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The sequential cellular changes in the local skin reaction produced in goats by *Glossina morsitans morsitans* infected with *Trypanosoma (Trypanozoon) brucei*

D. L. Emery, S. K. Moloo

Summary

Sequential biopsies of the skin reaction elicited in goats by *Glossina morsitans morsitans* infected with *Trypanosoma (Trypanozoon) brucei* were examined histologically to identify and to quantify the cellular populations involved in the reaction. The peak of the tissue response occurred 7–8 days after challenge with infected tsetse and preceded the initial detection of parasitaemia by 4–5 days. Microscopically, the cellular reaction was characterized initially by a marked infiltration of polymorphonuclear leucocytes (PMN) which were replaced by lymphoid cells. Plasma cells and macrophages were numerous during the decline of the skin reaction. Marked enlargement of, and germinal centre formation in the regional lymph node accompanied the development and regression of the chancre. The results suggest that the formation of a chancre is dependent on the skin thickness of the host at the site of tsetse challenge.

Key words: Trypanosoma (Trypanozoon) brucei; Glossina morsitans morsitans; goat; skin reaction; mice; chancre.

Introduction

Local skin reactions of delayed onset and of variable duration characteristically appear in man and rabbits as sequelae to the bite of tsetse infected with *Trypanosoma rhodesiense* (Fairburn and Godfrey, 1957; Willett and Gordon, 1957). This reaction is termed the chancre. Similar reactions have been reported following tsetse challenge of calves with *T. congolense* (Roberts et al., 1969; Luckins and Gray, 1978). In the chancre, trypanosomes undergo localized pro-

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liferation prior to invasion of the circulation as bloodstream forms. Trypanosomes are found in fluid expressed from the tissue lesions several days before they can be detected in peripheral blood (Willett and Gordon, 1957; Seed and Gam, 1967; Luckins and Gray, 1978).

In species in which it has been described it is not clear whether the chancre is a host reaction directed towards containment of the trypanosomes after their multiplication, or an immune response aimed at the prevention of their proliferation. As part of a study to assess the contribution of the chancre either to retard or to facilitate the dissemination of trypanosomes, this paper describes, from sequential skin biopsies, the detailed histology of the chancre induced by challenge of goats with tsetse infected with *T. (T.) brucei*. The development of the parasite in the tissue lesion and its systemic appearance are compared with the kinetics and intensity of the local cellular response from the host.

Materials and methods

Animals and infection

Goats: Adult male East African goats of mixed breed aged 12 months and weighing 20–25 kg were obtained from an area of Kenya which is free of trypanosomes and were housed in insect-proof isolation units and allowed free access to hay and water.

Mice: Male C57B1/6 mice aged 16 weeks were obtained from the ILRAD colony. These originated from stock obtained from Bomholtgard (Denmark).

Tsetse and trypanosomes. Stabilate *T. (T.) brucei* (ILRAD 20E3) was derived from STIB 247 after a passage of 4 days in irradiated mice. STIB 247 was prepared from a rat 28 days after infection with blood obtained on 3/12/71 from a naturally infected Kongoni in Serengeti National Park. Rats infected with *T. (T.) brucei* (20 E3) were killed when the parasitaemia was high and the blood collected from the heart was defibrinated. Teneral *Glossina morsitans morsitans* from ILRAD R_6 colony were fed in vitro upon this infected blood at 37° C through silicone membranes. They were subsequently fed in vivo on the flanks of goats every day, except Sundays and were kept individually in Geigy-1 cages. Between day 20 and 30 after the infected blood meal intake, the tsetse were allowed to probe on slides at 37° C, and those with metatrypanosomes in their saliva were used for subsequent challenge.

Experimental design: Three experiments were conducted. As a preliminary investigation to determine that a chancre develops as a result of infection with trypanosomes as distinct from the tsetse bite per se, a goat was bitten by three tsetse infected with *T. (T.) brucei* at different sites on the left flank, which had been clipped previously. On the contra-lateral flank, three uninfected tsetse were allowed to feed; these served as controls. The position of each 'bite' was marked. Following the successful induction in this goat of skin reactions at the sites of challenge with infected flies, six goats were each bitten with 4–6 tsetse infected with *T (T.) brucei* to evaluate sequentially the histopathology of the local response. Chancres resulted from 26 out of 30 bites and the remaining 4 sites were each located on different animals. An additional 4 goats were each bitten with 4–5 uninfected tsetse to provide control material.

In a third experiment to examine whether challenge with infected tsetse could provoke the development of a local skin reaction in laboratory animals, 5 C57B1/6 mice were each bitten on the belly by a tsetse infected with *T. (T.) brucei.*

Sampling techniques

Skin thickness and size of lymph nodes: Skin thickness at the site of challenge was measured daily with a pair of vernier calipers. The length and breadth of the regional prefemoral lymph node, which could be readily palpated subcutaneously, was also measured daily with calipers.

Parasitaemia: Daily blood samples were drawn from a peripheral ear vein (goats) or from the tail (mice) into EDTA-coated capillary tubes. Packed cell volume (PCV) or haematocrit, was measured and the buffy coat was examined under dark field microscopy for trypanosomes (Murray et al., 1977).

Skin biopsy: A biopsy of the site of challenge was taken from each of 3 goats (infected bites) and 2 goats (uninfected bites) 4 hours after the application of tsetse (day 0) and on each of days 2, 3, 4, 6, 7, 8, 12, and 20 after challenge. Goats were anaesthetized with 0.15 to 0.20 ml Rompun (Bayer) intravenously. The skin site was scrubbed, shaved and disinfected. A skin biopsy 2.5 cm $\times$ 0.5 cm including epidermis, dermis and panniculus carnosus muscle (cutaneous muscle) was excised and placed immediately in Bouin's or Mercuric Chloride-formal fixative. In each case, the biopsy was taken through the central area of the lesion and included normal skin at each extremity. Each chancre was biopsied only once, and the site was dusted with penicillin powder and covered with a sterile gauze dressing.

Quantitative histology

Skin sections 5 μm thick were cut from paraffin-embedded blocks and stained with either Giemsa or Mayer's haematoxylin and eosin. Sections were examined with a Leitz Orthoplan microscope using a 40$\times$ oil objective and a GW 10$\times$ eyepiece containing a calibrated graticule (Leitz-Wetzlar). Four sections from each biopsy were studied, and within each section, 4 representative fields (each 0.08 mm^2) were examined to enumerate the total numbers of cells and the numbers of each cell type per unit area (mm^2); because at certain stages of the infection, the cellular infiltrate appeared to be concentrated perivascularly, separate cell counts were performed in each field for perivascular sites and areas of dermal connective tissue distant from blood vessels. In each location, differential cell counts were performed on 200 cells. The number of trypanosomes in a field was scored as follows; +, 5–50 trypanosomes per mm^2; ++, 50–250 trypanosomes per mm^2; +++, 250–500 trypanosomes per mm^2; ++++, greater than 500 trypanosomes per mm^2.

Results

The clinical course of the chancre

In contrast to the absence of any discernible skin reaction in response to the bites of uninfected flies, chancres appeared in areas where infected flies had fed (Fig. 1). A discrete indurated cutaneous nodule formed at the site 4–5 days after challenge and rapidly increased over the next 3 days to present as a firm, inflamed and raised plaque accompanied by marked subcutaneous oedema, heat and pain. The skin surface at this time appeared brawny, and assumed a darker pigmentation. After 8 days the reaction became more diffuse and subsided gradually until the goats succumbed to the disease about 21 days after challenge. Skin thickness increased from 4 mm at the time of initial infection to 11 mm at the height of the response (Fig. 1), but the area had resumed close to normal dimensions when the host died.

The appearance of the chancre preceded by 4 to 6 days the first demonstration of trypanosomes in the blood (greater than 6–8 $\times$ 10^3 per ml) and the peak of the skin reaction had passed before parasitaemia was detected initially 9–10 days after challenge. The PCV declined steadily throughout the course of the infection (Fig. 1).

The chancre was accompanied by a pronounced enlargement of the

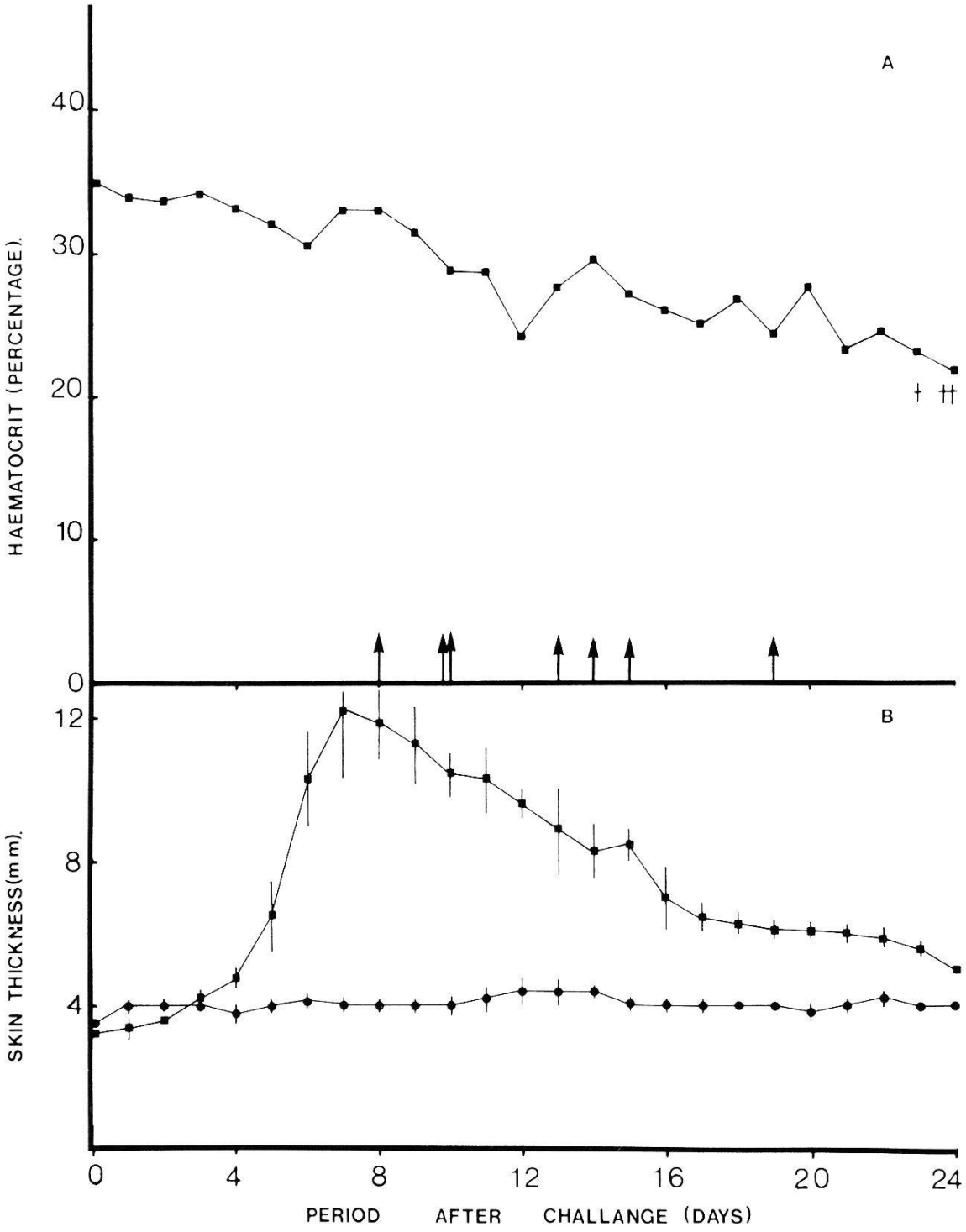

Fig. 1. A. Changes in haematocrit of 5 goats following infection with *T. brucei* (↑ = detection of parasitaemia; † = deaths). B. Changes in skin thickness at the site of challenge with tsetse infected with *T. (T.) brucei* (■ = sites of challenge with infected flies; ● = uninfected challenge sites).

regional prefemoral lymph node. A three-fold increase in volume above normal size was observed in the node at the peak of the response. At post-mortem examination of 5 moribund goats, the prefemoral lymph node draining the chancre weighed 12.4 ± 2.5 g (1 S.D.) and was significantly greater than the weight of 4.5 ± 1.6 g for the contralateral lymph node ($p < 0.01$).

Sequential histology of the tissue reaction

Although the majority of chancres appeared synchronously at 4 days after challenge, skin biopsies taken from chancres which appeared initially on the 5th day (2 out of 26) were grouped for histological analysis with the former samples, with a correction of +1 day for their belated development.

1. Detection of trypanosomes in tissue sections

Trypanosomes were first detected in histological sections prepared from sites of challenge 3 days after infection. Massive numbers of the parasites were found in the dermis and subcutis from 3–8 days until the peak of the tissue reaction. Thereafter, the number of trypanosomes declined, and parasites could not be detected in chancres taken from goats in the terminal stages of the disease. Trypanosomes were scattered throughout the inflammatory exudate between collagen bundles and focal accumulations of parasites were found at the bases of several isolated hair follicles. A small number were located in dermal lymphatics which were distended with amorphous proteinaceous material. Trypanosomes were not observed in superficial blood vessels in the biopsies. This was possibly due to the method of tissue fixation in which the intravascular contents were either lysed or eluted from the sections. The presence of trypanosomes in the chancre preceded by 5–7 days, the initial detection of parasitaemia in the peripheral circulation.

2. Quantitative changes in the cellular populations in the skin

The histological anatomy of normal caprine skin is shown in Fig. 2. In addition to the fibroblasts and reticulum cells responsible for the production of structural components, the dermis of ruminants contains a significant population of lymphocytes and plasma cells (Table 1) which probably represent the normal physiological response to epidermal microflora. A small proportion of eosinophils and mast cells are found commonly in connective tissue spaces, especially in areas adjacent to dermal blood vessels.

Following challenge with infected tsetse, the relative proportions and numbers of cells in dermal and perivascular locations remained relatively constant for the first 3 days after infection, except for a rapid absolute decline in the number of plasma cells and increases in the proportions of macrophages and eosinophils. Over the ensuing 4 days, a marked extravasation of polymorphonuclear leucocytes and mononuclear lymphoid cells occurred coincident with the morphological development of the chancre (Fig. 3). Both cell types appeared initially as perivascular accumulations, but within 24 h tissue infiltration was extensive. The cellular infiltration was accompanied by vascular congestion and inflammatory exudation which produced extensive dermal and subdermal oedema (Fig. 4). The structural integrity of the dermal collagen was disrupted by the reaction and many bundles appeared fragmented. At no stage was the

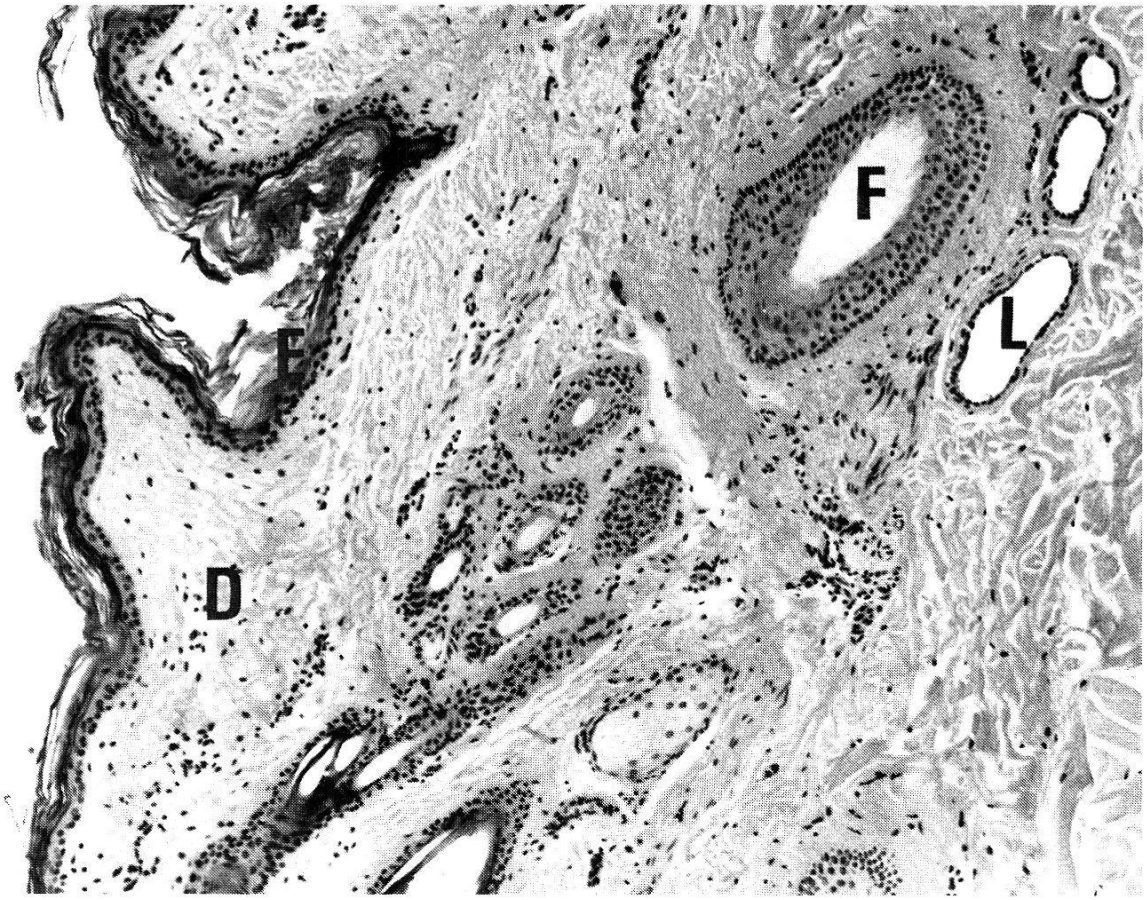

Fig. 2. Histology of normal caprine skin showing epidermis (E), dermis (D), hair follicles (F) and dermal afferent lymphatics (L) (H & E × 100).

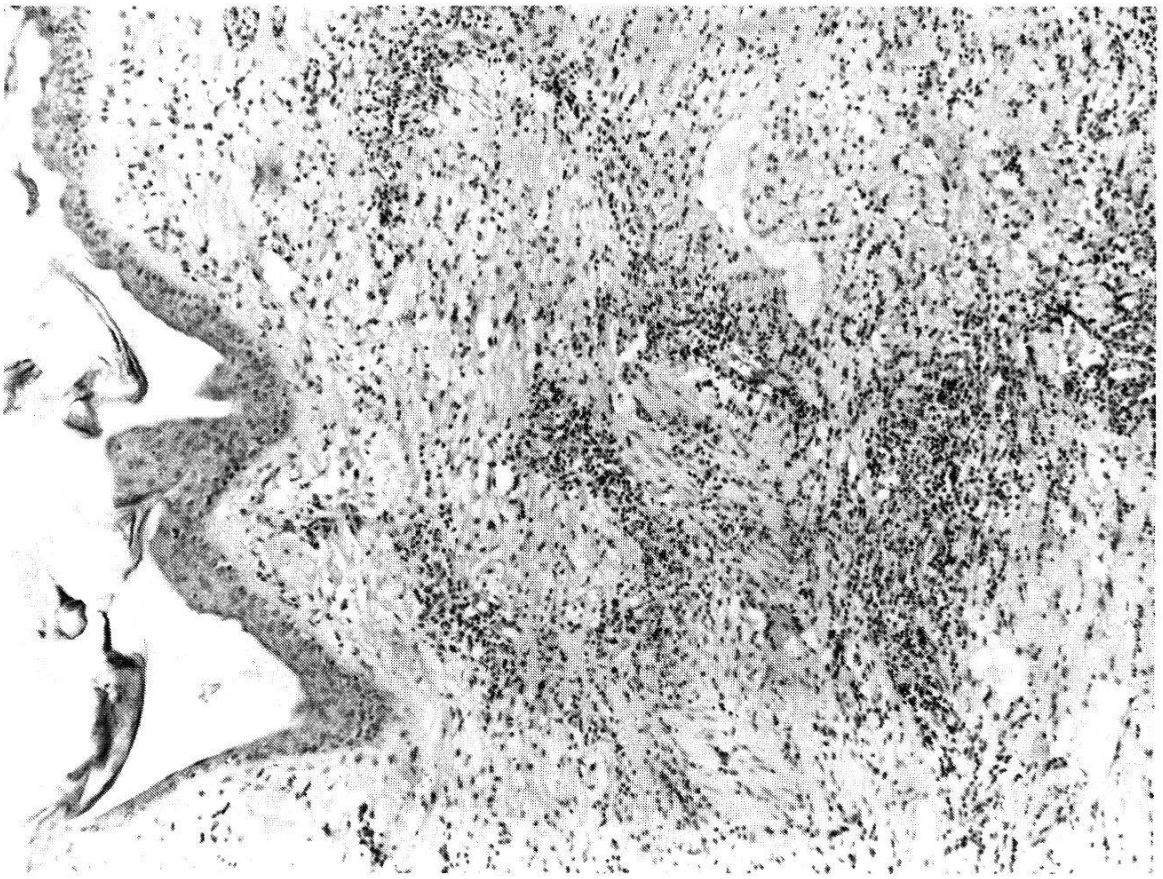

Fig. 3. The histology of the caprine chancre at the height of the tissue reaction 8 days after challenge (H & E × 100).

Table 1. Changes in cellular populations in the skin reaction elicited by *T. (T.) brucei*

Parameter	Period after challenge (days)									
	Contr.	0	2	3	4	6	7	8	12	20
a) The dermal connective tissue										
Total cells	517	689	566	922	1943	2288	2177	1796	2361	1218
Trypanosomes	–	–	–	+	++++	+++	+++	+++	+	–
PMN	<2.6	<3.6	2.9	<4.8	302	735	639	484	465	<6.3
Lymphocytes	150	186	123	268	846	652	722	633	857	328
Lymphoblasts	<2.6	<3.6	<2.9	<4.8	<10	24	<11	<9.3	<12	47
Plasma cells	177	200	65	91	131	84	61	93	171	303
Macrophages	21	36	50	48	111	202	165	93	49	29
Eosinophils	11	14	21	57	50	12	29	37	12	33
Tissue eosinophils	<2.6	<3.6	<2.9	24	<10	<12	<11	19	24	21
Mast cells	11	21	48	48	90	36	45	19	73	97
Others	186	257	268	426	481	644	586	484	698	406
b) The perivascular cellular population										
Total cells	517	713	972	1156	4120	2632	3247	2804	3296	3665
PMN	11	22	35	36	171	546	663	465	273	19
Lymphocytes	220	281	337	473	2.605	1.446	1.548	1.163	1.572	2.318
Lymphoblasts	2.7	3.7	5.0	6.0	214	55	67	145	238	342
Plasma cells	166	222	137	168	192	82	202	349	342	570
Macrophages	11	37	96	102	470	273	414	116	136	239
Eosinophils	5	15	25	78	43	14	57	29	34	38
Tissue eosinophils	2.7	3.7	5.0	6.0	21	14	17	58	17	19
Mast cells	11	30	71	60	107	27	34	29	68	114
Others	113	144	282	186	458	300	381	543	510	228

Numbers of each cell type are expressed per mm^2 in sections 5 μm thick. For further details, see "Materials and methods".

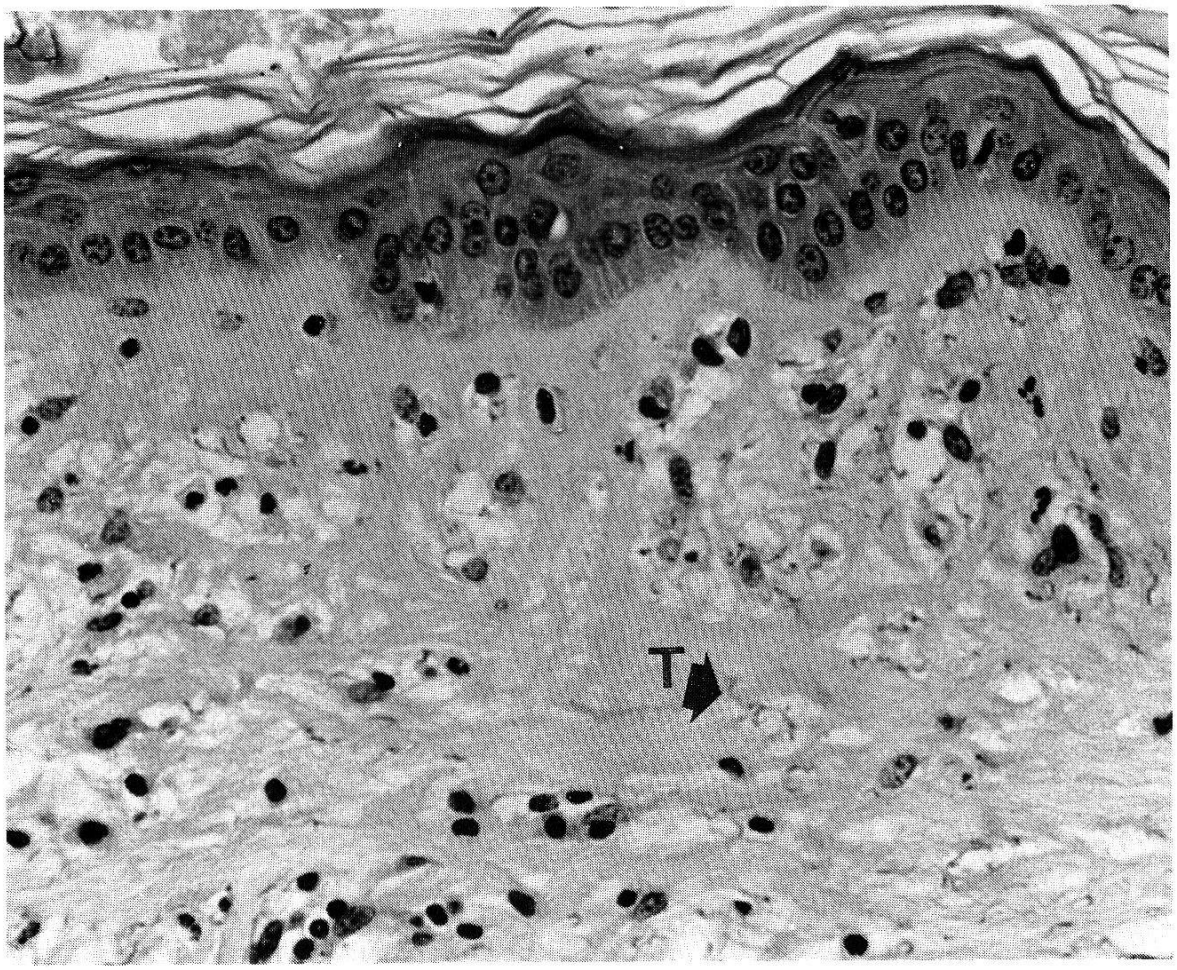

Fig. 4

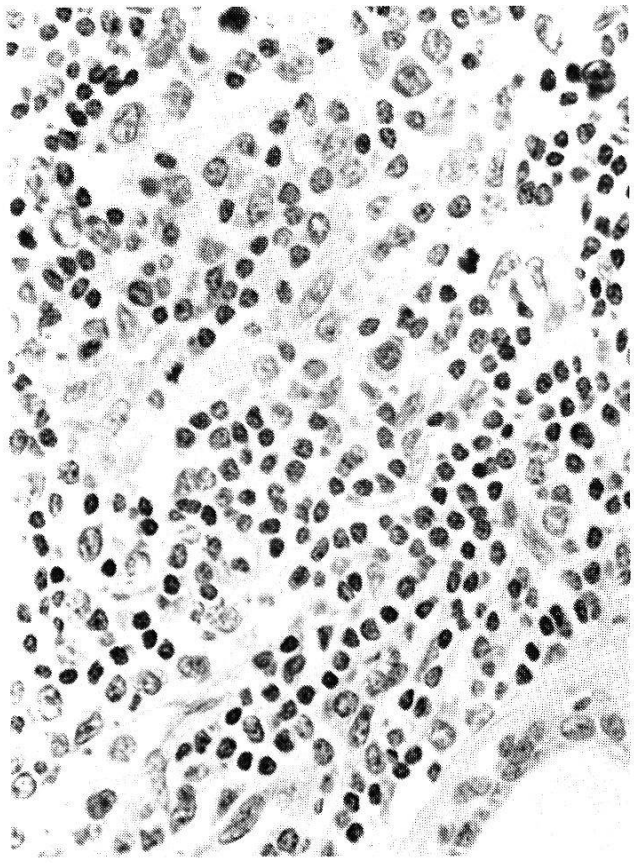

Fig. 5

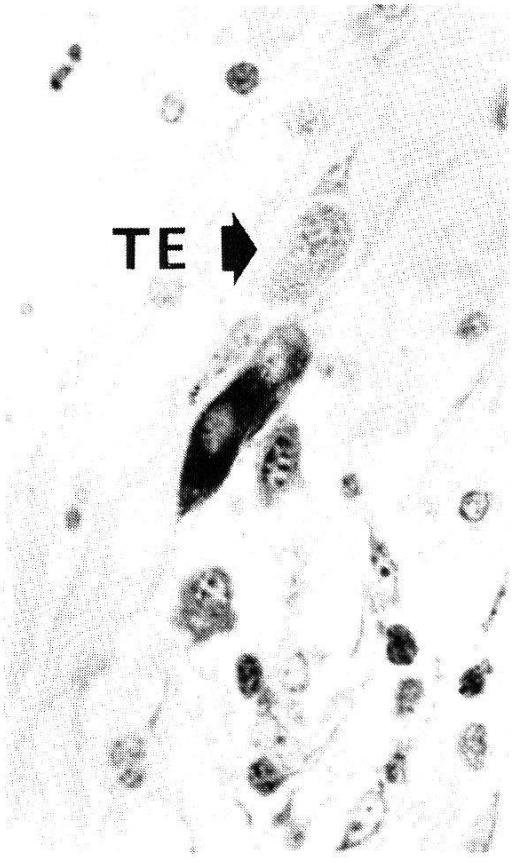

Fig. 6

tissue reaction so severe as to evoke cell death in any of the sebaceous glands or hair follicles in the skin. In contrast, the total cellularity and the proportions of the various cell types in biopsies sampled at similar intervals after the application of uninfected tsetse did not differ appreciably from that observed in normal skin, except in the terminal stages of the disease.

Lymphocytes and plasma cells: The lymphocyte was the predominant cell type contributing to the cellular infiltrate in the chancre. From a "resident" population of around 28–40% (150–250 per mm^2) of cells found in normal skin, a substantial extravasation and local proliferation of lymphoid cells occurred coincident with and following the PMN response (days 4–6). Initially larger numbers (550–2500 per mm^2) of small and medium lymphocytes (8–12 μm diameter) accumulated perivascularly around blood vessels in the dermis and underlying cutaneous muscle, but few lymphoblasts (>12 μm diameter) were found at this stage of the response. Within 24 h, lymphocytes were distributed throughout the dermal connective tissue, although their absolute numbers declined progressively from the deeper dermis towards the basal layer of the epidermis. From 6 days onwards, the numbers of lymphoblasts increased, although their distribution was confined to the immediate perivascular areas (Fig. 5). In addition, plasma cells became more numerous in the connective tissue particularly around dermal capillaries. Large numbers of lymphoblasts were still present in biopsies of the chancre sampled in the terminal stages of the infection, and small focal accumulations of plasma cells, some of which contained Russell bodies, remained scattered throughout the dermis after the chancre had subsided.

Macrophages: The presence of macrophages was not a feature of the early stages of the chancre. An increase in their absolute numbers was noted only during the peak and regressive stages of the tissue reaction, and their location was principally in the immediate vicinity of dermal venules. In the terminal stages of the infection, macrophages were also found in the dermal connective tissue, but rarely at any great distance from a capillary. In perivascular locations, macrophages appeared to associate with lymphoblasts. Trypanosomes were not observed to be ingested by macrophages in the chancre at any stage of the response.

Eosinophils: Eosinophils were infrequent in histological sections of the chancre prior to its decline. After day 8, they were found in increasing numbers (35–50 per mm^2) adjacent to dermal capillaries and scattered in the dermal connective tissue. They were less numerous in both the deeper layers of the dermis and around blood vessels in the panniculus carnosus muscles.

Fig. 4. The presence of *T. (T.) brucei* (T) in dermal connective tissue 4 days after challenge with infected tsetse flies (H & E × 250).

Fig. 5. Mononuclear cells and lymphoblasts located perivascularly in the dermis of the skin 10 days after challenge (H & E × 200).

Fig. 6. Tissue eosinophil (TE) in the caprine chancre 18 days after challenge (Giemsa × 800).

An unidentified cell type was found perivascularly and in the adjacent tissue spaces in skin biopsies taken from chancres which had subsided. It was distinguished in sections stained with Giemsa by the presence of fine pale eosinophilic granules filling an extensive pale cytoplasm. The clumps of chromatin were distributed evenly throughout a pale-staining oval nucleus (Fig. 6). The term "tissue eosinophil" was used tentatively to describe these cells, although their origin and functional relationship to eosinophil granulocytes was unknown. They were readily differentiated from the latter by the structure of the nucleus. Tissue eosinophils could be distinguished with difficulty by nuclear morphology in sections stained with haematoxylin and eosin, and did not share staining characteristics with mast cells, in that the granules of tissue eosinophils did not exhibit metachromasia after staining with Giemsa.

Mast cells: Mast cells were present in small numbers (15–20 per mm^2) in close proximity to dermal capillaries and in the adjacent dermis of normal skin. They were also found immediately beneath the adventitia of deeper cutaneous arteries and venules. During the development of the chancre, mast cells were seen less commonly, although their absolute numbers did not alter greatly. In the terminal stages of the disease and during the regressive phase of the chancre, increased numbers of mast cells appeared in skin biopsied from the sites of challenge with infected or uninfected tsetse flies, and in the skin remote from the sites of challenge. Basophilic leucocytes were not detected in normal caprine skin or during the development or regression of the chancre.

Challenge of mice with infected tsetse

To observe whether tsetse challenge of mice could produce chancres similar to those elicited in goats, infected tsetse were allowed to feed on the belly of mice. Parasites were first detected in the blood of the hosts 3 days after infection and all mice died between 3 and 5 days later. At no stage of the disease in these mice was a chancre detected macroscopically or histologically at the site of challenge. It was found after dissection of the site of challenge that the blood pool produced by the tsetse was located in the abdominal muscle and not in the subcutis.

Discussion

In the goat, the morphological features of the skin reaction which evolves as a sequel to the injection of metatrypanosomes with saliva during the feeding of tsetse infected with *T. (T.) brucei* closely resembles lesions observed following similar challenges with different trypanosomes in other species including man, rabbit and cattle (Fairburn and Godfrey, 1957; Willett and Gordon, 1957; Awad et al., 1969; Luckins and Gray, 1979). That the reaction represents a response directed towards the trypanosome is evidenced by the failure of bites

from uninfected tsetse flies to elicit any such reaction. Furthermore, it has been shown in rabbits that the intradermal inoculation of bloodstream trypanosomes provoked the development of a chancre identical to that resulting from challenge with infected tsetse flies (Awad et al., 1969; Seed et al., 1972).

The present study confirmed in the goat the results of investigations in other species, namely, that trypanosomes appear in large numbers in the chancre prior to their detection in the bloodstream. It is doubtful whether the localization of trypanosomes is necessary for a differentiative stage in the life cycle. Within minutes of an intradermal injection of the *T. (T.) brucei* metatrypanosomes into rats which do not exhibit a chancre, trypanosomes infective for secondary hosts are present in the bloodstream (Willett and Gordon, 1957). Furthermore, the demonstration of trypanosomes infective for rats in cardiac blood of rabbits within minutes of an intravenous injection of metacyclic trypanosomes dissected from tsetse flies (Willett and Gordon, 1957) argues against the necessity for trypanosomal maturation in the chancre. It is more likely that the ability to restrict the immediate spread of trypanosomes is a physiological property of the skin or skin thickness, as chancres are not a feature of the smaller laboratory animals, namely rat and guinea pig (Willett and Gordon, 1957). In thin-skinned animals, trypanosomes are probably deposited by the tsetse fly into the superficial muscular layers beneath the dermal tissue. This study demonstrated that chancres do not appear in mice challenged with tsetse infected with *T. (T.) brucei*, and that the trypanosomes are probably deposited in the musculature deep to the dermis. In this context, the cellular infiltration into the skin at the site of challenge with infected tsetse is similar in guinea pigs and rabbits, although a palpable chancre is not exhibited by the former host (Willett and Gordon, 1957). These differences may also indicate that laboratory rodents are not as well adapted biologically as the natural hosts to contend with experimental trypanosomiasis, although both *T. brucei* and *T. congolense* induced skin reactions in rabbits similar to those observed in the definitive hosts (Awad et al., 1969; Luckins and Gray, 1979). In this respect, it is not known whether *T. lewisi* and *T. musculi*, which are transmitted naturally by fleas between rats and mice respectively, induce chancres in their hosts. However, *T. cruzi*, which is transmitted by Reduviid bugs, elicits a chancre-like lesion (the "chagoma") in man (WHO report, 1974).

The differences between animal species in the behaviour of trypanosomes isolated from livestock highlight the problems of extrapolation of results from laboratory models to the larger domestic animals.

Histological studies of the chancre elicited by challenge of man with tsetse infected with *T. (T.) rhodesiense* have been described (Fairburn and Godfrey, 1957) and appeared similar to those observed in the present study. Detailed microscopic investigations of chancres produced in domestic livestock have been limited. In a brief report, Luckins and Gray (1979) described a mononuclear cellular infiltrate apparent from 8 days after challenge of calves with tsetse

flies infected with *T. congolense*, and a similar histological picture was also reported for sheep and rabbits (Luckins and Gray, 1978). The absence of a component of PMN in the studies in cattle may have been due entirely to the periods chosen for skin biopsy, as has been found in the present study, the PMN response occurred before the chancre had developed to any considerable size. This contention has been confirmed in more recent studies in cattle (Murray et al., 1979; Akol, in prep.). It is equally conceivable that the type of cellular reaction may be dependent for its timing and cellular composition on the species of both the trypanosome stock used for its elicitation, and the recipient of the challenge. In the goat, the skin reaction to *T. brucei* involves predominantly PMN, lymphocytes and plasma cells. Although macrophages are abundant in the sinuses of the draining lymph node, the increase in their absolute numbers in the chancre is less pronounced than the preceding cell types. Studies in cattle suggest that macrophages are more a feature of the cellular infiltration elicited by *T. congolense* in this species (Luckins and Gray, 1979).

The chancre is characterised by a latent period to its macroscopic appearance and can be classified under the broad category of delayed-type hypersensitivity (DTH) reactions. It is obvious from this and previous studies that the onset of the tissue response is extremely variable; it is probably dependent on the replication rate of the trypanosome involved and on the number inoculated (Willett and Gordon, 1957; Luckins and Gray, 1979). However, when a given trypanosomal stabilate is employed to elicit a chancre, the kinetics of the skin reactions are similar between animals from different host species. For example, the appearance of chancres following challenge with *T. congolense* is comparable in rabbits, cattle, sheep and goats (Luckins and Gray, 1979; Murray et al., 1979). Given the variability of onset of the chancre, it is pertinent to compare the histology of the chancre to similar skin reactions of delayed onset invoked by alternative procedures. By comparison with detailed descriptions of the human skin reactions attending contact allergy and resulting from the elicitation of classical DTH with protein antigens in sensitized individuals (Dvorak et al., 1974) the chancre is distinguished immediately from allergic dermatites classified as cutaneous basophil hypersensitivity by the absence of basophils from the former reaction. The failure in man of neutrophil granulocytes to participate in classical DTH response (Dvorak et al., 1974) also excludes the chancre from this category of local skin reactions.

The chancre appears to represent essentially a combination of an acute inflammatory response and an immunological reaction invoked by the presence of local trypanosomal proliferation, but delayed in appearance until sufficient numbers of the causal agent have been generated. That the parasite is the inductive stimulus for the reaction has been suggested by the production of skin lesions in guinea pigs inoculated intradermally with trypanosomal extracts (Seed, 1969). The elimination of the chancre and exacerbation of the severity of the disease in rabbits treated daily with hydrocortisone (Seed et al., 1972) is

compatible with the presence of inflammatory and immunological components in the chancre. Although the factors which restrict the trypanosomes to a local area are probably physiological, the appearance of the chancre represents the first major response of the host to the trypanosome. Therefore, the chancre is probably of considerable importance to the progress of the infection in the provision of antigen for priming of the draining lymph node(s). The chancre may also provide an ideal focus for the generation of variant parasites responsible for the initial parasitaemias in the host, as the studies of Luckins and Gray (1979) infer that antigenic variation of a given trypanosomal stock occurs within the chancre. Therefore, studies of trypanosomal variation within the chancre in the definitive host is critical to a complete appreciation of both the development of the parasite and the evolution of the host's response during the pathogenesis of trypanosomiasis. Studies are in progress to examine this latter component.

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