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A soluble factor from *Trypanosoma brucei rhodesiense* that prevents progression of activated human T lymphocytes through the cell cycle

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SUMMARY

African sleeping sickness is accompanied by a severe immunosuppression. As part of our efforts to examine the mechanisms by which this suppressive state is induced, we studied alterations in human T-lymphocyte function caused by *Trypanosoma brucei rhodesiense*. To this end, we used an *in vitro* system in which phytohaemagglutinin (PHA)-stimulated human peripheral blood mononuclear cells (PBMC) were cultured in a medium containing soluble, non-dialysable parasite products. We were able to demonstrate significant suppression of both lympho-proliferation and interleukin-2 receptor (IL-2R) expression. These effects were found to be dose-dependent and reversible after 48 hr of culture. The suppressive effects of living trypanosomes and the soluble parasite products on lympho-proliferation and interleukin-2 receptor expression were similar in that both precluded the entry of PHA-activated PBMC into the cell cycle. Eighty to ninety-eight per cent of the activated cells remained arrested in the G0/G1a (early G1) phase even 48 hr after stimulation, i.e. when last tested. Parasite-induced expression could not be overcome by the addition of recombinant human IL-2. These results suggest that immunosuppression associated with African trypanosomiasis may result from parasite-induced alteration of very early events during lymphocyte activation, leading to a virtually complete block in cell cycle progression and inhibition of IL-2R expression.

INTRODUCTION

Host immunosuppression associated with infections caused by African trypanosomes of the *brucei* group include T- and B-cell alterations in responsiveness to antigens and mitogens.[1–10] However, the mechanisms by which the parasite induces these suppressive effects have not yet been elucidated. Using an *in vitro* system in which *Trypanosoma brucei rhodesiense* was co-cultured with phytohaemagglutinin (PHA)-stimulated human peripheral blood mononuclear cells (PBMC), we were able to demonstrate markedly reduced [^{3}H]thymidine ([^{3}H]TdR) uptake and interleukin-2 receptor (IL-2R) expression, determined by flow cytometry (F. Kierszenbaum, S. Muthukkumar, L. A. Beltz and M. B. Sztein, manuscript submitted for publication).

Abbreviations: FITC, fluorescein isothiocyanate; IL-2, interleukin-2; IL-2R, IL-2 receptor(s); MFCh, mean channel number of the logarithm of fluorescence intensity; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline solution; PBS + BSA + azide, PBS containing 1% bovine serum albumin and 0·1% sodium azide; PHA, phytohaemagglutinin; PI, propidium iodide; rhIL-2, recombinant human IL-2.

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Whereas it is known that [^{3}H]TdR incorporation is related to the incorporation of this nucleotide into DNA, this measurement can be unrelated to cell proliferation in that it also represents non-S phase DNA synthesis, which does not represent cell proliferation.[11–13] Therefore, our finding that *T. brucei rhodesiense* impairs [^{3}H]TdR uptake by activated lymphocytes did not clarify whether the effect resulted from impaired DNA synthesis during the S phase or from cells being prevented from progressing from G0 and through the G1 pre-replicative phase. This question can be answered by using multiparameter flow cytometry, which enables determination of the proportions of cells in each of the cell cycle stages. We also asked whether the suppressive effect of *T. brucei rhodesiense* required physical contact with the lymphocyte or was mediated by a soluble parasite product(s). It will be shown in this paper that either the parasite or a soluble product(s) derived from it, which reproduces the suppressive effects of the trypanosome on IL-2R expression and lympho-proliferation, blocks entrance of PHA-stimulated PBMC into the cell cycle.

MATERIALS AND METHODS

Parasites

The KETRI-2285 strain of *T. brucei rhodesiense* used in this work, originally from the Kenya Trypanosome Research Insti-

tute, was kindly provided by Dr A. B. Clarkson, New York University, NY. The organism was initially stored under liquid nitrogen and then maintained by serial intraperitoneal passages in Crl-CD1 (ICR) Swiss mice (Charles River Laboratories, Portage, MI). Parasites were purified from the blood of mice infected intraperitoneally with $0{\cdot}5–1 \times 10^6$ organisms 2–3 days previously by chromatography through diethylaminoethyl-cellulose[14] using a buffer containing 103 mM Tris (pH 7·4), 1·5% glucose and 58 mM NaCl for column equilibration and elution. The eluted flagellates were washed twice by centrifugation with RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated (56°, 30 min) foetal bovine serum (FCS; Sigma Chemical Co., St Louis, MO), 100 IU/ml penicillin and 100 μg/ml streptomycin. This medium is referred to in the text as complete medium. Parasite concentrations were determined microscopically, using a haemacytometer, and adjusted to the desired level in the same medium. The suspensions consisted of 100% trypomastigotes, >99·9% of which were motile.

Preparation of T. brucei rhodesiense *supernatants (TRIF)*

Suspensions of *T. brucei rhodesiense* containing $1–5 \times 10^7$ parasites per ml in complete medium were incubated at 37° and 5% CO_2 for 16–24 hr and the culture supernatant sterilized by filtration through a 0·45-μm pore size filter. Aliquots of the filtrate were stored at −20° until used. These filtrates are referred to in the text as TRIF, for containing a *T. brucei rhodesiense* immunosuppressive factor(s). Aliquots of some TRIF preparations were dialysed (10,000 molecular weight cut off membrane) at 4° for 5 hr against 100 volumes of serum-free medium.

Preparation of PBMC

Blood was obtained from healthy volunteers. The PBMC were purified by centrifugation over a mixture of Ficoll–Hypaque of density 1·077 (400 ***g***, 20°, 30 min). After two washings with complete medium, the PBMC were resuspended, counted and adjusted to the desired concentration in the same medium. Cell viability, determined by trypan blue dye exclusion, was invariably >99%.

Lympho-proliferation assays

Cultures of PBMC in complete medium (96-well plates, 100 μl per well) were incubated at 37° and 5% CO_2 in the absence or presence of 5 μg/ml PHA (Sigma Chemical Co.), without or together with *T. brucei rhodesiense*, for various periods of time. Proliferation was evaluated in terms of incorporated radioactivity after addition of 1 μCi [^{3}H]TdR per well (specific activity 2 Ci/mmol; Amersham, Arlington Heights, IL). The concentrations of PBMC and parasites, and the duration of the [^{3}H]TdR pulses, are described in the Results section. In some assays, parasites were not included; instead, varying proportions of untreated or dialysed TRIF were substituted for the equivalent volume of complete medium. In pulse kinetics studies, [^{3}H]TdR was added at various times after initiation and the cultures were terminated 24 hr later. In all instances, the cultures were terminated by automated harvesting and processed for liquid scintillation counting. All assays were set up in quadruplicate.

Flow cytometric determinations of IL-2R expression

Cultures of PBMC in complete medium (24-well plates, 1 ml per well, $1{\cdot}25 \times 10^6$ cells/ml) were incubated at 37° and 5% CO_2 in the presence or absence of PHA (5 μg/ml) together with or without *T. brucei rhodesiense*. For single-colour analysis, the harvested cells were washed three times with phosphate-buffered saline solution, pH 7·2, containing 1% BSA and 0·1% sodium azide (PBS + BSA + azide) and were then incubated with fluorescein isothiocyanate (FITC)-anti-IL-2R monoclonal antibody (anti-CD25; Becton-Dickinson, San Jose, CA) at 0° for 30 min. Control cells were similarly incubated with FITC-normal mouse IgG to establish background fluorescence. The stained cells were washed two times with PBS + BSA + azide, fixed in 1% formaldehyde and stored in the dark at 4° until analysed using a FACScan flow cytometer (Becton-Dickinson). A minimum of 10,000 cells, gated on forward versus 90° light scatter to exclude erythrocytes, platelets, non-viable PBMC and *T. brucei rhodesiense*, were accumulated for each histogram. There was no detectable binding of FITC-anti-IL-2R monoclonal antibody to *T. brucei rhodesiense*. The percentage of positive cells was estimated against a background of cells stained with FITC-normal mouse IgG. Mean channel numbers of the logarithm of fluorescence intensities of the positive cell populations (MFCh) were used to compare the relative density of the relevant lymphocyte marker in the presence or absence of *T. brucei rhodesiense*. The logarithm of fluorescence intensities was distributed over four decades.

Cell cycle analysis

For cell cycle analysis, PBMC were incubated without or with PHA, in the absence or presence of *T. brucei rhodesiense*. The PBMC, parasite, mitogen concentrations and culture times are described in the Results section. In experiments in which reconstitution by recombinant human interleukin-2 (rhIL-2) was tested, 100 U/ml of this cytokine (i.e. the amount found to maximally support growth of the IL-2-dependent CTLL-2 cell line)[15] were added to the cultures at initiation. The rhIL-2 used in this study was a generous gift from Dr P. Sorter, Hoffman-LaRoche (Nutley, NJ). The cultures were terminated by harvesting and the cells were washed with cold, serum and dye-free RPMI-1640 medium. After centrifugation, the cells (1×10^6) were resuspended in the same medium and fixed by gradually increasing the concentration of cold ethanol from 25% to 70%. Samples were stored at 4° for at least 48 hr before being stained. Immediately before analysis, the cells were washed with Dulbecco's PBS (Gibco), centrifuged and resuspended in 500 μl of a freshly prepared mixture containing 18 μg/ml propidium iodide (PI; Calbiochem, La Jolla, CA) and 40 μg/ml ribonuclease A from bovine pancreas type I-AS (Sigma Chemical Co.) in PBS. After incubating this suspension at room temperature for 20 min, 50 μl of a freshly made solution of fluorescein isothiocyanate isomer I (FITC; Sigma Chemical Co.; 50 ng/ml) in 0·15 M NaCl were added, and the incubation was continued for 5 min.[16] The cells were immediately subjected to dual-parameter correlated flow cytometric analysis. FITC and PI fluorescences were collected through linear amplifiers. Erythrocytes, cell doublets, parasites, platelets and non-viable cells were excluded from analysis by setting an appropriate gate on forward versus side light scatter parameters. Thirty-thousand cells were collected for each sample. Two-parameter data, displayed as contour maps, were used to analyse the percentage

Table 1. IL-2 does not overcome the arrest of cell cycle progression caused by *T. brucei rhodesiense**

Culture condition	Time (hr)	Percentage of cells in G0/G1a	G1b	S	G2/M
PBMC	0	99·2	0·4	<0·4	<0·4
PBMC	15	91·9	8·0	<0·4	<0·4
PBMC + PHA	15	78·4	20·9	<0·4	<0·4
PBMC + PHA + *T. brucei rhodesiense*	15	97·7	2·0	<0·4	<0·4
PBMC + PHA + IL-2	15	64·5	35·1	<0·4	<0·4
PBMC + PHA + IL-2 + *T. brucei rhodesiense*	15	98·2	1·6	<0·4	<0·4
PBMC	24	88·3	11·6	<0·4	<0·4
PBMC + PHA	24	49·7	49·7	<0·4	<0·4
PBMC + PHA + *T. brucei rhodesiense*	24	98·7	1·1	<0·4	<0·4
PBMC + PHA + IL-2	24	50·4	49·9	<0·4	<0·4
PBMC + PHA + IL-2 + *T. brucei rhodesiense*	24	96·2	3·0	<0·4	<0·4
PBMC	48	96·3	3·1	<0·4	<0·4
PBMC + PHA	48	60·6	28·9	5·4	4·1
PBMC + PHA + *T. brucei rhodesiense*	48	94·4	3·3	1·7	0·6
PBMC + PHA + IL-2	48	69·9	19·8	7·2	3·1
PBMC + PHA + IL-2 + *T. brucei rhodesiense*	48	90·9	6·8	1·9	0·4

* PBMC ($1{\cdot}25 \times 10^6$ per ml) were incubated for the indicated amounts of time without or with 5 μg/ml of PHA in the absence or presence of 2×10^7 *T. brucei rhodesiense*/ml. Cells were stained and analysed as described in the Materials and Methods. This set of data is typically representative of two separate repeat experiments in which cells from different donors were used.

of cells in each of the phases of the cell cycle (i.e. G0/G1a; G1b; S and G2/M), as previously described.[11, 13, 17, 19] The following nomenclature was used: G0/G1a, quiescent plus cells in early G1, i.e. with less protein than S phase cells and diploid DNA content; G1b, cells in late G1, i.e. with protein content equal to that of S phase cells and diploid DNA content; S, cells with more than diploid but less than tetraploid DNA content; and G2/M, cells with tetraploid DNA content plus cells in mitosis.[11, 13, 17, 19]

RESULTS

Effects of *T. brucei rhodesiense* on the ability of PHA-stimulated PBMC to progress through the cell cycle

We studied whether *T. brucei rhodesiense* would suppress proliferation by blocking progression of PHA-stimulated PBMC through the cell cycle at a certain boundary or stage. The results shown in Table 1 indicate that the organism markedly inhibited not only entrance of cells into, but also subsequent cell progression through, the cell cycle. This effect was demonstrable 15 hr after activation (Table 1), i.e. at the earliest time that it was tested for. It should be noted that in the various repeat experiments the numbers of PHA-stimulated PBMC reaching the G1b phase in the presence of parasites at 15 hr represented between 2% and 20% of the cells in G1b found in parallel cultures lacking the trypanosome. Suppression became even more pronounced as additional culture time elapsed and more PHA-stimulated cells in the parasite-free cultures continued to enter the G1b, S and G2/M stages. During the remainder of the observation period, the proportion of stimulated PBMC exposed to trypanosomes which was found to be traversing

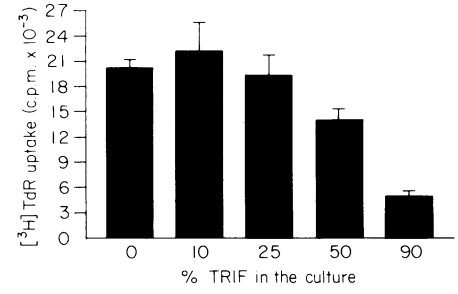

Figure 1. Titration of the suppressive effect of TRIF on proliferation by PHA-stimulated PBMC. The value obtained with PBMC alone was $2{\cdot}7 \pm 0{\cdot}5$ c.p.m. $\times 10^{-3}$. Bars represent the mean and vertical lines the standard deviation of quadruplicate values; [^{3}H]TdR pulse, 24–48 hr. The TRIF preparation used in this experiment was the filtrate of a suspension containing 5×10^7 parasites/ml incubated at 37° (5% CO_2) for 16 hr. Only the differences between the values obtained with 50% and 90% TRIF and the control value (PHA alone, 0% TRIF) were statistically significant ($P < 0{\cdot}05$). This set of data is typically representative of two separate repeat experiments in which cells from different donors were used.

through the cell cycle remained very low (<6% of the PBMC were in the G1b, S and G2/M phases). It is noteworthy that, in about half of our experiments, the mitogen-stimulated PBMC in G0/G1a stained more dimly with FITC (representing lower levels of cell protein content) when co-cultured with the parasite. The addition of rhIL-2 to parasite-PBMC co-cultures failed to overcome the suppressive effect of the parasite, as evidenced by the results of cell cycle kinetic experiments.

Table 2. Titration of the suppressive effect of TRIF on IL-2R expression by PHA-stimulated PBMC*

Culture condition	% TRIF	% IL-2R+ (MFCh)	% suppression
PBMC	0	3·9 (69)	
PBMC+PHA	0	47·3 (84)	
PBMC+PHA+TRIF	10	49·4 (88)	−4·4
PBMC+PHA+TRIF	25	47·0 (81)	0·6
PBMC+PHA+TRIF	50	40·6 (69)	14·2
PBMC+PHA+TRIF	75	37·1 (58)	21·6
PBMC+PHA+TRIF	90	25·2 (47)	46·7

* PBMC (1×10^6 per ml) were incubated for the indicated amounts of time without or with 5 μg/ml of PHA in the absence or presence of the indicated proportions of TRIF. The cultures were terminated at 15 hr and the cells were stained and analysed by flow cytometry as described in the Materials and Methods. This set of data is typically representative of two separate repeat experiments in which cells from different donors were used.

Table 3. Comparison of the suppressive effects of untreated and dialysed TRIF on lympho-proliferation by PHA-stimulated PBMC*

Culture condition	C.p.m. $\times 10^{-3} \pm$SD Exp. 1	Exp. 2
PBMC	0·5±0·4	0·8±0·6
PBMC+PHA	33·3±2·3	21·0±3·0
PBMC+PHA+untreated TRIF	8·1±0·8	4·0±0·7
PBMC+PHA+dialysed TRIF	4·4±0·8	8·3±1·1
PBMC+PHA+*T. brucei rhodesiense*	9·9±0·5	2·8±0·2

* PBMC ($1{\cdot}25 \times 10^6$ per ml) were incubated in complete medium alone or containing 90% of untreated or dialysed TRIF. Where present, the parasites were used at 2×10^7 organisms/ml. PHA was used at 5 μg/ml; [^{3}H]TdR pulse, 24–48 hr. This set of data is typically representative of two separate repeat experiments in which cells from different donors were used.

Table 4. Reversibility of the suppressive effect of TRIF on the proliferation of PHA-stimulated PBMC*

Culture condition	C.p.m. $\times 10^{-3} \pm$SD measured from 24–48 hr	48–72 hr	72–96 hr
PBMC	1·4±0·7	0·8±0·2	1·1±0·2
PBMC+PHA	18·2±1·5	18·4±2·8	23·7±4·3
PBMC+PHA+TRIF	7·8±1·2	17·6±1·8	23·9±2·5

* PBMC ($1{\cdot}25 \times 10^6$ per ml) were incubated in complete medium alone or containing 90% of untreated TRIF. PHA was used at 5 μg/ml. This set of data is typically representative of two separate repeat experiments in which cells from different donors were used.

Table 5. Delay by TRIF of the entrance of PHA-stimulated PBMC into the cell cycle*

Culture condition	Time (hr)	Percentage of cells in G0/G1a	G1b	S	G2/M
PBMC	0	94·5	5·3	<0·4	<0·4
PBMC	15	82·1	17·7	<0·4	<0·4
PBMC+PHA	15	50·1	49·5	<0·4	<0·4
PBMC+PHA+TRIF	15	97·7	1·9	<0·4	<0·4

* PBMC ($1{\cdot}25 \times 10^6$ per ml) were incubated for the indicated amounts of time in the presence of 5 μg/ml of PHA in the absence or presence of 90% TRIF. Cells were stained and analysed by flow cytometry as described in the Materials and Methods. This set of data is typically representative of two separate repeat experiments in which cells from different donors were used.

Suppressive effects of TRIF on PHA-stimulated PBMC

To establish whether *T. brucei rhodesiense*-induced suppression required physical contact between the organism and the PBMC, and whether a soluble parasite product(s) was involved in this effect, we determined both [^{3}H]TdR uptake and IL-2R expression by PHA-stimulated PBMC in the absence or presence of TRIF. As shown in Fig. 1, TRIF markedly reduced [^{3}H]TdR uptake in a concentration-dependent manner. The expression of IL-2R was also inhibited by TRIF, as evidenced by significant reductions in both the percentage of IL-2R$^+$ cells and the surface density of IL-2R molecules (represented by the MFCh values) (Table 2).

We considered the possibility that TRIF-mediated suppression might have been only apparent resulting from either parasite depletion of essential medium nutrients during TRIF preparation or competition between cold thymidine from disintegrated organisms and [^{3}H]TdR. However, TRIF preparations dialysed against fresh culture medium were as suppressive as non-dialysed aliquots (Table 3).

The suppressive effect of TRIF was reversible. In the various repeat experiments complete recovery of PHA-responsiveness was demonstrable either 48–72 hr or 72–96 hr after initiation of the cultures (Table 4).

We then examined whether TRIF would also block entrance of PHA-stimulated cells into the cycle. As can be seen in Table 5, this was indeed the case. At 15 hr, the pattern of distribution of cells in the various phases of the cell cycle was similar to that observed when *T. brucei rhodesiense* had been used (cf. Tables 1 and 5).

DISCUSSION

These results show that *T. brucei rhodesiense* and soluble products from it can arrest PHA-stimulated human lymphocytes in the G0/G1a phase of the cell cycle. The *in vitro* system used to document this effect was the same that helped us unveil the ability of this parasite to suppress IL-2R expression and lympho-proliferation (F. Kierszenbaum, S. Muthukkumar, L. A. Beltz and M. B. Sztein, manuscript submitted for publication) and appears to be a suitable model system for the

study of the pronounced immunosuppression seen in African trypanosomiasis.[1-10]

Incorporation of [^{3}H]TdR represents an approximation of lymphocyte stimulation but is hampered by some potential pitfalls. Demonstrated limitations of [^{3}H]TdR uptake include: (i) it does not provide data about the early events of lymphocyte activation prior to cell entry into the S period; (ii) significant [^{3}H]TdR incorporation is known to be associated with non-S phase DNA synthesis; (iii) the quantity of incorporated [^{3}H]TdR is influenced by the pool of endogenous precursors; (iv) endogenous precursors from dead cells compete for uptake; (v) bulk assays do not provide information about individual cells; and (vi) tritium emissions affect the ability of cells to divide.[11-13] For these reasons, it was important to establish whether the reduced [^{3}H]TdR incorporation caused by the presence of *T. brucei rhodesiense* truly reflected suppression of lymphocyte progression through the S phase. The answer to this question was derived from the present cell cycle studies which revealed that the parasite caused the stimulated PBMC to be arrested at the G0/G1a stage (Table 1). Therefore, suppressed [^{3}H]TdR incorporation did represent decreased S phase DNA synthesis because a large majority of the cells never reached this phase. The noted arrest in G0/G1a is in keeping with the suppression of IL-2R expression, caused by *T. brucei rhodesiense* (F. Kierszenbaum, S. Muthukkumar, L. A. Beltz and M. B. Sztein, manuscript submitted for publication), which is necessary for lymphocytes to progress through the G1 phase. However, these results do not rule out that events prior to IL-2R expression may be affected by the parasite.

Because IL-2 up-regulates IL-2R expression,[20-23] reduced IL-2 production in the presence of *T. brucei rhodesiense* would have explained the observed decrease in IL-2R expression. However, exogenous rhIL-2 did not restore responsiveness to the parasite-suppressed cells, i.e. it did not enable PHA-stimulated cells to enter the cell cycle (Table 1). This indicated that down-regulated IL-2 production was unlikely to be the mechanism by which *T. brucei rhodesiense* affected lympho-proliferation. This finding is in accordance with our previous observation that equal or greater levels of IL-2 activity were present in the supernatants of co-cultures of PHA-stimulated PBMC with the trypanosome than in parallel cultures lacking the organism (F. Kierszenbaum, S. Muthukkumar, L. A. Beltz and M. B. Sztein, manuscript submitted for publication).

Production of IL-2 and the interaction of this cytokine with its specific receptors (known to be expressed on the lymphocyte membrane approximately 10 hr after activation)[24] are critical events required for activated lymphocytes to be able to reach S, i.e. to traverse from early G1 (G1a) to late G1 (G1b).[25] In this context, our results, showing substantial cell accumulation at the G0/G1a stage, are in keeping with our previous finding that *T. brucei rhodesiense* suppresses IL-2R expression by PHA-stimulated PBMC in our system (Table 2). A similar effect was observed in this study when we used TRIF preparations (Table 5), suggesting that a soluble parasite product(s) mediated the suppressive effect. The possibility that the suppressive effect results from both physical parasite–PBMC contact and soluble mediator(s) active on the PBMC can not be ruled out by these results.

Preparations of TRIF which had been dialysed against fresh culture medium also suppressed PBMC responses to PHA, indicating that the molecular weight of the active molecule(s) must have been greater than 10,000 (Table 3). Activity demonstrable after dialysis makes it unlikely that consumption of essential medium nutrients by the parasite could have caused the noted suppression or that the reduced [^{3}H]TdR incorporation caused by TRIF could be due to excessive cold thymidine present in these preparations. Moreover, any cold thymidine present in TRIF could not have been responsible for its ability to suppress IL-2R expression, and excessive amounts of this nucleotide would not have allowed the suppressive effect to be reversible (Table 4). Such reversibility also ruled out a possible cytotoxic effect of TRIF.

We found that TRIF preparations not only reduced significantly lympho-proliferation (Fig. 1), but also the surface density of IL-2R on PHA-stimulated cells, evidenced by a decrease in fluorescence intensity of the IL-2R+ cell populations (Table 2). These results are consistent with the known fact that activated lymphocytes expressing low levels of IL-2R have a diminished capacity to progress through the cell cycle.[26]

The protein content of PBMC from cultures containing the parasite was lower than that observed in the absence of the organism, suggesting that inhibition of protein synthesis might be implicated in the mechanism whereby *T. brucei rhodesiense* induces immunosuppression. We are studying this possibility.

Our flow cytometric analysis of the cell cycle places the time of the process(es) targeted by *T. brucei rhodesiense* at 15 hr or earlier. The flagellate might alter calcium ion fluxes, phosphatydil inositol turnover, transcription of genes for IL-2R or other key proteins involved in T-lymphocyte activation. These possibilities are being investigated in our laboratories.

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