

Role of the chancre in induction of immunity to tsetse-transmitted *Trypanosoma (Nannomonas) congolense* in goats

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ABSTRACT

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Local skin reactions (chancres) developed in goats at the sites of deposition, by tsetse flies, of metacyclics of *Trypanosoma congolense*. The chancres developed much faster and were more pronounced when ten infected tsetse were allowed to feed on a spot as compared to only one fly per spot. The initial host cellular reaction in the chancre was predominantly polymorphonuclear, followed at the peak of development of the chancre by a predominantly lymphoblastic and plasmacytic reaction. Trypanosomes were found in various stages of division as well as degeneration in chancre biopsies taken at various days post-infection (p.i.). Most of the trypanosomes recovered from the chancre tissue fluid were found to bear the same variable surface glycoprotein (VSG) epitopes as the corresponding metacyclics for as long as 13 days p.i., as revealed by indirect immunofluorescence using mouse anti-metacyclic VSG hyperimmune sera and monoclonal antibodies. Immunization of goats with metacyclic trypanosomes, by exposure to infected tsetse bites followed by treatment of the infected goats on day 13 p.i., gave rise to the development of protection to homologous tsetse-transmitted challenge, whilst immunization by intravenous inoculation of the metacyclics did not induce such protection. Chancre formation would thus appear to be vital for the induction of comprehensive immune recognition of the metacyclic variable antigen repertoire deposited in the skin by infected tsetse, and hence development of protective immunity.

INTRODUCTION

A localized reaction (chancre) in the skin of a susceptible host to the presence of proliferating trypanosomes is an established feature of the pathology of human and animal trypanosomiasis (Fairbairn and Godfrey, 1957; Bol-

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ton, 1965; Roberts et al., 1969; Emery and Moloo, 1980, 1981; Barry and Emery, 1984).

Development of the chancre occurs within a few days after the susceptible host has been bitten by infected tsetse, the reaction reaching a peak during the second week before declining to undetectable levels during the third week (Morrison et al., 1985). The size and the kinetics of development of the reaction in goats and cattle differ among species of trypanosomes: the reaction is most severe and occurs earlier following *Trypanosoma brucei* infection (Emery and Moloo, 1980; Barry and Emery, 1984); it is less severe, or on occasions as severe as *T. brucei*, in infections with *T. congolense*, while the reaction in *T. vivax* infections is least dramatic (Emery and Moloo, 1981). Moreover, induction of the chancre also differs amongst trypanosome stocks within a single species. For example, the Kilifi isolates of *T. congolense* (Masake et al., 1987) and some stocks of *T. vivax* (Morrison et al., 1985) are not associated with chancre formation.

The chancre not only serves as a site for establishment of infection but also as a focus for multiplication and persistence of trypanosomes before their dissemination into the bloodstream (Akol and Murray, 1982). Furthermore, it provides a "meeting place" for the first contact between the trypanosome antigens and the host's immune system (Emery and Moloo, 1981; Akol and Murray, 1985). That the chancre could serve as a site for sensitization of the host's immune system is indicated by the presence of large numbers of lymphoblasts and plasma cells, some of which contain Russell bodies (Akol and Murray, 1982) in the later stages of chancre development. Akol and Murray (1985) also demonstrated that the development of protective immunity to an homologous tsetse-transmitted challenge depended on the timing of treatment in relation to the development of the chancre. Animals treated 15 days or more after infection displayed complete acquired immunity, while those treated earlier than day 15 displayed partial or no immunity to homologous challenge.

A possible explanation for the above observation is that the dividing trypanosomes in the chancre might retain the protective surface antigen epitopes of the corresponding metacyclics, so that the longer the treatment is delayed, the better the priming of the immune response by the antigens of the dividing trypanosomes in the chancre. The objective of this study was, therefore, to investigate the antigenic relationship between variable antigen types (VATs) of the metacyclics and those of the trypanosomes within the chancre in goats, so as to elucidate the role of the chancre in the induction of immunity to tsetse-transmitted *T. congolense*.

MATERIALS AND METHODS

Animals

Castrated adult East African Galla crossbred goats weighing 20–25 kg were used. They were purchased from the Kumanchu location in the Mukogodo

Division of Laikipia District of Kenya, an area known to be tsetse- and trypanosomiasis-free. The goats were treated with long-acting antibiotics, cocidiostats and anthelmintics on arrival. They were later treated for ectoparasites (by dipping) and placed under quarantine for one month, during which time they were vaccinated against contagious caprine pleuropneumonia and foot and mouth disease.

Ten-week-old male of female BALB/c mice used in this study were bred at ILRAD.

Trypanosomes

One clone and one stock of *Trypanosoma (Nannomonas) congolense* were used. The clone, *T. congolense* IL 1180 (ILNat 3.1), was derived (Nantulya et al., 1984) from STIB 212, an isolate from a lion in the Serengeti area of Tanzania (Geigy and Kauffmann, 1973), while the stock, *T. congolense* IL 2642, was isolated from a cow at the Government Farm Institute, Busoga, in 1962 (Akol and Murray, 1985).

Tsetse flies

Glossina morsitans centralis were from the ILRAD tsetse colony (Moloo et al., 1985).

Infection and identification of infected tsetse

Three capillaries of each trypanosome stabilate (containing about 1×10^7 trypanosomes/ml) were thawed and suspended in 3 ml of cold sterile phosphate-buffered saline glucose (PSG), pH 8.0, and injected intramuscularly into a goat. Peripheral blood from the goat was examined daily for trypanosomes by the buffy coat technique (Murray et al., 1977). Five days after initial detection of parasites, 200 teneral male tsetse were infected by allowing them to feed daily for 25 days on the infected goat, after which tsetse with mature infections were identified by the warm-slide probe method of Burt (1946). The tsetse flies were thereafter maintained by feeding on rabbits.

Preparation of mouse anti-metacyclic hyperimmune sera

Infected tsetse were fed on BALB/c mice (five flies per mouse) on days 0, 3, 6, 9 and 12, and the mice were then treated with Berenil^(R) on day 14. The mice were subsequently fed upon by five infected tsetse once weekly for at least 4 weeks, starting 10 days after treatment, and bled 4 days after the last feed. The sera obtained were heat-inactivated at 56°C for 30 min, and tested for antibody activity by the indirect immunofluorescence antibody test (IFAT) performed on metacyclics in infected tsetse salivary probes, as described by Nantulya et al. (1980).

Derivation of anti-metacyclic monoclonal antibodies

Monoclonal antibodies of the VATs of metacyclics were derived following the procedure described by Nantulya et al. (1983). The immunoglobulin classes and subclasses of the antibodies were determined by double immunodiffusion using commercially prepared antimouse immunoglobulin antisera (Meloy, Springfield, VA), following the procedure described by Ouchterlony and Nilsson (1986).

The relationship between metacyclics and chancre trypanosomes

Mouse anti-metacyclic hyperimmune sera and mouse anti-metacyclic monoclonal antibodies were tested by IFAT (Nantulya and Doyle, 1977) on both the homologous and heterologous metacyclics as well as on trypanosomes from the chancres in goats infected by tsetse bites, and the percentage of stained parasites was determined.

Extraction of trypanosomes from chancres

Infected tsetse were allowed to feed on shaved flanks of the goats and subsequent development of chancres was monitored. Trypanosomes were aspirated from the chancres by syringe and needle. A small volume (0.2 ml) of 10% EDTA in PBS, pH 7.2, was flushed into the chancre and then aspirated. The aspirate was placed in 0.5 ml cold sterile PSG, pH 8.0. In two of the goats, the chancre was excised, after local infiltration with Xylocaine^(R), and macerated in 5 ml cold PSG, pH 8.0, and the animals were killed by exsanguination. The trypanosome suspensions obtained by either method were separated from contaminating blood by anion exchange chromatography (Lanham and Godfrey, 1970). The purified trypanosome suspensions were formaldehyde-fixed and tested for reactivity with anti-metacyclic antibodies by IFAT (Nantulya and Doyle, 1977).

Comparison of the level of immunity induced by tsetse bites and by intravenous inoculation of metacyclics

Two groups of goats were infected with *T. congolense* IL 2642. The first group of eight (Group A) was infected using five infected tsetse per animal. The second group of twelve (Group B) was infected by intravenous injection of 1.0 ml suspension of metacyclic trypanosomes obtained from salivary probes of infected tsetse (ten infected tsetse were probed into 10 μ l PSG, pH 8.0, and made up to 1.0 ml with more PSG, pH 8.0). All the goats were monitored daily for parasitaemia, and in the case of the first group the skin thickness at the bite sites was measured with Vernier calipers. On day 13 p.i. all the goats were treated when parasitaemic with 7 mg kg⁻¹ Berenil. 21 days post-treatment, all the goats, including four uninfected control goats (Group C) were each challenged with five tsetse infected with homologous trypanosomes. Chancre reactions and parasitaemia were monitored daily for 30 days,

after which all parasitaemic animals were treated with Berenil. The animals were subsequently challenged using tsetse infected with the other *T. congolense* clone (IL 1180) and observed for parasitaemia for 30 days, after which the animals were treated and the experiment terminated.

RESULTS

Specificity of mouse hyperimmune sera for T. congolense metacyclics

At dilutions of 1:50, 1:100 or 1:200, mouse hyperimmune sera against *T. congolense* IL 1180 or IL 2642 metacyclics stained >99.9% of the homologous metacyclic population, while at dilutions of 1:400 and 1:800 there was heterogeneity in the staining characteristics of the metacyclics. At 1:400 and 1:800 dilutions, mouse antiserum to metacyclics of *T. congolense* IL 1180 stained 66.7% and 0%, while that against *T. congolense* IL 2642 stained 80% and 45.5% respectively. There was no cross-reactivity between metacyclics of the two *T. congolense* populations.

Heterogeneity of metacyclic VATs as revealed by the monoclonal antibodies

The monoclonal antibodies against *T. congolense* IL 1180 and IL 2642 were cross-tested by IFAT against the two metacyclic populations. The proportions of metacyclic VATs recognized by each monoclonal antibody and the immunoglobulin subclasses of the antibodies are given in Table 1. The results obtained confirmed the heterogeneity of metacyclic VATs observed with hyperimmune serum.

TABLE 1

Proportion of *Trypanosoma congolense* IL 1180 and IL 2642 metacyclics recognized, in the indirect immunofluorescence antibody test, by monoclonal antibodies

Monoclonal antibody	Derived against metacyclics of	Immunoglobulin subclass	% metacyclics recognized in	
			IL 1180	IL 2642
1180/46.3.7	IL 1180	IgG _{2a}	17.0±0.8 ¹	0
1180/46.5.14	IL 1180	IgG _{2a}	33.1±1.6	0
2642/31.13.16	IL 2642	IgG ₃	0	18.0±2.2
2642/31.14.10	IL 2642	IgG ₃	0	10.2±2.0
2642/28.15.22	IL 2642	IgG ₃	0	31.7±2.4

¹Mean ± standard deviation.

Reactivity of mouse anti-metacyclic antisera with chancre trypanosomes

The VATs of the parasites from the chancres were compared, by IFAT, with the VATs of the corresponding metacyclic population. Table 2 shows that VAT-specific anti-metacyclic hyperimmune sera recognized a high percentage of trypanosomes from the chancre.

Kinetics of the development of chancres

Local skin reactions developed at the sites of tsetse bites as early as day 7 p.i., depending on the number of tsetse fed per site (Fig. 1). Goats that re-

TABLE 2

Proportion of trypanosomes in chancres from goats recognized at antibodies to homologous *Trypanosoma congolense* metacyclics

Mouse hyperimmune serum to metacyclics of	% chancre trypanosomes stained in populations harvested on post-infection days indicated					
	8	9	10	11	12	13
IL 1180	97.5 ± 3.9 ¹	94.7 ± 5.1	84.0 ± 6.5	79.8 ± 5.2	76.5 ± 5.0	76.2 ± 2.5
IL 2642	98.2 ± 3.8	95.7 ± 4.2	85.0 ± 5.3	77.1 ± 3.4	N.D.	N.D.

¹Mean ± standard deviation.

N.D. = not determined.

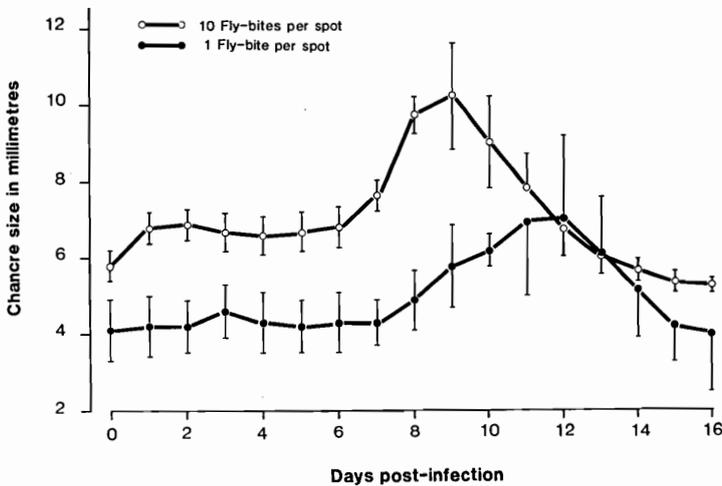


Fig. 1. Kinetics of daily development of chancre in goats infected with tsetse-borne *Trypanosoma congolense*.

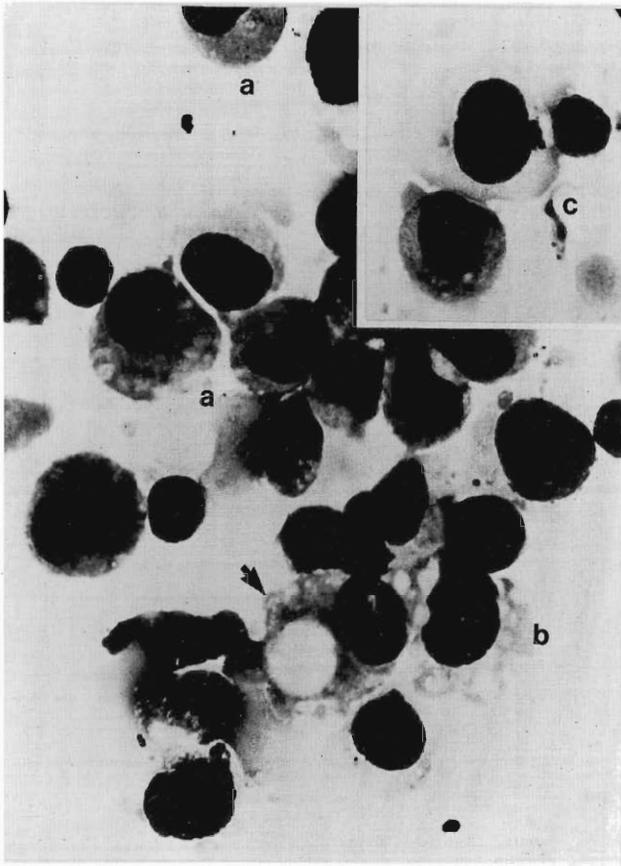


Fig. 2. Giemsa-stained smear of aspirate of chancre taken on day 11 post-infection, showing numerous plasma cells (a), one containing a Russell body (arrow). Vacuolated macrophage (b), and (inset) a dividing trypanosome (c). Magnification: $\times 1500$.

ceived ten fly bites per site had a marked increase in skin thickness at the bite site on day 7 p.i. compared with those that received one fly bite per spot ($P < 0.002$). Giemsa-stained smears of aspirates of the chancres taken on day 8 p.i. contained numerous small lymphocytes and a few large lymphocytes and lymphoblasts. There were also numerous neutrophils, but few macrophages and very few trypanosomes. The aspirates taken on day 11 p.i. contained predominantly large lymphocytes, lymphoblasts and plasma cells, some of which contained Russell bodies. There were numerous macrophages, trypanosomes and very few neutrophils (Fig. 2). Histopathological lesions included an initial diffuse interstitial oedema, congestion and cellular infiltration (mostly neutrophils and small lymphocytes) of both the papillary and

TABLE 3

Development of immunity in goats previously infected by tsetse bite or by intravenous inoculation with metacyclics of *Trypanosoma congolense* stock IL 2642

Group	Number of goats	Mode of immunization	Number of animals with chancres	Number of animals parasitaemic	Homologous challenge 21 days after treatment	Number of animals with chancres	Number of animals protected ¹	Number of animals parasitaemic after heterologous (IL 1180) challenge
A	8	Tsetse challenge	8/8	8/8	Tsetse	0/8	7/8	8/8
B	12	Intravenous inoculation	N.A.	12/12	Tsetse	12/12	0/12	12/12
C	4	Uninfected (controls)	N.A.	N.A.	Tsetse	4/4	0/4	4/4

N.A. = not applicable.

¹Parasitaemia not detected (complete protection).

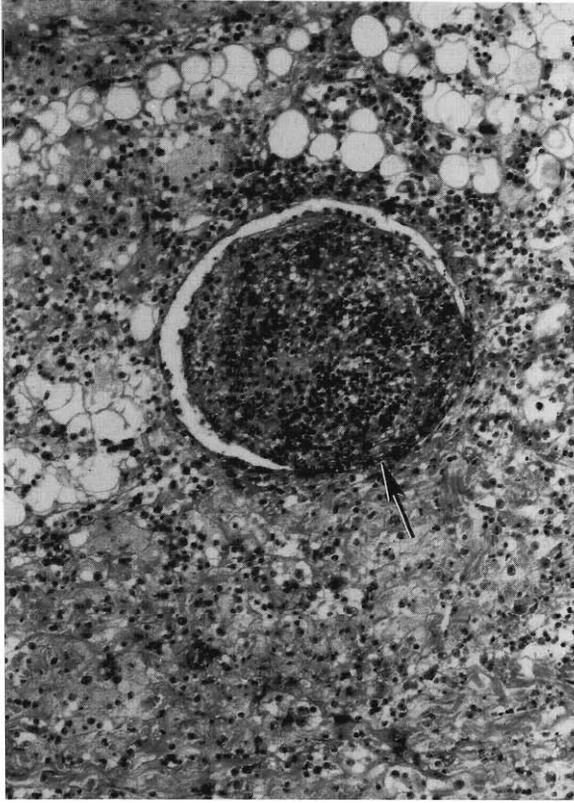


Fig. 3. Histology of day 8 post-infection chancre showing severe and diffuse cellular infiltration of the reticular dermis (mostly neutrophils), oedema, fibrin exudation and lymphatic thrombosis (arrow). H & E, $\times 375$.

the reticular dermis. There were microthrombi in capillaries and lymphatics (Fig. 3) and trypanosomes in distended lymphatics and within connective tissue. At day 11 p.i. the chancre showed an apparently normal epidermis, mildly oedematous capillary dermis, congestion and focal interstitial mononuclear cellular infiltration.

Induction to immunity of homologous tsetse challenge

The results in Table 3 show that chancres did not develop in the eight goats previously infected (i.e. immunized) with metacyclics by tsetse bite, and that seven of the animals were completely protected against homologous tsetse challenge. In contrast, chancres developed in all the twelve goats previously immunized with metacyclics by the intravenous route and all the animals became parasitaemic, as did the control animals.

Upon cross-challenge with heterologous parasites (*T. congolense* IL 1180)

all the animals, including the controls, developed chancres and became parasitaemic.

DISCUSSION

Mouse hyperimmune sera prepared against metacyclic VATs of the two *T. congolense* populations revealed no cross-reactivity, indicating that the two populations belonged to different serodemes. Thus, these results lend further support to the suggestion that metacyclics of any given *T. congolense* serodeme are characteristic of the serodeme in terms of VAT composition (Nantulya et al., 1980). At high dilutions of anti-metacyclic hyperimmune serum, heterogeneity in the metacyclic VATs was observed. This heterogeneity in the metacyclic VATs was confirmed by the staining characteristics obtained with anti-metacyclic monoclonal antibodies. The heterogeneity of *T. congolense* metacyclic VATs is in agreement with the observations by Crowe et al. (1983) who reported that there were twelve different metacyclic VATs in the *T. congolense* serodeme they studied.

The development of the chancre was due to the proliferating trypanosomes in the skin. Numerous trypanosomes were found inside the lymphatics and within connective tissue, in varying stages of division and degeneration. Histopathological lesions were similar to those reported by previous workers (Emery and Moloo, 1980, 1981; Barry and Emery, 1984).

The present work has also shown that trypanosomes found in the chancres express VATs of the corresponding metacyclics, and that the metacyclic VAT-expressing trypanosomes multiply and persist in the chancre for several days after tsetse bite. The persistence and multiplication of surface antigen-bearing metacyclic trypanosomes in the chancre would appear to be vital for the development of a comprehensive immune recognition of the metacyclic VATs deposited in the skin by the tsetse fly. We have indeed been able to demonstrate that whereas immunization with metacyclics by tsetse bite readily induced complete protection against homologous tsetse challenge, the goats immunized by intravenous inoculation with metacyclics developed parasitaemia upon challenge, as did the non-immunized goats. This observation would explain why the development of protective immunity to an homologous tsetse-transmitted challenge depends on the timing of treatment in relation to the development of chancre (Akol and Murray, 1985). Animals treated on day 15 p.i. displayed complete acquired immunity to homologous challenge; those treated between days 10 and 12 exhibited partial immunity, while the animals treated on days 5 or 10 were fully susceptible. On the basis of our findings, it would also follow that *T. congolense* stocks that do not induce chancres may not readily give rise to acquired immunity to tsetse challenge. This indeed would appear to be the case. Immunity to metacyclic challenge was difficult to induce in goats exposed to tsetse infected with *T. congolense* stocks from

Kilifi which do not produce chancres (Masake et al., 1987). Likewise, our findings would explain the difficulties encountered in the induction of protective antibody responses to *T. vivax* metacyclics (Vos et al., 1988) as *T. vivax* does not produce significant chancre reactions (Morrison et al., 1985).

In conclusion, this study has demonstrated that the chancre is a site for the proliferation and persistence of trypanosomes that bear the same variable surface glycoproteins (VSGs) as the corresponding metacyclics. Persistence of the metacyclic VSGs in the skin would appear to be vital for comprehensive immune recognition of the full metacyclic VAT repertoire to occur. This may reflect superior processing and presentation of the VSGs to the host immune system by the Langerhans cells and macrophages in the skin and the draining lymph nodes.

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