Parasite kinetics and cellular responses in goats infected and superinfected with *Trypanosoma congolense* transmitted by *Glossina morsitans centralis*

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*Trypanosoma congolense* infected tsetse were fed on the flanks of goats at sites drained by the prefemoral lymph node. The efferent lymphatic of this lymph node was surgically cannulated and the lymph was collected daily and examined for appearance of parasites, lymph flow and cells. Trypanosomes were detected in the lymph 4 days after infection, which was 2 days prior to the appearance of the local skin reaction or the presence of parasites in the blood. Once the animal became parasitaemic, trypanosomes were found to recirculate in the lymphatic system, appearing in the lymph of the contralateral lymph node 11 days after infection. In goats infected with *T. congolense* and superinfected 12 or 13 days later with a different tsetse-transmitted *T. congolense* serodeme, parasites belonging to the second serodeme were apparently delayed in their development in the skin and appeared up to 7 days later in the efferent lymph when compared to control animals. This delay in development might have implications for field situations where superinfections frequently occur: it might result in limiting the number of serodemes of *T. congolense* an animal can be infected with at any one time.

Key words: *Trypanosoma congolense*; Goats; Interference; Superinfections; *Glossina morsitans centralis*; Lymph cannulation; Parasite kinetics; Serodeme

Introduction

Animals in tsetse-infested areas are under constant challenge by tsetse flies carrying various trypanosome species or serodemes. In these situations, superinfections of animals are known to occur frequently (Masake et al., 1987). Recently, interference in the establishment of tsetse-transmitted superinfection of animals infected with *Trypanosoma congolense* has been reported to occur in cattle (Morrison et al., 1982), goats (Dwinger et al., 1986) and rabbits (Luckins and Gray, 1983). The interference phenomenon was assessed by the failure to develop clinically detectable local skin reactions, an absence of rise in parasitaemia following superinfection, a delay in antibody response to the superinfecting trypanosomes, and a lack of immunity to subsequent challenge following treatment. However, it was not clear from these studies whether establishment of the second parasite population was completely or
partially arrested. Therefore, investigations were undertaken to collect efferent lymph from the lymph node draining the site of the tsetse bite and to study the kinetics of parasite appearance in the lymph and to monitor changes in lymph flow and cellular responses following superinfection.

Materials and Methods

Experimental animals

Adult male East African/Galla cross-bred goats of 20–30 kg body weight were obtained from an area in Kenya which is free of trypanosomiasis. The goats were treated against intestinal helminths and coccidia as previously described (Dwinger et al., 1987). Three weeks later they were housed in fly-proof isolation units, where they had free access to hay and water with a limited supply of concentrates. For subinoculation of lymph and blood samples and for trypanosome neutralization tests, Swiss outbred mice, 8 to 10 weeks old and of either sex, were obtained from the ILRAD breeding colony.

Trypanosomes

Trypanosoma congolense IL 1587 was a clone prepared from trypanosomes isolated from a dog in Nairobi, Kenya, in 1976 (Dwinger et al., 1989).

T. congolense ILNat 3.1 was a clone derived from STIB 212, which was isolated from a lion in the Serengeti area, Tanzania (Geigy and Kauffmann, 1973). The two T. congolense clones belong to two distinct serodemes as defined by serodeme-specific DNA probes (P. Majiwa, pers. comm.) and metacyclic variable antigen type (V.M. Nantulya, pers. comm.).

Tsetse flies

Glossina morsitans centralis originating from the ILRAD colony were fed daily from the teneral stage onwards on infected goats and kept at 25°C and 70% relative humidity. Tsetse were examined for infection as described by Dwinger et al. (1989). Tsetse harbouring infective forms were fed on the experimental animals as previously described (Dwinger et al., 1987).

Experimental design

One or 2 days after surgery, the goats received five to six bites at skin sites located in a half circle 2 cm anterior, dorsal and posterior to the skin incision (ipsilateral) necessary for cannulation of the lymphatics or on the flank opposite the one bearing the cannula (contralateral side). The sites were marked with a felt-tipped pen. All the tsetse flies used for superinfection were fed 3 days later on one mouse each, to determine whether they were still infective. The mice were then checked twice weekly for trypanosomes for one month or until positive, by microscopic examination of tail blood. The details of infection and superinfection (where applicable) of the nine experimental goats are shown in Table 1. Three weeks after superinfection, goat No.
### Table 1
Summary of infections and superinfections of nine goats infected with *Trypanosoma congolense* transmitted by *Glossina morsitans centralis*

<table>
<thead>
<tr>
<th>Goat number</th>
<th>Infected on D₀ with</th>
<th>Day of surgery</th>
<th>Site of infection in relation to position of cannula</th>
<th>Superinfected with</th>
<th>Day of super-infection</th>
<th>Site of super-infection in relation to position of cannula</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>T. congolense</em> IL1587</td>
<td>D₁</td>
<td>ipsilateral</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td><em>T. congolense</em> IL1587</td>
<td>D₁</td>
<td>ipsilateral</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td><em>T. congolense</em> ILNat 3.1</td>
<td>D₃</td>
<td>ipsilateral</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td><em>T. congolense</em> ILNat 3.1</td>
<td>D₁</td>
<td>ipsilateral</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td><em>T. congolense</em> IL1587</td>
<td>D₁₂</td>
<td>ipsilateral</td>
<td><em>T. congolense</em> ILNat 3.1</td>
<td>D₁₃</td>
<td>ipsilateral</td>
</tr>
<tr>
<td>6</td>
<td><em>T. congolense</em> IL1587</td>
<td>D₁</td>
<td>contralateral</td>
<td><em>T. congolense</em> ILNat 3.1</td>
<td>D₁₂</td>
<td>ipsilateral</td>
</tr>
<tr>
<td>7</td>
<td><em>T. congolense</em> IL1587</td>
<td>D₁</td>
<td>contralateral</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>8</td>
<td><em>T. congolense</em> IL1587</td>
<td>D₁</td>
<td>contralateral</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>9</td>
<td><em>T. congolense</em> IL1587</td>
<td>D₁</td>
<td>contralateral</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA = not applicable.

6 was treated with 7 mg/kg diminazene aceturate (Berenil, Hoechst AG, Frankfurt, F.R.G.) by intramuscular injection and challenged 21 days later with *T. congolense* ILNat 3.1 infected tsetse.

### Sampling technique

**Surgical procedure**

The efferent lymphatic duct from the prefemoral lymph node was cannulated in 9 goats. The surgical technique, originally described for sheep (Hall, 1967) and modified for goats (Emery et al., 1980), has been described in detail elsewhere (Dwinger, 1985). The lymphatics arising from the prefemoral lymph node were chosen for surgical cannulation, because of their easy access in goats and because the skin area being drained is readily accessible to tsetse bites and subsequent skin measurements.

**Lymph**

Lymph was collected at 4 to 6 h intervals and the volumes measured to calculate the lymph flow rate. On one occasion each day 1–2 ml of fresh lymph were collected into a heparinised tube. This lymph was used for subinoculation of mice, neutralization tests in mice, for counting lymphocytes and trypanosomes, and to make Giemsa-stained cytospin smears. The output of lymphocytes per ml of lymph was determined by counting cells in a haemocytometer. At the same time, trypanosomes were enumerated. When no parasites were found in the counting chamber, lymph was spun at 500 x g for 10 min and the sediment was examined for the presence of trypanosomes. Lymphocytes with a diameter greater than 12 μm were classed as lymphoblasts (Emery et al., 1980). The percentage of lymphoblasts present in the
lymph was calculated by examining Giemsa-stained cytospin smears microscopically with the use of an eyepiece containing a calibrated graticule with a grid division (Leitz, Wetzlar, F.R.G.). Cytospin smears were made by spinning 0.1 ml of lymph and 0.4 ml of phosphate saline glucose (PSG), pH 8.0, for 8 min at 800 x g in a Cytospin centrifuge (Shandon Elliott, U.K.). The preparations were air dried and fixed with methanol.

**Blood**

2 ml blood samples were collected daily from the jugular vein into EDTA-coated vacutainer tubes. From goat No. 9, blood was collected from the peripheral ear vein at the same time. During the first 8 days after infection, blood samples were subinoculated into mice. Packed red cell volume percent (PCV) was measured and the buffy coat was examined for trypanosomes using phase-contrast microscopy (Murray et al., 1977). The number of trypanosomes was estimated by a scoring method as previously described (Paris et al., 1982). Blood samples negative for trypanosomes were re-examined using the hematocrit centrifuge technique (Woo, 1970).

**Skin**

The development of chancres was assessed by measuring skin thickness at the bite sites with vernier callipers daily for a period of 16 to 18 days.

**Subinoculations into mice**

From each goat, 0.1 ml lymph samples and 0.2 ml blood samples were inoculated daily by intraperitoneal or intravenous injections into each of a total of four mice. The mice were monitored for parasites in the blood as described above. Subinoculations were continued until trypanosomes were detected microscopically in the lymph and the blood.

**Neutralization tests**

Following superinfection with *T. congolense* ILNat 3.1, the appearance of these parasites in the lymph was established by neutralizing the primary infection with immune serum against *T. congolense* IL 1587. Immune serum consisted of pooled sera taken 21 and 28 days after infection of three goats with *T. congolense* IL 1587 following tsetse transmission. Lymph was diluted with medium consisting of PSG, pH 8.0, and 10% (w/v) foetal bovine serum (FBS) to achieve a final concentration of 2 x 10^3 trypanosomes per ml. Equal amounts of test lymph and immune serum were added and incubated at 4°C for 30 min. Groups of six mice were each inoculated by intraperitoneal injection with 0.2 ml of the mixture. To serve as controls, groups of mice were inoculated with test lymph following incubation with PSG. Lymph collected from a goat with a primary infection of *T. congolense* ILNat 3.1 was mixed with immune serum against *T. congolense* IL 1587, incubated and then inoculated into mice to serve as an additional control. Neutralization tests were performed on lymph collected on days 1, 3, 4, 7, 8, 9, 10 and 11 after superinfection of goat No. 6 and on days 1, 5, 8 and 11 after superinfection of goat No. 5. Mice were checked for parasites as described above.
Breakthrough populations were investigated as follows: Teneral tsetse flies were allowed to feed on parasitaemic mice and then maintained on clean rabbits. Twenty one days later the infected tsetse were identified and permitted to probe singly through the netting of their tubes onto a polytetrafluoroethylene coated multispot slide (Wellcome, Essex, U.K.) preheated to 37°C. The metacyclets extruded were examined for the presence of variable antigen types (VAT) belonging to the superinfecting ILNat 3.1 serodeme by the indirect immunofluorescent antibody test (IFAT) using monoclonal antibodies against ILNat 3.1 metacyclic VATs. The IFAT was carried out on the fresh unfixed salivary probe materials as described by Nantulya et al. (1980). The monoclonal antibodies were raised against metacyclic forms of \textit{T. congolense} ILNat 3.1 following conventional methods (Nantulya et al., 1983). Metacyclic trypanosomes from tsetse infected with \textit{T. congolense} IL 1587 served as controls.

Results

\textit{Parasite kinetics, lymph flow and cellular responses in goats following a primary infection}

\textit{Skin thickness changes.}
Changes in skin thickness in goats infected with \textit{T. congolense} IL 1587 (Fig. 1) or with \textit{T. congolense} ILNat 3.1 were similar to those described previously by Dwinger et al. (1986).

\textit{PCV values}
During the first 18 days of infection, PCV values decreased from 36% to 24% (Fig. 1).

\textit{Trypanosomes in lymph and blood}
Parasites were detected in the lymph by mouse subinoculation in the two goats infected with \textit{T. congolense} IL 1587 4 days after infection. Trypanosomes were detected by direct microscopy in undiluted lymph at 7 days after infection in goat No. 1 and at 5 days after infection in the sediment of lymph of goat No. 2 following centrifugation. By day 7 parasite numbers had reached $2 \times 10^5$ per ml and thereafter fluctuated between $10^4$ and $10^6$ per ml in goat No. 1 (Fig. 1).

By subinoculating blood intravenously into mice, trypanosomes were detected generally earlier (4 days in the case of goat No. 1) than was possible by microscopic examination (Fig. 1).

Time of detection and parasite kinetics in lymph and blood of the goats Nos. 3 and 4, infected with \textit{T. congolense} ILNat 3.1, was similar to that described above for animals infected with \textit{T. congolense} IL 1587.

In goats 6, 7, 8 and 9 infected with \textit{T. congolense} IL 1587 on the contralateral flank, parasites were detected on day 6 after the tsetse bites by subinoculation of jugular blood into mice. Lymph was found to contain parasites several days later in the four goats involved, e.g. parasites were detected in the lymph of the contralateral flank of goat No. 6 on 11 days after the tsetse bite, using subinoculation into mice (Fig. 2).
Fig. 1. Parasite kinetics, lymph flow and cellular response in efferent lymph from the prefemoral lymph node of a goat (No. 1) in relation to the development of local skin reactions, parasitaemia and anaemia following the bites by tsetse infected with *T. congolense* IL 1587. *A* and *B*: △ = changes in skin thickness (mean of 6–7 sites) following infection with *T. congolense* IL 1587 transmitted by tsetse; ○ = number of trypanosomes (*T. congolense* IL 1587) per ml of lymph; ● = initial detection of trypanosomes in lymph by subinoculation into mice; ▯ = day of infection with *T. congolense* IL 1587; * = initial detection of trypanosomes in blood by subinoculation into mice; ■ = changes in packed red cell volume percentage (PCV); ○ = number of trypanosomes per ml of blood. *C*: - - - = lymph flow; - - - = lymphocyte output. *D*: - - - = percentage blast cells (cells with a diameter greater than 12 μm) in lymph.

**Lymph flow and cellular output**

In goats bitten by infected tsetse on the ipsilateral flank the lymph flow increased from 2 ml/h on the day of infection to nearly 5 ml/h 12 days later (Fig. 1). Large numbers of lymphocytes appeared in the lymph between 12 and 16 days after infection constituting an 180% increase in cellular output. The percentage of lymphoblasts increased from initially negligible levels to 34% of the total output on day 14 (Fig. 1).

In lymph drained from the contralateral flank (goat No. 6) a tenfold increase occurred in the output of total lymphocytes during the first 3 days after infection (4 days after surgical intervention), while the lymph flow did not alter significantly during the first 10 days (Fig. 2). Lymphoblasts constituted between 2 and 4% of the total cellular output on the contralateral side from day 4 onwards following primary infection.
Fig. 2. Parasite kinetics, lymph flow and cellular response in efferent lymph from the prefemoral lymph node of a goat (No. 6) in relation to the development of local skin reactions and parasitaemia following the bites by tsetse infected with T. congolense IL 1587 and 12 days later superinfected with T. congolense ILNat 3.1 transmitted by tsetse. The goat was infected on the flank contralateral to where the cannula was situated and superinfected on the ipsilateral flank. A and B: △ = changes in skin thickness (mean of 6-7 sites) following infection with T. congolense IL 1587 transmitted by tsetse; ▲ = changes in skin thickness (mean of 6 sites) following superinfection after 12 days with T. congolense ILNat 3.1 transmitted by tsetse; ○ = day of infection with T. congolense IL 1587; ● = day of infection with T. congolense ILNat 3.1; ○ = number of trypanosomes (T. congolense IL 1587) per ml of lymph; □ = number of trypanosomes (T. congolense IL 1587 and ILNat 3.1) per ml of lymph; ● = number of trypanosomes per ml of blood. C: — = lymph flow; — = lymphocyte output. D: — = percentage blast cells (cells with a diameter greater than 12 μm) in lymph.

Parasite kinetics, lymph flow and cellular responses in goats infected and superinfected with different T. congolense clones

Skin thickness changes
No detectable increase in skin thickness was observed following challenge of infected goats with tsetse infected with T. congolense ILNat 3.1 (Fig. 2).

PCV values
On the day of infection goat Nos. 5 and 6 had PCV values of 35 and 33%, and these had subsequently decreased 3 weeks later to 32 and 21%, respectively. A control animal cyclically infected with T. congolense ILNat 3.1 showed a decrease in PCV from 32% on the day of infection to 20% 3 weeks later.
Trypanosomes in lymph and blood
During the first days following superinfection, parasites in the lymph were completely neutralized by immune serum against *T. congolense* IL 1587 and none of the mice inoculated became parasitaemic. Control mice inoculated with test lymph mixed with PSG all became positive for trypanosomes. Similarly, control mice inoculated with lymph mixed with the heterologous immune serum, all became positive.

Breakthrough populations of the neutralization tests were transmitted through tsetse and then analysed using monoclonal antibodies directed specifically against metacyclic VATs of the superinfecting *T. congolense* ILNat 3.1. Using this technique parasites belonging to the serodeme used for superinfection were detected in the efferent lymph of goat No. 6 by day 7 after superinfection and in the lymph of goat No. 5 by day 11 (Fig. 2). Trypanosome numbers started increasing in the efferent lymph of goat No. 6 from 8 days after superinfection onwards to levels of $10^5$ parasites per ml (Fig. 2). In neither one of the two goats could a rise in parasitaemia be detected following superinfection with *T. congolense* ILNat 3.1 (Fig. 2).

Lymph flow and cellular output
The lymph flow increased temporarily in both animals 1 to 4 days after superinfection (Fig. 2c). The cellular output was raised in one of the goats (No. 6) at 7 days after superinfection accompanied by an increase in the percentage of blast cells from 4% on day 1 to 7% on day 7 after superinfection (Fig. 2).

Immunity to challenge following trypanocidal treatment
Following trypanocidal treatment, goat No. 6 proved to be immune to challenge by tsetse infected with the serodeme used for superinfection. Unfortunately, goat No. 5 could not be challenged.

Discussion
Following infection of goats with tsetse-transmitted *T. congolense*, parasites leave the site of the tsetse bite before a local skin response is apparent. The major route of dissemination is via peripheral lymph, lymph nodes and central lymph to the bloodstream. Detection of *T. vivax* and *T. brucei* in efferent lymph of goats has also been reported to occur prior to the development of the chancre or detection of parasitaemia (Emery et al., 1980; Barry and Emery, 1984) suggesting a similar route of dissemination. In the same way, parasites reach the bloodstream in *T. congolense* infected cattle (Akol and Murray, 1986). To our knowledge no investigations have been reported using lymph cannulations in goats infected with *T. congolense*.

It is likely that dilution of parasites in the large blood volume is responsible for their late detection in the bloodstream compared to the early detection in lymph. Moreover, lymph draining from the prefemoral lymph node passes through several other nodes before reaching the thoracic duct (Getty, 1975) and this might also delay parasite dissemination.

Following infection, a slight increase in lymph flow and lymphocyte output and a dramatic rise in the relative number of lymphoblasts coincided with the formation of the chancre. Small numbers of parasites were found consistently in lymph from the contralateral flank. This finding was confirmed in several goats by cannulating
lymphatics in the flank opposite the infected side and subinoculating the collected lymph in mice. Apparently *T. congolense* multiplies at the site of inoculation by the tsetse fly (Luckins and Gray, 1978) and in the regional lymph node (Luckins and Gray, 1979). In addition, the results of the present study suggest that organisms present in the blood can return to extravascular sites from where they are redirected via the lymphatics to the blood. Barry and Emery (1984) found *T. brucei* recirculating in peripheral lymph of goats, and Akol and Murray (1986) detected *T. congolense* in lymph collected from the prefemoral lymph node of a calf 15 days after infection on the opposite flank. Detection of large numbers of *T. congolense* in the efferent lymph ipsilateral to the site of the tsetse challenge and the consistent detection of small numbers of organisms in lymph on the contralateral side is contrary to earlier reports where these trypanosomes were absent or very scarce in the lymph of infected rats or sheep (Ssenyonga and Adam, 1975; Tizard et al., 1978). However, in the latter report parasites were inoculated directly into the bloodstream. The results of the present study indicate that *T. congolense* can not be regarded as strictly a blood parasite as has also been previously suggested by Luckins and Gray (1979).

Investigation of lymph of *T. congolense*-infected goats following superinfection with a different *T. congolense* serodeme showed a delay in the appearance of the superinfecting population. The delay ranged from 3 days (goat No. 6) to 7 days (goat No. 5) compared to the time of first parasite appearance in the lymph of goats with a single primary infection. Following superinfection, lymph flow increased over the initial 4 days, but lymphocyte output and relative number of blast cells hardly changed. The number of lymphoblasts did not increase following superinfection of the flank opposite the initial infection site, despite the presence of trypanosomes in the lymph. Both observations suggest a low antigen load following superinfection.

Previous reports (Morrison et al., 1982; Luckins and Gray, 1983; Dwinger et al., 1986) have shown that no local skin reactions and no rise in parasitaemia occur in the majority of cattle, rabbits or goats, following superinfection. Antibodies against the superinfecting parasites were not detected in goats, suggesting a complete arrest of the second infection either in the skin or in the circulation. Our investigations have shown that the growth of the trypanosomes used for superinfection was delayed in the skin. The result of this delay in development is that small numbers of parasites are present in the lymph and blood, but do not cause an obvious increase in detectable parasites in these compartments or an accelerated decrease in PCV levels. Moreover, it seems that the number of parasites from the superinfecting clone are often too few, in comparison to the large number of organisms from the primary infection, to prime the host in an immunologically detectable way. However, in some cases the superinfecting clone elicits a response sufficient to render the animal immune to subsequent homologous challenge as was observed in a previous study (Dwinger et al., 1986) and in the present study in goat No. 6, which showed a relatively short delay of 3 days in parasite development. Moreover, in studies in cattle a neutralising antibody response was readily detectable to metacyclic trypanosomes of the superinfecting clone, but slight responses were detected when the animals were challenged with bloodstream forms (Morrison et al., 1982).

Speculations on the mechanisms involved in the interference phenomenon have been discussed previously (Dwinger et al., 1986, 1989). Recent reports suggest that interference between development patterns of parasite stocks not only occurs
between different serodemes of the same trypanosome species but in certain cases between different species (Dwinger et al., 1989).

The significance for the situation in the field of the inhibition of parasite development, both in the skin and in the bloodstream, is that it might limit under natural challenge the number of serodemes of *T. congolesense* an animal can be infected with at any one time.

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