Clin. exp. Immunol. (1981) **43**, 583–589.

The *in vitro* response to mitogens of leucocytes from cattle infected with *Trypanosoma congolense*

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(Accepted for publication 19 September 1980)

SUMMARY

The *in vitro* proliferative response of bovine leucocytes from peripheral blood to LPS, PWM and Con A was monitored during the course of infection with *T. congolense*. The stimulatory effect of LPS and PWM on PBL was significantly decreased in infected cattle while the reduction of stimulatory response to Con A was less marked. There was a high background proliferation of unstimulated leucocytes from infected cattle. This increase in background counts correlated with the parasitaemia in that both features were abolished by treatment with the trypanocidal drug *Berenil* (4,4′-diamidino-diazoaminobenzene-di-acetamide acetone). Co-cultivation of trypanosomes with PBL from normal cattle did not depress the responses of the latter to mitogens, and trypanosomes maintained in a cell-free medium failed to incorporate ^{14}C-thymidine under the conditions used.

INTRODUCTION

In experimental African trypanosomiasis of laboratory animals, a profound reduction in DNA synthetic responses of leucocytes to mitogens occurs (Corsini *et al.*, 1977; Jayawardena & Waksman, 1977; Jayawardena, Waksman & Eardley, 1978; Roelants *et al.*, 1979; Goodwin *et al.*, 1972; Murray *et al.*, 1974; Albright, Albright & Dusanic, 1977; Pearson *et al.*, 1978). Most of these investigations have been carried out in mice and rats except for the recent work in bovidae reported by Sollod & Frank (1979). The latter authors failed to demonstrate a decrease in DNA synthesis of leucocyte cultures from infected cattle in response to PHA and PWM stimulation on the basis of total ^{3}H-thymidine incorporation. We therefore decided to study these responses in detail by taking into account both total DNA synthesis and stimulation indices in leucocytic cultures of *T. congolense*-infected cattle.

MATERIALS AND METHODS

Parasites. Trypanosoma congolense IL311 is a stabilate derived from stock GUTR-37 isolated from a clinically ill cow in Gambia. The trypanosomes used to infect the cattle were grown in irradiated rats.

Animals. Forty boran steers aged 9–11 months weighing approximately 200 kg were obtained from an area known to be free of trypanosomiasis and were screened for antibodies to *Babesia spp.*, *Theileria spp.*, *Anaplasma spp.* and *Trypanosoma spp.* by immunofluorescence (Wilson, 1969) and enzyme-linked immunosorbent assay (Luckins, 1977) before use.

The cattle were housed in an insect-proof accommodation and fed on hay and concentrates.

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0099-9104/81/0300-0583$02.00

Experimental design. Two groups of boran cattle were used in two different experiments. In the first experiment (Experiment A), 11 out of 16 boran steers were inoculated intravenously with 1×10^5 viable *T. congolense* while the remaining five were maintained as uninfected controls. In the second experiment (Experiment B), 12 out of 24 boran cattle were inoculated intravenously with 1×10^5 *T. congolense*. The remaining animals were maintained as uninfected controls. Six of the infected and six of the uninfected cattle from Experiment B were treated with the trypanocidal drug, Berenil (4,4′-diamidino-diazoaminobenzene-di-acetamide acetate; Hoechst), at a dose of 7 mg/kg body weight administered intramuscularly 25 days after infection.

Daily samples of venous blood were collected from experimental animals into EDTA-containing tubes or heparinized, 60-ml disposable syringes and were examined for the presence of *T. congolense* and the response of peripheral blood leucocytes (PBL) to mitogens was measured at weekly intervals.

Course of parasitaemia. Daily samples of EDTA-containing jugular vein blood from the experimental cattle (Experiment A) were examined for the presence of *T. congolense* in the buffy coat under dark-ground, phase-contrast microscopy (Murray, Murray & McIntyre, 1977). To quantitate the actual numbers of parasites per millilitre of blood, 5 μl of blood were mixed with 45 μl of Ziehl Nielsen–Carbol fuchsin-staining solution (Murray & Morrison, 1979) and the numbers of parasites were counted using a haemocytometer.

The response of peripheral blood leucocytes (PBL) to mitogens. Experiment A: At weekly intervals after infection, blood samples, volume 50 ml, were withdrawn from the jugular vein into syringes containing 5 iu/ml heparin (Novo Industri, Copenhagen, Denmark). The leucocytes were washed three times in heparinized Hanks' balanced salt solution (HBSS) (5 iu heparin/ml) and centrifuged at 500 ***g*** for 5 min. The cells were resuspended at a concentration of 2×10^6 leucocytes per ml of culture medium (RPMI 1640) supplemented with 10% heat-inactivated fetal calf serum (Gibco, batch K202191D), 20 μg/ml glutamine, 100 iu/ml penicillin, 100 μg/ml streptomycin and 10 mM HEPES buffer. Leucocytes from each animal were set up in the presence of either pokeweed mitogen (PWM), concanavalin A (Con A) (Pharm Industrie, Villeneuve-la-Gorenie, France) or medium alone. Triplicate cultures were initiated by the addition of 4×10^5 lymphocytes in 200 μl of medium to either 2 μg PWM or 0·5 μg Con A (Pearson *et al.*, 1979) in 50 μl medium or 50 μl medium alone, in a 96-well microtitre plate (Sterilin, Middlesex, England). The plates were covered with loose-fitting lids and incubated at 37°C in a humidified atmosphere of 5% carbon dioxide in air. Blastogenesis of cultured PBL was assessed by the addition of 0·02 μCi of ^{14}C-labelled thymidine in 20 μl medium (methyl ^{14}C-thymidine, Radiochemical Centre, Amersham) after 72 hr of incubation. Cultures were incubated for a further 18 hr before each well was harvested into glass-fibre filters using a Titertek cell harvester. The filters were dried and placed into glass scintillation vials containing 5 ml scintillation fluid consisting of 3% PPO (w/v) and 0·3% POPOP (w/v) in toluene. Each sample was counted in a Tri-carb liquid scintillation spectrometer (Packard). Mean counts of triplicate samples were determined and stimulation indices (SI) were calculated as the ratio of:

$$\text{SI} = \frac{\text{mean counts per minute (c.p.m.) of stimulated cultures}}{\text{mean c.p.m. of unstimulated cultures from the same animal}}$$

Experiment B: The PBL were prepared in the same manner as in Experiment A and stimulated with mitogens as described by Pearson *et al.* (1979). Blastogenesis in the cultured PBL was assessed by addition of 0·5 μCi ^{125}I-uridine (Radiochemical Centre, Amersham) to appropriate wells after 24, 48, 72 and 96 hr of incubation. The cultures were incubated for a further 4 hr and were harvested as described in the first experiment. The dried filters were placed into plastic vials and each sample was counted in an Auto-gamma scintillation spectrometer (Packard, Model 5360) and stimulation indices were determined.

Statistical analysis. The Mann–Whitney non-parametric test was used for the statistical analysis of the data (Snedecor & Cochran, 1976).

Effect of trypanosomes on the in vitro *growth of leucocytes from normal cattle.* Trypanosomes were isolated from heparinized whole blood on DEAE cellulose columns as described by Lanham (1968) and 2×10^5 parasites were incorporated into cultures with PBL from normal cattle. Control cultures contained varying numbers of trypanosomes (10–50 trypanosomes per well) or normal

PBL alone and both were cultured in RPMI 1640 growth medium in which ^{14}C-thymidine was incorporated.

Labelling of surface membrane and of lymphocytes. Anti-bovine immunoglobulin conjugated with fluorescein isothiocyanate was used to investigate the surface-labelling characteristics of lymphocytes from cattle examined in Experiment B as described by Pearson *et al.* (1979).

RESULTS

Trypanosomes appeared in the blood circulation of infected cattle on day 3. The first peak of parasitaemia was attained by day 10 (approximately 5×10^5 parasites/ml). Following a transient drop in the parasitaemia a second peak was seen on day 14 which was of a higher magnitude ($1{\cdot}1 \times 10^6$ parasites/ml). Subsequent parasitaemic peaks were of lower magnitude and fell gradually up to the end of the experiment (Fig. 1).

Experiment A

The responses to stimulation with Con A on peripheral blood leucocytes (PBL) from normal and infected cattle, as indicated by stimulation indices (SIs), are shown in Fig. 2a. On the basis of SIs, leucocytes from normal cattle responded better to stimulation with Con A than did leucocytes from infected cattle when tested on days 21 and 28 after infection ($P > 0{\cdot}05$).

PWM induced stimulation of DNA synthesis in cultures of PBL from both uninfected and infected cattle as shown in Fig. 2b. These results are similar to those obtained from cultures stimulated with Con A except that the differences between infected and uninfected cattle were manifested early (day 7) and were more pronounced.

Experiment B

The responses to stimulation with LPS and Con A of PBL from normal and infected cattle of Experiment B, as indicated by best SI and c.p.m. on different days of peak response, are shown in Table 1. On the basis of SI, leucocytes from uninfected cattle exhibited better proliferative responses than did PBL from infected cattle. However, the depressed responses of PBL from the latter group were restored to normal levels 25 days following treatment of the hosts with Berenil while the SI of PBL from untreated cattle remained low.

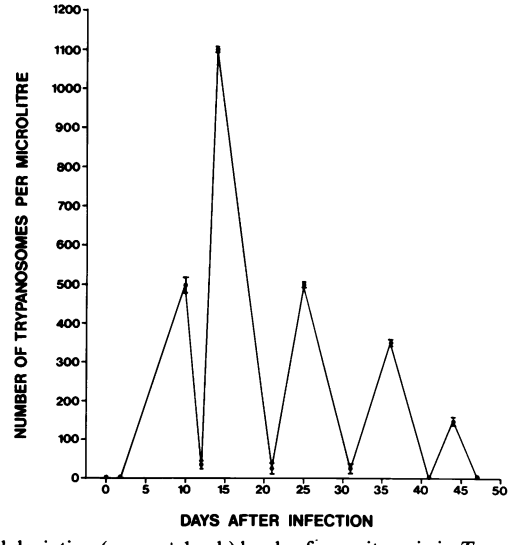

Fig. 1. Mean and standard deviation (mean $\pm$ 1 s.d.) levels of parasitaemia in *T. congolense*-infected cattle. The readings were obtained from 11 infected cattle.

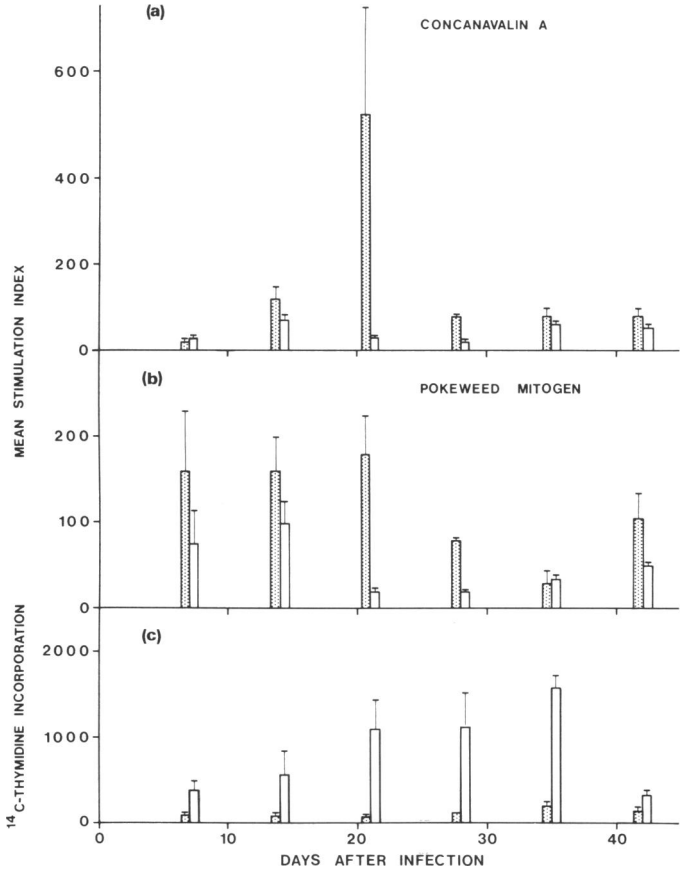

Fig. 2. (a) The proliferative response to concanavalin A of PBL from uninfected and *T. congolense*-infected cattle. The results are expressed as mean stimulation index ± standard error from cultures established with PBL of five uninfected and 11 infected cattle and harvested after 72 hr in culture (Experiment A). (■) PBL from uninfected cattle, (□) PBL from *T. congolense*-infected cattle. (b) The proliferative response to pokeweed mitogen of PBL from normal and infected cattle. The results were computed and expressed as in (a) (Experiment A). (c) The incorporation of ^{14}C-thymidine by unstimulated PBL from uninfected and *T. congolense*-infected cattle. The results are expressed as mean ^{14}C-thymidine counts per minute ± standard error from cultures established with PBL of 16 cattle and harvested after 72 hr in culture (Experiment A). Symbols as in (a).

On close examination of the data from both experiments, it was noted that the level of DNA synthesis in unstimulated cultures of the PBL from infected cattle was significantly higher than in unstimulated cultures of PBL from uninfected cattle ($P > 0{\cdot}01$, Fig. 2c).

The proliferative response to mitogens of leucocytes from uninfected animals was not significantly altered by the incorporation of live trypanosomes in the culture medium (Table 2). In addition, cultured trypanosomes failed to incorporate ^{14}C-thymidine.

The proportion of cells labelled with anti-bovine Ig and PNA did not change during the course of infection.

DISCUSSION

A study of the proliferative response of leucocytes from infected cattle to mitogens revealed a decrease in DNA synthesis in response to Con A, PWM and LPS in terms of stimulation indices

Table 1. Changes in mitogenic response of peripheral blood leucocytes in *T. congolense*-infected cattle (Experiment B). The results are expressed as mean counts (± standard error) per minute of ^{14}C-thymidine in cultures from 12 animals per point

		Days after infection			
Mitogen	Cattle	0	18–21	40–45	20–25 days after Berenil treatment
LPS	Infected	24·7±4·7*	12·8±1·3	8·4±1·8	19·5±6
		229±41·4†	2,134±316·5	4,153±1,430	920±77
	Control	23·2±6·2	21·9±3·4	21·3±3·6	17·7±3·4
		417±170	795±136·4	1,424±258·8	1,252±381
Con A	Infected	382·8±36·4	210·9±33·3	150·7±28·4	250·3±33·7
		191±37	883±166·2	849±150·5	436±89·3
	Control	265·9±48·9	268·9±49·6	495·2±115·1	463·7±156·6
		321±61·3	673±109	222±44	302±89·5

Data expressed as * stimulation index (SI) and † background count.
LPS = lipopolysaccharide, Con A = concanavalin A.

Table 2. Effect of trypanosomes on the *in vitro* growth of normal peripheral blood leucocytes. The results are expressed as mean counts (±standard error) per minute of ^{14}C-thymidine incorporated in cultures from two normal cattle

Culture	Unstimulated	Concanavalin A	Pokeweed mitogen
Lymphocytes + trypanosomes	278±45*	19,748±1,040	22,213±884
	1±0·5†	66±10	83·5±8·5
Lymphocytes alone	348±89	25,230±980	22,794±90
	1±0·6	72·5±14·2	65·5±9·0

Data expressed as * c.p.m. and † SI.

(SIs) but not in total ^{14}C-thymidine incorporation. The unstimulated leucocyte cultures of PBL from the infected cattle incorporated higher quantities of ^{14}C-thymidine when compared to their counterparts in the uninfected animals. In addition, introduction of live trypanosomes into leucocyte cultures from uninfected animals did not alter the background incorporation of ^{14}C-thymidine. Further, trypanosomes maintained in a cell-free culture containing ^{14}C-thymidine did not incorporate significant quantities of the isotope after 24 hr.

The SIs evaluated here reflect the number and degree of mitogen-induced proliferation of leucocytes over their unstimulated proliferative state. Therefore, the low SIs seen in *T. congolense*-infected cattle may suggest a reduced proliferative response to the mitogens used. This suggestion would be satisfactory if the background leucocytic DNA-synthesis in the unstimulated proliferative state is ignored. In the light of the latter state, it is apparent that the unstimulated cultures of infected animals have a higher number of cells in a proliferative state. Hence, it is impossible to say that leucocytes from infected animals demonstrate a reduced proliferative response to mitogens. It may be that there are fewer numbers of leucocytes responding to mitogens and those cells that are

undergoing proliferation prior to exposure to mitogens do not react further to PHA, PWM and LPS. There is indirect evidence in support of this contention (Corsini *et al.*, 1977; Murray *et al.*, 1974).

Recently, Anderson *et al.* (1979) demonstrated that purified T cell blasts (originally exposed to 5 μg/ml Con A) were unresponsive to Con A. In addition, work on mice in this laboratory (Masake, Musoke & Nantulya, unpublished) has shown that PBL and splenic leucocytes taken from days 6 to 12 following administration of sheep or horse red blood cells have reduced proliferative responses to Con A, LPS and PWM. Therefore, we suggest that cells undergoing DNA synthesis are incapable of responding to mitogen stimulation by further proliferation and differentiation.

We have suggested that the unstimulated cultures from infected animals have a higher number of cells undergoing DNA synthesis when compared to those of the uninfected cattle. The possible explanation for the existence of additional numbers of proliferating cells in the unstimulated leucocyte cultures of the infected animals could be the result of prior stimulation of leucocytes by other mitogenic agents such as trypanosome-derived mitogenic factor (Esuruoso, 1976; Assoku & Tizard, 1978) and to trypanosome antigen.

A similar phenomenon has been demonstrated in mice (Corsini *et al.*, 1977; Murray *et al.*, 1974). Corsini *et al.* (1977) found high background proliferation in splenic cultures from *T. brucei*-infected mice while Murray *et al.* (1974) demonstrated high ^{125}I-UdR incorporation by lymph nodes of *T. brucei*-infected mice. In the latter case, the high background reduced the stimulation index in mice sensitized with oxazolone since the ^{125}I-UdR incorporation was similar for both infected and normal mice sensitized with oxazolone.

It is evident that trypanosomes contribute either directly or indirectly to the high background proliferation. This suggestion is further supported in the present study and in the work of Roelants *et al.* (1979) by the disappearance of high background counts following elimination of the parasites from circulation either through self-cure or treatment with Berenil. The direct effect of trypanosome-derived mitogens, however, is rather doubtful since normal leucocytes mixed with varying numbers of trypanosomes *in vitro* failed to acquire high background counts.

In view of the high background counts observed in the unstimulated cultures of PBL from infected cattle, it is evident that there was no reduction in the proliferative response of leucocytes from infected cattle to Con A, LPS and PWM. This is in agreement with the work of Sollod & Frank (1979).

The authors are grateful to Messrs F. McOdimba, C. Attinda, T. Tenywa and Ms Asmina Jiwa and Leah Agina for their technical assistance; to Drs A. J. Musoke, V. Nantulya, D. L. Emery and F. Rurangirwa for their valuable comments and advice on the manuscript.

ILRAD publication serial number 129.

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