 28 °C DE52) and those propagated at 35 °C (TC 35 °C) resembled both grossly and histologically the reactions caused by the tsetse-transmitted parasites (Bolton, 1965; Roberts et al., 1969; Luckins and Gray, 1978; Gray and Luckins, 1979, 1980; Emery et al., 1980; Akol and Murray, 1982).

Reactions induced by the unpurified mixed population propagated at 28 °C (TC 28 °C) differed macroscopically and histologically from those caused by the metacyclic forms purified from the mixed population or those propagated at 35 °C. The proportionately lower percentage increase in skin thickness at corresponding dose levels and the diffuse nature of the reactions produced by the mixed population might be attributed to the low numbers of metacyclic trypanosomes present in this preparation, estimated at less than 4% (Hirumi et al., 1982). In further contrast to the chancre-like reactions induced by the two populations of metacyclic trypanosomes, the skin reactions caused by the mixed populations became ulcerated and necrotic with intense neutrophil infiltration and necrotizing vasculitis. This outcome was possibly due to the presence of the high proportion of epimastigote and procyclic forms (Hirumi et al., 1982). The ability of these uncoated parasites to activate the alternative pathway of complement (Ferrante and Allison, 1983) with subsequent generation of chemotactic factors (Fearon, 1979) might explain the presence of large numbers of neutrophils in the lesion and may have contributed to the intense inflammatory reaction caused by the unpurified mixed population propagated at 28 °C. It might be that the variation in the intensity of the inflammatory reaction induced even in the same animal by different tsetse infected with *T. congolense* is due to the fact that different flies extrude varying numbers of trypanosomes including epimastigotes, during feeding (Otieno and Darji, 1979).

In conclusion, *T. congolense* parasites propagated in vitro with the morphological appearance of the tsetse-derived metacyclic forms, were also shown to possess the biological activity of the latter. The metacyclic trypanosomes propagated in vitro were infective to cattle and were able to induce skin reactions that were similar, macroscopically and histologically, to those caused by the tsetse-transmitted forms of the parasite.

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