Mitogenicity of autolysates of *Trypanosoma congolense*[1]

R. K. G. Assoku[2] and I. R. Tizard

Department of Veterinary Microbiology and Immunology, University of Guleph, Guelph (Ontario, N1G 2W1, Canada), 16 June 1977

Summary. Autolysates of *Trypanosoma congolense*, in subcytotoxic amounts, were found to be highly mitogenic in vitro for the spleen cells of normal mice. Significant amounts of [^{3}H]-thymidine were also incorporated by the responding spleen cells of nu/nu (athymic) mice. In contrast, the spleen cells of cyclophosphamide-treated mice were unresponsive. The findings suggest that a potent B-cell-mitogen is generated by the autolysing *T. congolense* organism.

Immunosuppression resulting from infection with the pathogenic African trypanosomes is well-documented[3-6] but mechanisms involved in the development of this immunological phenomenon still remain unclear. A paradoxical finding which has greatly contributed to the difficulty in the elucidation of this phenomenon is the simultaneous association of a highly active immunological and mononuclear phagocytic system[7] with an immunosuppressed state to other antigens. Various hypotheses have been advanced to explain this phenomenon, and one of the most attractive has been that B-cells capable of recognizing the introduced antigen may be depleted as a result of polyclonal B-cell activation[5,6,8]. Recent reports have thus stressed the close functional and pathogenetic relationship between the high IgM-levels, polyclonal B-cell-activation and immunosuppression in African trypanosomiasis[9,10]. Whether a trypanosome-derived mitogen plays any significant role in this immunosuppression, as well as in the spontaneous polyclonal B-cell-responses in this disease, has yet to be resolved.

We have shown that *Trypanosoma congolense* generates, on autolysis, certain free fatty acids and lysolecithin which were potently cytotoxic and haemolytic[11,12]. We have also suggested recently that these trypanosomes-derived factors may play a significant role in the pathogenesis of African trypanosomiasis[13]. In this communication, we present evidence to show that autolyzed trypanosome-derived factors, in subcytotoxic amounts, are potently mitogenic in vitro for mouse spleen cells. This important characteristic was first detected during our studies on the effects of these factors on mitogen(PHA and LPS)-stimulated lymphoblastogenesis, when it was observed that the factors very significantly ($p < 0.01$) stimulated mouse splenic culture cells to incorporate thymidine (table).

The trypanosome-derived factors were prepared as described previously[11,12] and a standard, subcytotoxic dose of 4.5 µg/ml 10^6 splenic lymphocytes was established using a quantitative adaptation of the trypan blue dye exclusion method. Spleen cell cultures were carried out[14] in sterile flat-bottomed Falcon plastic tubes (No. 2054, B-D Falcon, California, USA) in quadruplicate aliquots of 3 ml/tube containing 15×10^6 responding splenic lymphocytes in RPMI-1640-antibiotic supplemented medium. Cells were cultured with or without a known T- or B-cell-mitogen (PHA or LPS, respectively; Difco), or with the trypanosome-derived factors for 48 h at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. 18–24 h before harvesting, 1 µCi of tritiated-thymidine (sp. act. 6–7 Ci/mmole; New England Nuclear) was added to each tube. Cells were harvested, processed and [^{3}H]-thymidine incorporation into lymphocyte nucleoprotein determined by liquid scintillation spectroscopy[14].

The table summarizes the data of a number of experiments in which the stimulatory effects on murine spleen cells of a standard, subcytotoxic amount on the trypanosome-derived factors and optimal amounts of known T- and B-cell-mitogens (PHA and LPS, respectively), were examined. As can be seen, the addition of the *T. congolense* autolysate promoted a cellular stimulation reflected in significantly increased ($p < 0.01$) thymidine uptake (table, experiment A). Stimulation by PHA and LPS was generally greater than this, but in some experiments [for example, the response of the athymic, nude mice (vide infra)], the mitogenic effect of the trypanosome-

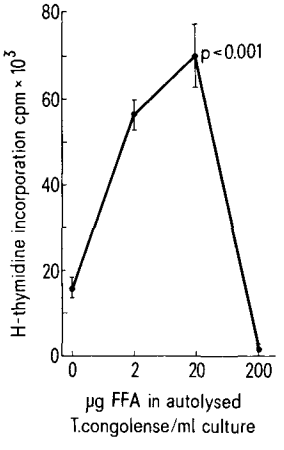

Fig. 1. Dose-response curve showing the incorporation of [^{3}H]-thymidine after stimulation of normal mouse spleen cells. p-value given for the optimal stimulatory concentration of autolyzed *T. congolense*. Vertical bars represent SE of the mean responses of quadruplicate cultures.

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derived factors was distinctly greater than that of the known B-cell-mitogen (LPS). However, coincubation of either the PHA or LPS with the trypanosome-derived factors resulted in reduced lymphoproliferative response to either PHA or LPS alone (table, experiment B). This reduction could be due to the fact that optimal concentrations of PHA and LPS were used in the cultures, and mitogenic effects decrease sharply when such mitogens are used in supraoptimal doses[14,15].

A similar autolysate from *Trypanosome lewisi*, a non-pathogenic organism, produced only minimal blastogenic response, and this preparation has been found to contain significantly reduced amounts of free fatty acids[12].

The experiments reported above were carried out repeatedly under constant environmental conditions, and the unequivocal demonstration in vitro of mitogenic stimulation of the spleen cells of normal mice by the trypanosome-derived factors supports the hypothesis that some of the effects of trypanosomal infection, such as immunosuppression and synthesis of nonspecific immunoglobulins, might be associated with the elaboration by the parasite of a mitogen which stimulates lymphoid cells to blast transformation[6,8,10].

A dose-response study clearly indicated that as little as 2 μg of the trypanosome preparation significantly ($p < 0.02$) stimulated spleen cell suspension containing 5×10^6 cells/ml of culture (figure 1); increased doses of up to 20 μg/ml of culture caused augmented responses ($p < 0.001$). Higher concentrations of the factor, however, resulted in declining responses until, at the highest concentration used (200 μg/ml cell culture containing 5×10^6 cells/ml), no responses occurred (figure 1); trypan blue dye exclusion test indicated 100% mortality of these responding cells.

In order to determine the class of cell on which these trypanosome-derived factors might act, spleen cells from both athymic, nude (nu/nu) and cyclophosphamide-treated mice were separately cultured with either the trypanosome factors, AHA or LPS. As illustrated in figure 2, highly significant ($p < 0.001$) mitogenic responses by the cells of athymic mice to both the trypanosome-derived factors and LPS occurred, which clearly suggested that the trypanosome factors behaved as a B-cell-mitogen. This observation was further supported by lack of response to the trypanosome-factors by the spleen cells of mice pretreated with cyclophosphamide. These points are in agreement with the findings of Esuruoso[9] and Campbell (as cited by Mansfield and others[16]), but are in conflict with those of Mansfield and colleagues[16], whose mechanically-disrupted *T. congolense* and *T. brucei* extracts were found to be mitogenic in vitro for rabbit but not mouse spleen lymphocytes. The possibility that the stimulation observed in our experiments was due to the presence of a heterologous serum factor in the cultures[17] was also highly unlikely because none of the control culture preparations produced any significant stimulation (see the table and figure 1).

Immunosuppression is closely associated with African trypanosomiasis[3–6]. Whether a trypanosome-derived mitogen plays a significant role in this phenomenon, as well as in the spontaneous polyclonal B-cell-responses, remains to be determined. Nevertheless, there is now increasing evidence, both direct and indirect, to support the postulate that a trypanosome-derived B-cell-mitogen stimulates B-cell clones to undergo differentiation[6–8], thus resulting not only in unregulated clonal expansion and secretion of immunoglobulins with numerous specificities, but also in clonal exhaustion and/or paralysis so that the induction of specific antibody responses is severely depressed. Thus, background IgM-responses to many antigens recognizable by B-cells are increased in trypanosome-infected animals[10], and trypanosome infection therefore probably induces polyclonal B-cell-activation. Indeed, a parallel has recently been drawn[10] between the polyclonal activation of trypanosome infections and that produced by LPS[18], the secreted polyclonal products being suggested to account for the increased Ig-levels seen in trypanosome infections[10].

The responses of normal mouse spleen cells to known mitogens and autolyzed *T. congolense* and *T. lewisi*

Experiment	Culture addition/or 'stimulant'*	[^{3}H]-TdR uptake (cpm ± SEM of 4 replicate determinations)	p***
A	Phytohaemagglutinin (PHA)	47312.53 ± 1279.36	–
	PHA + autolyzed *T. congolense*	43227.60 ± 2911.16	–
	T. congolense (AT congo)	14464.90 ± 678.30	0.01
	None	4304.20 ± 120.20	
B	*Salmonella typhimurium* lipopolysaccharide (LPS)	144660.03 ± 43425.82	–
	LPS + AT congo	82896.25 ± 8570.60	–
	AT congo	56535.40 ± 7814.06	0.002
	None	10531.93 ± 377.94	
C	AT congo	22423.67 ± 2064.28	0.001
	None	528.93 ± 125.51	
D	Autolyzed *T. lewisi***	3479.20 ± 891.35	0.05
	None	528.93 ± 125.51	

*PHA, LPS and AT congo were used, respectively, at a concentration of 10 μl, 200 μg and 60 μg/culture of 15×10^6 splenic cells. **Same aliquot amount as autolyzed *T. congolense* did not cause any significant stimulation. ***Significance of difference between cultures with and without 'stimulant' (using Student's t-test).

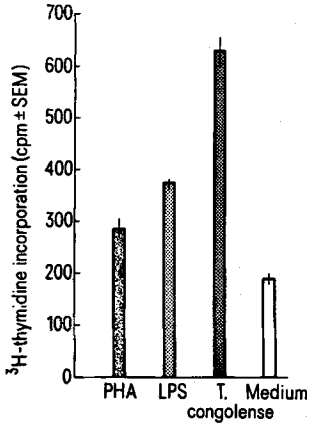

Fig. 2. In vitro effects of PHA, LPS and autolyzed *T. congolense* on the spleen cells of athymic (nude: nu/nu) mice. PHA, LPS and autolyzed *T. congolense* (T.congo) were used, respectively, at a concentration of 5 μl, 100 μg and 20 μg/culture tube of 2×10^6 cells.

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We essentially agree with the hypothesis that the polyclonal activation of B-cells in the presence of a continuous trypanosome infection is likely to result in a progressive depletion of antigen-reactive B-lymphocytes[10], since these cells would then be activated to change into secretor cells, perhaps with little or no accompanying proliferation. In this way the immunosuppression in trypanosome infections may be explained by the depletion of B-cells capable of recognizing the introduced antigen. Indeed, a number of B-cell-mitogens have recently been shown to completely suppress primary immune responses in vivo to sheep erythrocytes[19]. Our demonstration of the in vitro mitogenic activity of these trypanosome products lends concrete support to the above hypothesis. Finally, if both activation and immunosuppression are polyclonal, it would be expected that the antibody responses to later-appearing trypanosome antigenic variants would also be impaired. Recent findings[10] lend some support to this view.

Other theories, such as enhancement of T-suppressor cell function during trypanosomiasis[7], must be considered in the light of recent compelling evidence that suppressor cells were involved in the immunological hyporesponsiveness observed in African trypanosomiasis[20]. Yet still another relevant and possible mechanism of immunosuppression could be that cells stimulated in vivo with the mitogen could release lymphokines[21] (e.g. interferon[22]), which would then block (or stimulate) other essential cell lines likely to play a prominent role in primary immune response, as has indeed recently been postulated[23] to explain the complex phenomenon of immunosuppression. We have, however, recently failed to detect increased interferon levels in *T. congolense* infected calves (Dr B. Rouse, personal communication).

In conclusion, our findings indicate that, under optimal conditions of culture, the *T. congolense*-derived factors are potently mitogenic in vitro for splenic lymphocytes from normal and congenitally athymic mice. We feel that it would also be worthwhile to investigate the possibility of demonstrating this mitogenic effect on the lymphoid cells of cattle and man, the 2 species in which the natural disease is most important. We would also like to know which fraction of the trypanosomal factor produces this effect and its role in the pathogenesis of the natural disease. We are currently investigating all these possibilities.

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Small lymphocyte production and lymphoid cell proliferation in mouse bone marrow[1]

S. C. Miller, M. Kaiserman and D. G. Osmond[2]

Department of Anatomy, McGill University, Montreal, Quebec H3A 2B2 (Canada), 17 June 1977

Summary. In mice given ^{3}H-thymidine systemically during temporary circulatory occlusion of one hind limb, comparison of the labeling of rapidly-renewing small lymphocytes in the tibial marrows demonstrated that these cells were locally produced. Labeling by ^{3}H-thymidine infusion revealed that many marrow large lymphoid cells, presumptive small lymphocyte progenitors, had a marked proliferative activity and rapid turnover which varied according to cell size, was maximal in young mice and declined with increasing age.

Inbred mice have been used widely in immunological studies and the bone marrow has been strongly implicated as a primary site of production of rapidly-renewing, virgin B small lymphocytes[3,4], but the myelogenous origin of such cells has not been formally proven. The present studies, utilizing radioautography with ^{3}H-thymidine, were therefore designed to demonstrate whether or not the rapidly-renewing small lymphocytes in mouse marrow are locally produced, and to examine the proliferation of presumptive progenitor cells in this species.

^{3}H-thymidine was administered systemically to C3H mice while being excluded from the tissues of one hind limb by temporary circulatory occlusion (figure 1). The appearance of labeled cells in the tibial marrow of both hind limbs was then compared. At all sampling intervals after ^{3}H-thymidine injection, a marked disparity existed between the small lymphocyte labeling index in the marrow of the 2 limbs. The customary rapid increase in small lymphocyte labeling was shown only in the marrow that had initial access to the injected ^{3}H-thymidine (figure 1), indicating that the accumulation of labeled small lymphocytes was dependent upon the initial labeling of locally situated precursor cells. If the labeled marrow small lymphocytes were blood-borne immigrants they would be expected in similar numbers and labeling intensities in both tibial marrows, because each limb had access to labeled, circulating cells for up to 3 days. A transitory appearance of small numbers of weakly labeled small lymphocytes in the initially occluded marrow (figure 1) can be attributed to the local reutilization of circulating ^{3}H-thymidine labeled products. That the temporary vascular stasis had not, in itself, impaired the local renewal of marrow small lymphocytes was demonstrated by subjecting mice to the standard limb occlusion procedure shortly after ^{3}H-thymidine had been administered and cleared from the circulation: 48 h after ^{3}H-thymidine injection the labeling index of

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2 Correspondence to Dr D. G. Osmond, Department of Anatomy, McGill University, 3640, University Street, Montreal, Quebec H3A 2B2, Canada.
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