2003

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SUMMARY

The first visible response in livestock to the bite of a trypanosome-infected tsetse fly is the formation of a localized skin reaction, also known as a chancre. This is an inflammatory response in the skin associated with swelling and an influx of cells. It is thought to be associated with an acquired immune response to the injected metacyclic trypanosomes. In this study, we examined the role of T lymphocytes in the development of the inflammatory response, by depleting cattle of T cell subpopulations and monitoring the development of chancres. Depletion of CD4 cells, but not CD8 cells, resulted in a significant reduction in chancre formation, confirming that an acquired response mediates the inflammatory response. In addition, it was established that the CD4 T cells mediate the generation of memory for immunity to a homogueous re-challenge. The inflammatory response in the skin did not affect further progress of the infection.

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INTRODUCTION

After the bite by a trypanosome-infected tsetse fly, metacyclic forms establish in the skin where they differentiate into bloodstream forms and spread to the vascular system (1–3). The major route of dissemination is via the draining lymph node, and trypanosomes bearing metacyclic antigens are observed in the afferent lymph, but not in the efferent lymph where only bloodstream forms are detected (4). The first visible sign of a response to the parasite in the mammalian hosts (ruminants, man, rabbits) is the appearance a few days later of a local skin reaction or chancre at the infected bite-site (5,6), which always precedes the presence of parasites in the blood. In cattle, the local skin reactions can measure up to 10 cm in diameter and are red, hot, oedematous and painful. The swelling is caused initially by a massive influx of polymorphonuclear cells at the bite site, followed by infiltration of lymphoid and macrophage cells, and reaches a peak during the second week, after which time it subsides to undetectable levels a week later. Lymphocyte subpopulations have been monitored in the skin at the site of the chancre in sheep, and initially a rise in B- and all T-cell populations (CD4, CD5, CD8) was observed, with a marked increase of the CD4/CD8 ratio (7). Degranulating mast cells have been observed 5 days after infection (8). Chancres are the consequence of a local inflammatory response against the parasite, but not tsetse salivary products, as trypanocides abrogate the chancre (9) and intradermal inoculation of *in vitro* cultured metacyclic forms will induce chancres (2,10–12). The onset, size and duration of the chancre correlate with the number of metacyclic parasite forms that are inoculated into the skin. As tsetse flies inject a higher number of metacyclics of *Trypanosoma brucei* strains than they do of *Trypanosoma congolense* and *Trypanosoma vivax* (13), the most severe chancres are observed with *T. brucei* infections, followed by *T. congolense*, while only a small nodular reaction is seen with *T. vivax* (14,15). Intradermal

inoculation of bloodstream forms can also induce detectable chancres, suggesting that the inflammatory reaction is not due to any specific metacyclic product (11).

When infected tsetse flies were fed on an animal with an ongoing trypanosome infection, no chancres were formed and no superinfection was established, irrespective of whether a homologueous or heterologous trypanosome serodeme was used (16,17). This phenomenon is called interference, because it is not the result of an immunity, but needs the presence of an active infection. When the animal was treated after the first infection with a trypanocidal drug before the challenge infection, chancres were formed and infection was established in the blood (7,16). However, if the challenge infection after the trypanocidal treatment was given with the homologueous trypanosome serodeme, reactions did not occur, no parasitaemia was observed in the blood and the animals showed solid immunity (9,18,19). This suggested that an immune response is generated in the chancre, directed against the variable antigen type (VAT) of the metacyclic forms. Timing of the trypanocidal treatment after the primary infection showed that establishment of immunity correlated with the time of chancre formation (9). The capacity of lymph and serum to neutralize infectivity of the homologueous trypanosomes after establishment of a chancre, suggested that the host mounted an antibody response against the metacyclic trypanosomes. This antibody response coincided with the achievement of immunity (4,9). This is corroborated by the presence of a large number of trypanosomes in the chancre (1–3) and the demonstration that the percentage of trypanosomes with the metacyclic VATs remained high in the chancre (20,21) and the afferent lymph, but not in the efferent lymph (4). Furthermore, immune animals could still be infected by injecting bloodstream forms, suggesting that the immunity was only raised at the level of the metacyclics (20). In those trypanosome strains, such as *T. vivax*, that did not produce chancres, immunity was much more difficult to induce (22).

Depletion of T-cell subpopulations in cattle by use of mouse monoclonal antibodies has been demonstrated (23) and complete depletion of CD4 and CD8 T cells in lymphoid tissues was possible for a time span of 2–3 weeks (24), which is long enough to see the formation and disappearance of the chancre. In this paper we analysed the role of T-lymphocyte subpopulations in the development of the chancre and in the establishment of immunity to metacyclics, by comparing untreated and *in vivo* depleted cattle.

MATERIALS AND METHODS

Cattle

Indigenous trypanosusceptible Boran cattle (*Bos indicus*) or West-African trypanotolerant N'Dama cattle (*Bos taurus*),

all born and raised at the breeding farm of the institute, were used. Their ages ranged from 6 to 18 months. Experimental procedures and animal management protocols were undertaken in accordance with the requirements of the Institute Animal Care and Use Committee.

Tsetse flies

Tsetse flies (*Glossina morsitans centralis*) were bred in the institute, and fed on goats infected with *T. congolense* clones IL-1180, IL-13E3 or IL-2079 as previously described (11). IL-1180 and IL-2079 were derived from STIB-212 and STIB-249, respectively, and isolated from a lion in the Serengeti (25). Both were antigenically different (26). IL-13E3 (9) was derived from an isolate made from an infected cow in Busoga in 1962.

Infections and chancre formation

In all experiments, five tsetse flies that were infected with *T. congolense* clone IL-1180 or IL-13E3 were allowed to bite on the shaved left flank of the animal. The spots on the skin on which the flies had fed were marked by ink. Formation of chancres was monitored by measuring double skin thickness with vernier callipers, before the bite and then daily from days 8–14 post infection. Results were expressed as the skin thickness of the chancre on the day with highest swelling minus the skin thickness of the same site before the fly bite.

Appearance of parasitaemia in the blood was monitored by dark ground phase contrast microscopic examination of the buffy coat (27). The cattle were treated when packed cell volume (PCV) dropped below 13%, or at the end of the experiment after 6 weeks of infection, by deep intramuscular injection with diminazene aceturate (Berenil®; Hoechst, Frankfurt, FGR) at a dose of 7 mg/kg body weight to eliminate the infection.

In the re-challenge infection experiment, seven cattle were infected with *T. congolense* clone IL-13E3 and 6 weeks later they were treated. The animals were re-challenged with the homologueous clone 1 years after the trypanocidal treatment. The re-challenged cattle were treated 3 weeks after the tsetse-fly bite, and a second re-challenge infection with an unrelated *T. congolense* clone (IL-2079) was carried out 2 months later. Animals were treated after 1 month.

T-cell depletion

CD4 and CD8 T-cell subpopulations were depleted in two different experiments, respectively, using monoclonal antibodies to BoCD4 (IL-A11) and BoCD8 (IL-A105) according to the method described in detail previously (24). A total of 23 mL of filter-sterilized ascitic fluid from monoclonal

Table 1 Schedule of monoclonal antibody administration to obtain complete depletion of CD4$^+$ or CD8$^+$ cells

Day before infection	Quantities of ascitic fluid administered i.v., with 1-h intervals
−3	25 µL, 25 µL, 50 µL, 50 µL, 100 µL, 200 µL
−2	50 µL, 200 µL, 500 µL, 2 mL
−1	100 µL, 1 mL, 5 mL, 14 mL

antibodies IL-A11 or IL-A105, containing, respectively, 5·2 and 8·1 mg antibody/mL, was injected i.v. using the schedule summarized in Table 1, and leaving at least 1 h between each injection.

Progress of depletion of the T-cell subpopulations was monitored in blood by flow cytometry as described previously (24).

Data analysis

Statistical analysis of variance of skin thickness was performed using Genstat software. Differences were considered significant when $P < 0.05$.

RESULTS

CD8 depletion

In a first experiment, chancre development was compared between *Bos indicus* cattle that were either completely depleted for CD8 cells or non-depleted. Four cattle were depleted with monoclonal antibody IL-A105, starting 2 days before infection, and five animals were left untreated. CD8-positive cells were completely removed from blood of the treated animals. They reappeared around week 2 (Figure 1) and their percentage in blood leucocytes remained at 1% until
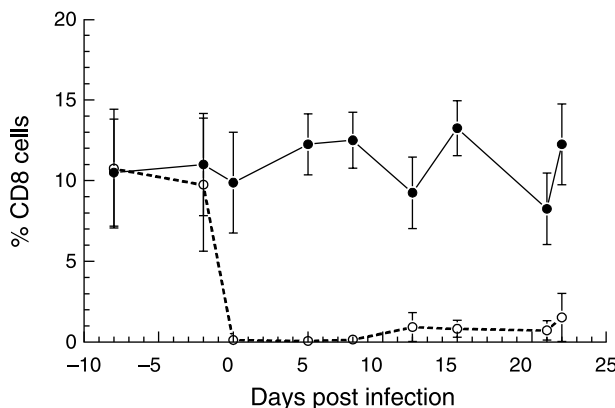
Figure 1 Percentage of CD8-positive cells in blood of depleted (mean of four with standard deviations) and non-depleted (mean of five) cattle.

week 3. In the non-depleted animals, the percentage fluctuated between 8 and 13%. On day 0, all animals were bitten by five tsetse flies infected with *T. congolense* clone IL-1180. Chancres developed on almost every bite site in the control group (24/25 chancres, one negative site on Ind221) and in the CD8-depleted group (19/20 chancres, one negative site on Ind225). The skin thickness at the chancres varied within and between animals (Table 2), but no statistically significant differences were observed between the CD8-depleted and non-depleted groups. Parasitaemia developed in all cattle and the period between infected tsetse-fly bite and detection of parasites in the two groups was not significantly different (12·8 ± 0·4 vs. 13·5 ± 1·3 days, respectively).

CD4 depletion

The role of CD4 T-cells in the development of the chancre was assessed in a second experiment, in which three *Bos taurus* cattle and three *Bos indicus* cattle were depleted for CD4

Table 2 Effect of CD8 depletion on chancre formation at the site of the tstetse bite

Animal	Trypanosome serodeme	Depleted T cells	Mean increase skin thickness[a]	Day to detection of parasitaemia[b]
Ind113	IL-1180	–	8·8 ± 1·8	13
Ind220	IL-1180	–	3·2 ± 0·1	14
Ind221	IL-1180	–	6·0 ± 0·6	12
Ind222	IL-1180	–	6·1 ± 0·8	16
Ind223	IL-1180	–	4·3 ± 0·1	16
Ind218	IL-1180	CD8	4·2 ± 0·4	14
Ind219	IL-1180	CD8	4·6 ± 0·2	16
Ind224	IL-1180	CD8	9·7 ± 0·7	16
Ind225	IL-1180	CD8	3·9 ± 0·6	14

[a]In mm (means and standard deviation of five bite sites).
[b]Day after infection that first parasites were detected in the blood.
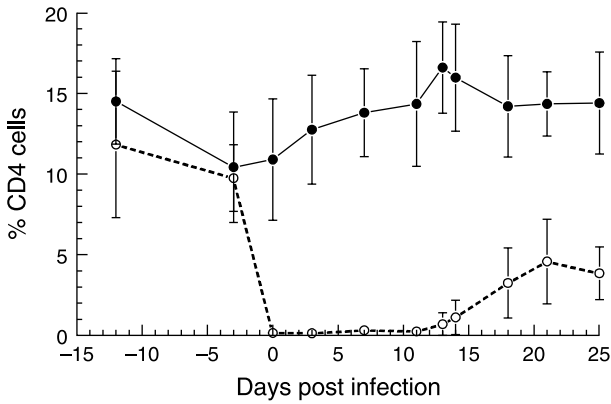
Figure 2 Percentage of CD4-positive cells in blood of depleted (mean of six with standard deviations) and non-depleted (mean of four) cattle.

cells, and compared with two non-treated animals of each breed. The CD4 cells were successfully removed from the blood, but reappeared after 2 weeks (Figure 2). By week three, an average of 3–4% of CD4 cells were found in the animals. On day 0, each animal was bitten by 10 tsetse flies infected with *T. congolense* clone IL-1180, five on each flank.

The results in Table 3 show a significant reduction ($P < 0.05$) in chancre formation in the CD4-depleted group. The mean increase in skin thickness was 3.3 ± 1.1 mm in the non-depleted, while only 1.3 ± 1.5 mm in the CD4-depleted cattle. However, variation within the depleted group was large, with one animal having typical chancre formation (Ind92) and another having no chancres (Tau93). The time of first appearance of trypanosomes in blood did not significantly differ between the non-depleted and CD4-depleted groups (14.2 ± 1.8 vs. 15.1 ± 1.2 days, respectively) and infection was established in all animals.

Re-challenge experiments

A primary infection with *T. congolense* clone IL-13E3 was established in seven cattle, four of which were depleted for CD4 cells (two *Bos indicus* and two *Bos Taurus*) and three controls were not depleted. The number of chancres in the three control animals was 14 out of 15 bites, indicating that the animals were sensitive to IL-13E3 metacyclics. In the depleted animals, only five chancres were observed out of 20 bites, and all five occurred on the same animal. But the increase in skin thickness in this depleted cow was only about half of that in the non-depleted controls (Table 4). Differences between the two groups were statistically significant ($P < 0.005$). The time of detection of trypanosomes in the blood did not significantly differ between the non-depleted (13 ± 0 days) and CD4-depleted group (12.3 ± 1.1 days). Six weeks into the infection, trypanocidal therapy was given to all animals to clear infection.

After a period of 1 year, the same cattle were tested for their reactivity to a homologueous infection. Skin thickness differed significantly ($P < 0.05$) between the two groups. The control group that was not depleted in the primary infection developed a weak skin swelling (2.3 ± 0.3 mm) during the re-challenge. This is less than the swelling in the primary infection (5 ± 1 mm) and suggests that, in this experiment, some degree of memory was established during the primary infection. This memory was not sufficient to produce sterile immunity and prevent re-infection. The group that was depleted for CD4 cells before the primary infection developed typical chancres after the homologueous re-challenge (6.9 ± 2 mm), suggesting that no immunity had been established during the primary infection.

A second re-challenge infection was carried out to ensure that an unrelated *T. congolense* serodeme (IL-2079) was not affected by the previous T-cell depletion. Both groups

Table 3 Effect of CD4 depletion on chancre formation at the site of the tstetse bite

Animal	Trypanosome serodeme	Depleted T cells	Mean increase skin thickness[a]	Day to detection of parasitaemia[b]
Ind88	IL-1180	–	3.0 ± 0.2	15
Ind91	IL-1180	–	3.6 ± 0.2	14
Tau89	IL-1180	–	4.6 ± 0.1	12
Tau92	IL-1180	–	2.0 ± 0.2	13
Ind87	IL-1180	CD4	1.75 ± 0.3	13
Ind90	IL-1180	CD4	0.48 ± 0.09	12
Ind92	IL-1180	CD4	4.00 ± 0.1	13
Tau90	IL-1180	CD4	1.00 ± 0.1	13
Tau91	IL-1180	CD4	0.44 ± 0.03	13
Tau93	IL-1180	CD4	-0.01 ± 0.03	13

[a]In mm (means and standard deviation of 10 bite sites).
[b]Day after infection that first parasites were detected in the blood.

Table 4 Effect of CD4 depletion on formation of chancres after multiple infections

Animal	1st infection IL-13E3		2nd infection IL-13E3		3rd infection IL-2079	
	Depleted T cells	Mean increase skin thickness[a]	Depleted T cells	Mean increase skin thickness[a]	Depleted T cells	Mean increase skin thickness[a]
Tau49	–	6·1 ± 1·6	–	2·5 ± 0·3	–	11·4 ± 2·0
Tau50	–	4·6 ± 2·8	–	2·4 ± 1·9	–	11·1 ± 4·5
Ind349	–	4·3 ± 1·0	–	1·9 ± 1·7	–	4·3 ± 3·6
Tau47	CD4	0·6 ± 0·6	–	4·1 ± 2·1	–	12·7 ± 0·4
Tau48	CD4	0·5 ± 0·7	–	7·5 ± 3·6	–	4·0 ± 1·8
Ind300	CD4	0·2 ± 0·4	–	6·9 ± 2·1	–	6·0 ± 2·9
Ind301	CD4	2·6 ± 0·6	–	9·1 ± 3·1	–	8·2 ± 2·6

[a]In mm (means and standard deviation of five bite sites).

reacted strongly and the mean increases in skin thickness were very similar: 7·7 ± 3·7 mm in the group that was CD4-depleted in the primary infection and 8·9 ± 4·0 mm in the control group.

DISCUSSION

There was a large variation in the extent of chancre reduction in the CD4-depleted cattle, with both extremes encountered: one animal having no chancres at all and another developing near-normal chancres. One explanation is that this variation is due to possible differences in the degree of CD4^{+} cell elimination in the skin. No correlation between the time of reappearance of CD4 cells in the blood and chancre formation was found in the depleted animals. The extent of cell depletion in the skin after *in vivo* administration of monoclonal antibody has never been tested before and may be harder to achieve than depletion in blood or lymphoid tissues. In an attempt to improve local depletion of CD4 cells in the skin, one animal (not part of this experiment) was additionally depleted by subcutaneous injection of 5 mL of CD4 ascitic fluid at the site of infection. Although the swelling was not as high as that in non-depleted animals, skin thickness was still increased after tsetse-fly bite, suggesting that complete inhibition of chancre formation may not be dependent on removal of CD4 cells from the skin alone. However, only immunohistology of skin sections could unambiguously demonstrate a relationship between remaining CD4 cells in skin and chancre formation. Another explanation is that differences in the individual's immune status could account for the variations observed amongst animals. Other cell populations, such as γδ-T lymphocytes, may be able to compensate for the depleted CD4^{+} T cells, or macrophages and other antigen-presenting cells may directly stimulate an inflammatory response. These cell populations, including CD8^{+} T cells, have been demonstrated in chancres (7).

The re-challenge experiment suggested that memory is mediated by CD4^{+} T lymphocytes. Indeed, the control animals produced typical chancres during the first infection, and developed little swelling of the skin after a homologueous re-challenge. This suggests that non-depleted animals developed some degree of memory during the primary infection which diminished the inflammatory response against a homologueous re-challenge, as suggested by (9) and (20). It is possible to obtain sterile immunity to a homologueous infection (18), but the period of 1 year between treatment and re-challenge in our study may have been too long. In contrast, animals that lacked CD4^{+} cells formed no, or less severe, chancres during the first infection, but produced typical chancres during the homologueous re-challenge. The absence of CD4^{+} T cells prevented the development of an inflammatory response in the skin and, as a consequence, prevented a build-up of memory. These animals were thus susceptible to a homologueous re-challenge. Depleted and non-depleted animals alike were still equally susceptible to a heterologous challenge.

The results show that *in vivo* depletion of CD4^{+} T cells before inoculation of trypanosomes by tsetse-fly bite resulted in a significant reduction of chancre formation. This did not affect the establishment of infection and presence of trypanosomes in blood. This suggests that CD4^{+} T lymphocytes play a major role in the initiation of inflammation and the formation of a chancre, and in establishment of memory to a homologueous challenge. In contrast, removal of CD8 cells did not have any effect on the degree of chancre development. These studies confirm that CD4^{+} T cells, but not CD8^{+} T cells, are critical for a VAT-specific response and memory associated with the primary histologic response. CD4^{+} helper cells and IFN-γ have been shown to be critical for the protection of mice against *T. b. rhodesiense* infections (28,29). In other murine models, CD8^{+} T cells have been shown to modulate trypanosome growth after non-specific activation by a trypanosome molecule, TLTF, and secretion

of INF-γ (30,31). It is obvious that in our host–parasite model, this non-specific activation does not contribute to the inflammatory response in the skin and to induction of memory. Also, CD8 cells did not contribute to parasite control and pathogenesis during a systemic infection (32).

The formation of the chancre in response to inoculation of metacyclic trypanosomes by tsetse-fly bite and induction of homogueneous immunity is, to a large degree, dependent on the CD4$^+$ T lymphocyte population. However, the size of the chancre does not necessarily influence the progress of infection into the blood and trypanosomosis in cattle.

ACKNOWLEDGEMENTS

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