THE MECHANISMS OF IMMUNOSUPPRESSION IN RATS INFECTED BY *TRYPANOSOMA LEWISI*

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ABSTRACT

Trypanosoma lewisi infections are characterized by immunosuppression. This study was done to clarify the defect in the action of the immmunological cells which is responsible for this phenomenon. Infected rats taken at different days of the infection were as able as normal rats to respond immunologically to the T-independent antigen polyvinylpyrrolidone, indicating that a B cell defect is not responsible for suppression of the immune system. Stimulation of spleen cells from infected rats with Concanavalin A showed that T cells were suppressed during the exponential phase of the infection. Production of the T cell soluble factor Interleukin 2 was suppressed at days 7 and 20 of the infection. Since the existence of an adherent suppressor cell was demonstrated with mixed cell cultures it is hypothesized that suppression of T cell activity is due to a defect in Il 2 production induced by the action of a suppressor macrophage. A colateral study has indicated that bloodstream or insect forms of the parasite are equally infective in naive rats by the oral or intraperitoneal routes of inoculation. This result indicates that transmission of *Trypanosoma lewisi* can occur equally from the insect flea vector, or as a result of cannibalism.

ABREGE

Les infections causées par *Trypanosoma lewisi* se caractérisent par une immunosuppression. Cette étude a été mise sur pied dans le but d'identifier l'origine de cette carence par l'étude de l'activité des cellules impliquées dans le système immunitaire. Les rats infectés, indépendamment du temps de l'infection, ont démontré autant de capacité que les rats normaux à répondre de façon immunologique à l'antigène T-indépendant polyvinylpyrrolidone indiquant ainsi une activité saine et normale des cellules B d'animaux infectés. La stimulation mitogénique des splénocytes de rats infectés avec la Concanavaline A a démontré une suppression des cellules T durant la phase exponentielle i.e. au jour 7 de l'infection. De plus, la production du facteur soluble Il 2 sécrété par les cellules T est supprimée aux jours 7 et 20 de l'infection. Puisque l'existence de cellules adhérentes suppressives a été démontrée, l'hypothèse d'une suppression de l'activité des cellules T due à une défaillance au niveau de la production de Il 2, elle-même induite par l'action de macrophages suppressifs, est émise. Une étude collatérale a indiqué que les formes sanguines ou de l'insecte du parasite sont egalement infectieuses lorsqu'inoculees soit oralement ou par injections intrapéritonéales, dans les rats normaux. Ces résultats proposent que la transmission de *Trypanosoma lewisi* se produise par l'entremise de l'insecte vecteur ou soit la résultante du phénomène de cannibalisme.

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INTRODUCTION

Suppression of the ability of the immune system to respond to a given antigen occurs in trypanosome-induced infections, as in most, if not all, parasitic infections. The mechanism by which this immunodepression is induced has not been fully identified, but several hypotheses have been proposed to explain the phenomenon. (1) a break in the link through which T and B lymphocytes cooperate (Terry *et al*, 1973), (2) the action of suppressor cells (Ramos, Schadtler-Simon & Ortiz-Ortiz, 1979), (3) clonal exhaustion of antibody-producing cells induced by intense polyclonal activation (Hudson *et al*, 1976), or (4) the direct action of the parasite, or its soluble products, on the immediate precursors of antibody-producing helper cells (Albright, Albright & Dusanic, 1978) Whatever mechanism(s) operates to suppress, the functional common denominator of all is to permit the parasite to establish and to survive in the normally immunocompetent host.

The objective of this study was to investigate the cause of immunosuppression during trypanosome-induced infections, using as a model the specific parasite of the rat *Trypanosoma lewisi*. This host-parasite association is an excellent model for a study of the immunobiology of trypanosomiasis: *T. lewisi* is a natural parasite of rats and this host-parasite association resembles that of the more pathogenic species with the advantage of not showing the phenomenon of antigenic variation

The results of this study are presented in 4 main self-

contained chapters or sections: the first, as a follow-up of the report of St-Charles, Frank & Tanner (1981) will discuss the capacity of infected animals to produce specific antibody against a helper-T cell-independent antigen (polyvinylpyrrolidone); the results which will be presented will give an insight on the induction of antibody-producing cells (B cells) in the infection. The second chapter will report the results of the blastogenesis of spleen cells recovered from infected animals at different stages of the infection, when stimulated with the specific mitogens Concanavalin A and Lipopolysaccharides. Since the role of the lymphokines Interferon and Interleukin 2 are important in the control of other trypanosome infections, we studied the capacity of spleen cells to produce Il 2 during the infection; the results of this investigation are described in the third section. These sections address the problem of understanding the immunological mechanism(s) underlying the induction of immunosuppression in infections by *Trypanosoma lewisi*. The results described in the fourth and last chapter are of a colateral study with a different objective: a re-examination of the proposal (Hoare, 1972) that the transmission of *T. lewisi* occurs by oral inoculation of the parasite in the rat. Comparisons are made in that study with infections obtained by intraperitoneal inoculations of the parasite in rats. Although the immunological implications of these latter studies were not investigated, the results which were obtained present interesting new evidence concerning the natural transmission of the parasite; they are, thus, introduced into this thesis.

It is hoped that the results of this study will clarify an understanding of how trypanosomes can establish and maintain themselves in immunocompetent animals.

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CHAPTER 1

THE ANTIBODY RESPONSE OF RATS INFECTED BY *TRYPANOSOMA LEWISI* TO THE T-INDEPENDENT ANTIGEN POLYVINYLPYRROLIDONE.*

* for submission to Immunology.

Summary. The ability of spleen cells from rats infected with *Trypanosoma lewisi* to mount a secondary response to the helper-T cell-dependent antigen sheep red blood cells (SRBC) has been previously studied. Results of that study showed that the B cell response to SRBC was significantly suppressed both *in vivo* and *in vitro*. It was therefore of interest to determine in the present study the capacity of antibody producing B cells to respond immunologically to the helper-T cell-independent antigen polyvinylpyrrolidone (PVP). Normal and infected rats at day 7, 12, 20, 30 and 40 of the infection were immunized intraperitonealy with 0,1 ml of 10^9 tannic acid-treated SRBC onto which PVP was adsorbed. The animals were killed on the 7th day of the immunization and the blood plasma was analysed for antibody production to the PVP. The results indicated that normal and infected rat were fully capable of responding immunologically with an antibody response to the PVP. On the basis of this study, it is concluded that immunosuppression during infection by *T. lewisi* is not due to a dysfunction of B cells.

INTRODUCTION

Mechanisms by which parasites baffle the immune system to permit their own survival in the host for extensive periods of time were, and still are, the prime interest of numerous research groups. Under normal circumstances, the immune system of an animal into which an organism is introduced will be stimulated to attack the antigen by generating, for example, specific antibodies (Roitt, Brostoff & Male, 1985); activation depends, with only a few exceptions, on the collaboration of specific T and B cells. Immune responses to the majority of antigens (T-dependent antigens) requires that this collaboration takes place; there are few antigens (T-independent antigens), however, which can stimulate B cells directly to form antibodies, without requiring an interaction with T-helper cells. It is well known that in trypanosome infections (Murray *et al*, 1973; Albright, Albright & Dusanic, 1977; Reed, Roters & Goidl, 1983) the normal action of the immune system against both types of antigens is compromised.

The immune system of rats infected with *Trypanosoma lewisi* is significantly suppressed in its ability to respond to the T-dependent antigen sheep red blood cells (SRBC; St-Charles, Frank & Tanner, 1981). These authors showed that the secondary plaque-forming cell (PFC) antibody response to SRBC was suppressed in infected spleen cells and in primed normal rat splenocytes cultured with live, freeze-thawed, sonicated or heat-treated trypanosomes. Although this report suggested that infections by *T. lewisi* are regulated through the mechanism of

immunodepression, the results of these authors did not clarify the nature of the defect which the parasite produced in the immune system. The following study was intended to fill some of this gap by an investigation of the capacity of infected animals to produce antibodies following immunization with the T-independent antigen polyvinylpyrrolidone (PVP).

MATERIALS AND METHODS

Maintenance of parasite

The parasite was provided by Dr. Pierre Viens, Département de Microbiologie et d'Immunologie, Université de Montréal, in September 1978 and originated from the parasite bank of the London School of Hygiene and Tropical Medicine. *Trypanosoma lewisi* has been maintained in our laboratory by serial passage in normal Fisher-344 inbred rats (Charles River Canada Inc., St-Constant, Qué.). Trypanosomes were obtained on the 12th day of the infection by cardiac puncture from the blood of infected donors, using heparinized syringes. The blood was centrifuged twice: at 160 x g for 10 minutes to sediment the leucocytes and red cells, and then at 140 x g for 15 minutes to pellet the trypanosomes. The latter were resuspended in cold Hanks' Balanced Salt Solution (HBSS) and counted without staining in a Neubauer heamacytometer under phase constrast illumination (400x). One tenth ml of a suspension of 1 x 10^8 trypanosomes / ml in HBSS was injected intraperitonealy (i.p) into normal rats to elicit an infection.

Antigen

Polyvinylpyrrolidone (PVP; M.W. 10,000; Lot No. 320-0770) was obtained from Sigma Chemicals Company, St. Louis, MO. Trypomastigotes for antigen extraction were recovered from day 15 infected donors, filtered through a DE52 cellulose column equilibrated with pH 8,0 PBSG (Lanham, 1968), centrifuged at 1000 x g / 15 min at 4°C and resuspended in 10mM Tris-HCl buffer (pH

8,0) containing 4mM of the enzyme inhibitor phenylmethyl-sulfonyl fluoride (Sigma, St. Louis, MO.). The trypanosomes were then disrupted at 0°C with 150 kc ultrasounds (Blackstone Ultrasonics Inc, Shelfield, PA.), and centrifuged at 120 000 x g at 4°C for 1 hour in a Beckman Model L 3-50 ultracentrifuge (Beckman Instruments Inc, Palo Alto, CA.). The dye-binding estimation method of Bradford (1976) was performed to determine the protein concentration of the sonicated extract.

Immunization

PVP was bound to sheep red blood cells (SRBC) for immunization, according to the method of Roller & Trainin (1974). Briefly, freshly-drawn SRBC in Alsever's solution were washed twice in normal saline at 2500 rpm g for 5 minutes and made up to a concentration of 2,5% in phosphate buffered saline, pH 7,2 (PBS). Equal volumes of 2,5% SRBC and 1:40,000 tannic acid (M.W. 1701.18; Lot No 786873; Fisher Scientific Company) in PBS were incubated for 10 minutes at room temperature. Tannic acid-treated SRBC were washed three times and made up again to 2.5% in saline; an equal volume of this cell suspension and a 0.1 mg/ml solution of PVP in PBS, pH 6,4 was incubated for 10 minutes at room temperature. Final washes of PVP-tannic acid-treated SRBC were made in normal saline, the cells were counted and made up to a concentration of 1×10^9 cells / ml in saline. Volumes of 0,1 ml of this suspension were used for the intraperitoneal immunization of normal and infected rats at day 7, 12, 20, 30 and 40 of the infection. Seven days after immunization, the rats were anaesthetised with CO_2 and the blood was harvested by cardiac puncture

with heparinized syringes. The blood was centrifuged once at high speed (1400 x g for 15 minutes) to obtain the plasma, which was then tested by ELISA for the presence of specific antibodies to the parasite and to the PVP antigen.

Assay for antibody production

ELISA (Enzyme-Linked Immunosorbent Assay) was performed 7 days after immunization with PVP on the plasma from normal non-immunized (NNI), normal immunized (NI) and infected-immunized (II) rats by the method of Voller *et al* (1979). Briefly, 96 wells of flat-bottomed microtitre plates (Beckton-Dickinson Labware, Oxrand, CA.) were coated overnight at 4°C with 0,1 ml of 10 µg of PVP / ml of carbonate buffer (pH 9,6) or with 0,1 ml of 5 µg of the trypomastigote extract / ml of buffer. Plates were washed 3 X with PBS-TWEEN (phosphate buffered saline, pH 7,2; TWEEN-20, Nutritional Biochemicals Corporation, Cleveland, OH; 1 TWEEN-20: 2000 PBS) and filled with 0,1 ml of BSA-PBS-TWEEN (1g of bovine serum albumin / 100 ml of PBS-TWEEN; BSA was obtained from Sigma; Lot No. 115F-0644) and incubated for 1/2 hour at 37°C to block any remaining protein-binding sites in the plastic wells. After another series of three washings, plasma diluted in BSA-PBS-TWEEN was dispensed in the antigen-coated wells and incubated for 1 hour at 37°C. The plates were washed again and incubated for 1 hour at 37°C with the enzyme-conjugate peroxidase-labelled anti-rat-IGg1 (1:1000 in BSA-PBS-TWEEN, Miles-Yeda Ltd, Israel). Final washes were performed and 10 minutes after the addition of the substrate solution ABTS (Sigma) +3% H_2O, the optical density was read at 405 n.m. using a Titretek Multiscan MC ELISA plate reader

(Flow Laboratories McLean, VA.).

Statistical analysis was done by the Student's *t* test (Snedecor & Cockran, 1967).

RESULTS

The results presented in Figure 1.1 indicate that infected animals, regardless of the time of the infection, are equally or more capable of responding immunologically to the SRBC-PVP antigen when compared to normal uninfected immunized rats. A significant peak ($p < 0,01$) antibody response was observed at day 30 of the infection. This day corresponds to the end of the infection, when the number of trypanosomes in the blood of rats is declining to eventually disappear (Figure 1.1).

To evaluate the specific antibody response to the parasite of these infected and immunized rats, ELISA was performed with the same plasma as above but using the trypomastigote extract as the antigens. Figure 1.2 indicates that infected animals produce a good antibody response against the trypomastigote antigens with very high yields at days 27, 37 and 47 ($p < 0,01$ & $p < 0,001$). When superimposed on the parasitaemia, the curve (O , Figure 1.2) shows a maximum response near the end of the infection, after the recovery of infected animals.

Figure 1.1

Parasitaemia of rats infected with *T. lewisi* and anti-PVP antibody production in these animals .

(●) Parasitaemia x 10^6 / ml +/- S.E.

(○) Reciprocical anti-PVP antibody titre +/- S.E. of infected rats

(- -) Reciprocical anti-PVP antibody titre +/- S.E. of normal uninfected rats

Statistical analysis by the Student's t test:

** $p < 0,01$

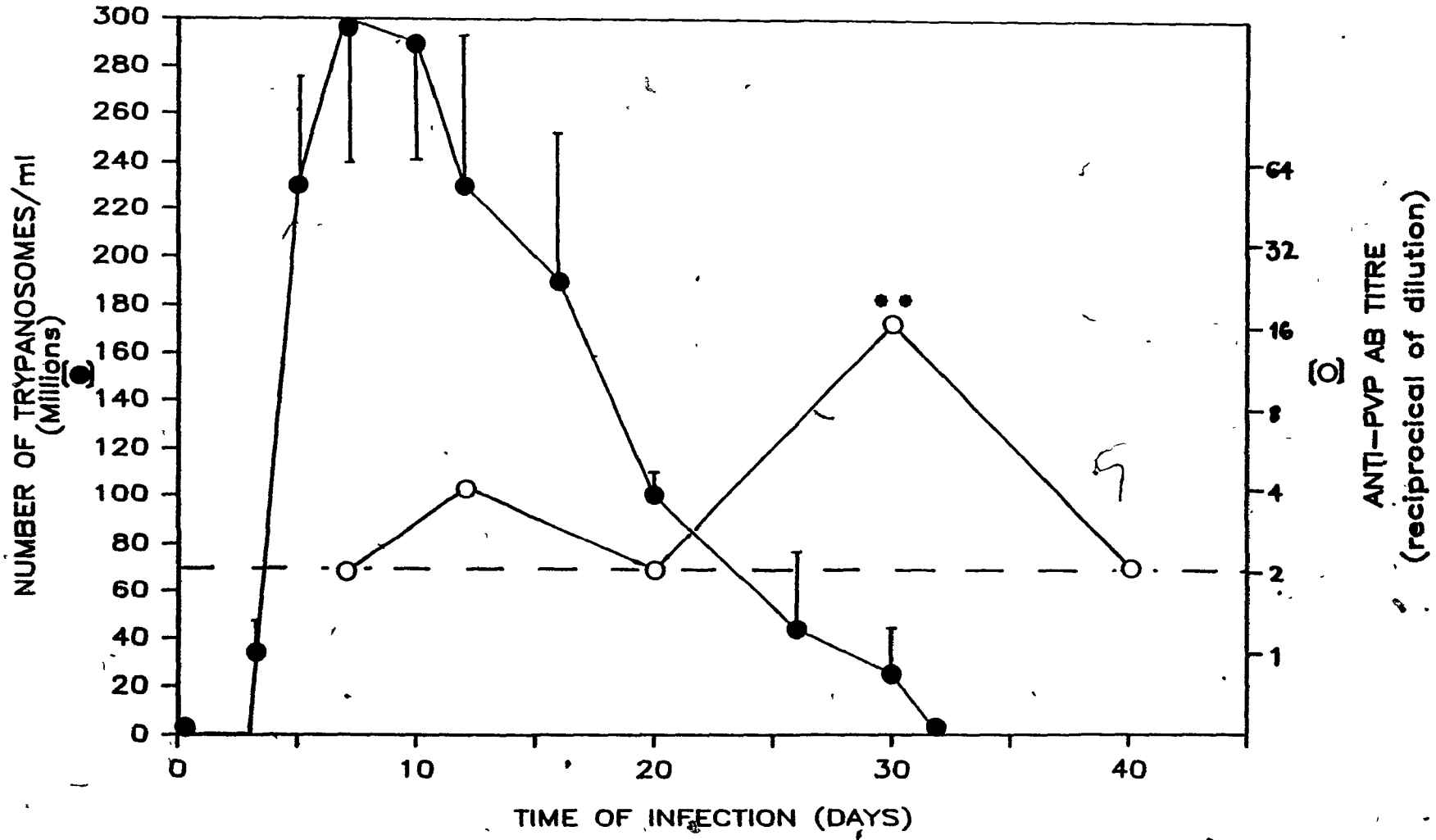

NUMBER OF TRYPANOSOMES/ml (Millions)
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
0
10
20
30
40
TIME OF INFECTION (DAYS)
64
32
16
8
4
2
1
ANTI-PVP AB TITRE (reciprocal of dilution)

Figure 1.2

Parasitaemia of rats infected with *T lewisi* and anti-trypomastigote antibody production in these animals

(●) Parasitaemia x 10^6 / ml S E

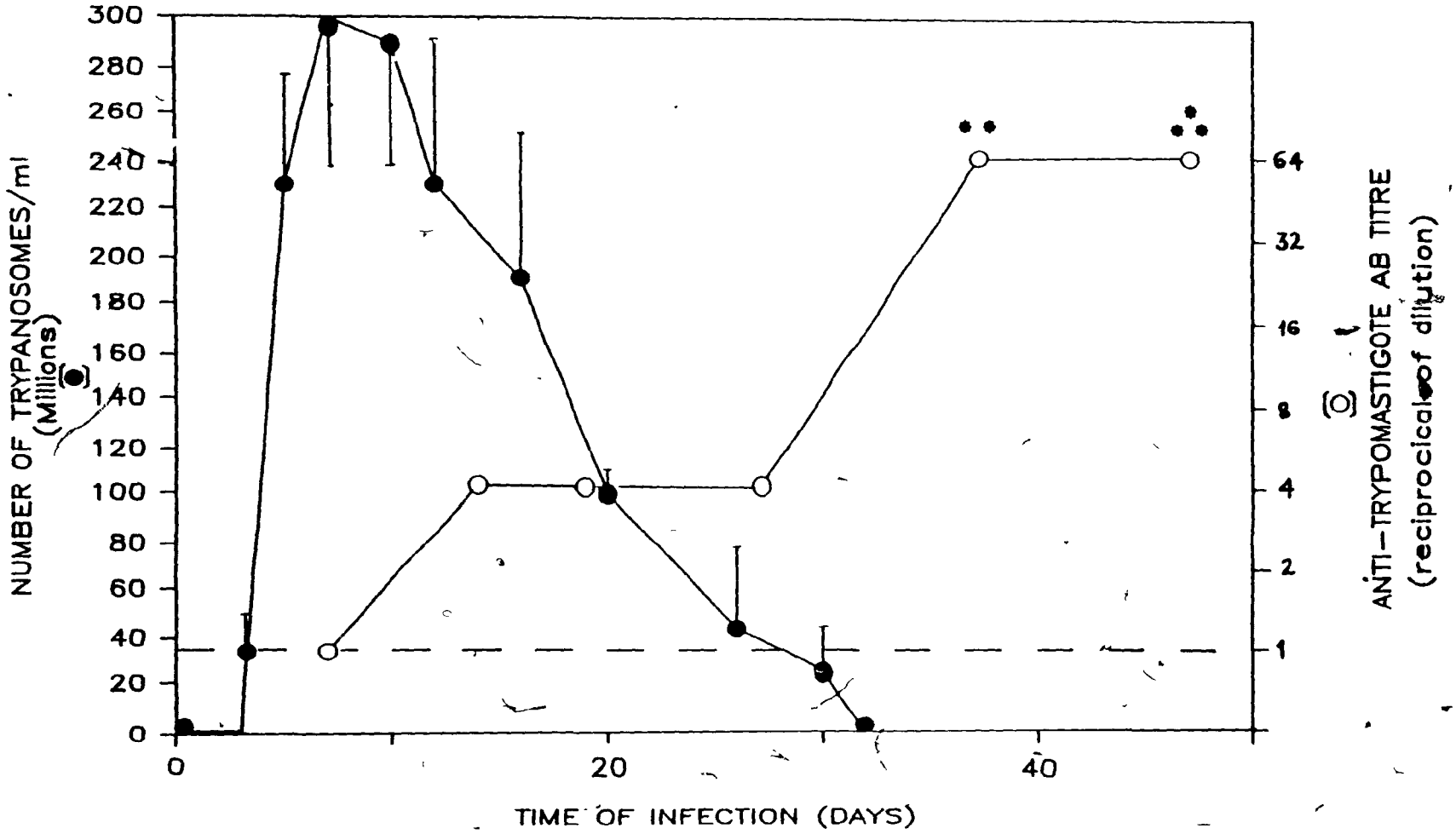
(○) Reciprocical anti-trypomastigote antibody titre +/- S E of infected rats

(– –) Reciprocical anti-trypomastigote antibody titre +/- S E of normal uninfected rats

Statistical analysis by the Student's t test

** $p < 0,01$

*** $p < 0,001$


NUMBER OF TRYPANOSOMES/ml
(Millions)
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
0
20
40
TIME OF INFECTION (DAYS)
64
32
16
8
4
2
1
ANTI-TRYPOMASTIGOTE AB TITRE
(reciprocal of dilution)

DISCUSSION

St-Charles *et al*, 1981 demonstrated a suppressed anti-sheep erythrocyte antibody production by spleen cells from rats infected by *T. lewisi*, both *in vivo* and *in vitro*. This state of immunosuppression was demonstrated from beginning to end of the infection; it could even be demonstrated after the infected animals had completely recovered. Paradoxically, results obtained in this study indicate that infected animals are very good producers of antibodies against trypanosomes (figure 1.2). The control of infection by *T. lewisi*, as the antibody response following immunization with SRBC, is controlled by a thymus-dependent mechanism. Indeed, antithymocyte serum-treated and infected rats suffer fulminating infections, more than half of which are fatal (Spira & Greenblatt, 1970) The peculiar paradox of immunosuppression in immunocompetent animals is also characteristic of a large variety of parasite infections (Terry *et al*, 1973; Tanner & Faubert, 1974, Mansfield & Bagasra, 1978)

One other mystery in rats infected by *T lewisi* lies in the presence of a significant yield of trypanosome-specific antibodies, long after the disappearance of the parasite from the blood and recovery of the animal. One is tempted to hypothesize that trypanosomes continue to be present in the animal after recovery from the parasitaemia, continuing to stimulate the specific anti-parasite antibodies. Viens *et al* (1972) have demonstrated *T. musculi* in the *vasa recta* of the kidneys after the parasite was eliminated from blood.

Reports of the immunological impairment of T and/or B cells, or macrophages, of the spleen of parasite-infected animals have been widely published. In *Trypanosoma cruzi* infections (Chagas' disease), both T and B cells are suppressed in their ability to respond to T-dependent and independent antigens by the action of trypanosome-induced suppressor cells (Clinton *et al*, 1975; Ramos *et al*, 1978; Cunningham & Kuhn, 1980; Reed *et al*, 1983); *T. brucei* also induces suppressor cells (Jayawardena, Waksman & Eardley, 1970; Eardley & Jayawardena, 1977). *T. musculi* infections have shown a similar suppression of T and B lymphocyte responses (Albright *et al*, 1977). *T lewisi*, on the other hand, does not affect the activity of B lymphocytes (figure 1.1) since anti-PVP antibodies were present in the plasma of infected rats all through the course of the infection. Significantly, B cells from infected rats were as efficient as, or more efficient, than the B cells of normal uninfected control animals in the production of antibodies against the thymus-independent antigen PVP. A similar situation has also been reported for laboratory infections by *T. rhodesiense* where B cell activity remained normal during the course of infection in spite of a functional loss of T cells; B lymphocyte function failed, however, at the stage preceeding death (Mansfield *et al*, 1978)

The results of this study indicates two interesting and important facts which characterize the immunobiology of infections with *T. lewisi:* (1) the immunological response of infected animals to parasite antigens is intact throughout the infection in spite of the fact that the same animals are unable to respond

to the heterologous T-dependent antigen SRBC (St-Charles *et al*, 1981); and (2) the results of the studies described in this chapter suggest that immunosuppression in this infection is due to a T cell defect, since B lymphocytes function normally to make antibodies to the T-independent antigen PVP.

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CHAPTER 2

THE DEPRESSED RESPONSE OF SPLEEN CELLS FROM RATS INFECTED BY *TRYPANOSOMA LEWISI* TO CONCANAVALIN A STIMULATION: GENERATION OF A POPULATION OF SUPPRESSOR MACROPHAGES.*

* for submission to Immunology.

Summary. Rats infected by *Trypanosoma lewisi* are depressed in their ability to respond to sheep red blood cells (SRBC), but it has been shown earlier that infection does not appear to affect the capacity of B cells to make antibodies. To study the target for immunosuppression further, the synthesis of DNA by spleen cells recovered from *T. lewisi* infected rats to stimulation by the mitogens Concanavalin A (ConA) and lipopolysaccharide (LPS) was examined The response to ConA mitogen was depressed only for spleen cells taken from infected rats on day 7; the response to LPS was normal at this time Spleen cells from animals at days 12, 20 and 35 of the infection and normal uninfected rats were equally capable of responding to both mitogens. Mixed-cell cultures of normal spleen cells with splenocytes from day 7 infected rats indicated that the immunosuppression in these infected animals is due to suppressor adherent cells. These studies suggest strongly that infection by *T. lewisi* induces a defect in the T-lymphocyte cell population and that, furthermore, infection stimulates the generation of a population of suppressor macrophages.

INTRODUCTION

Trypanosoma lewisi depresses the capacity of the immune system of infected rats to respond to SRBC both *in vitro* and *in vivo* (St-Charles, Frank & Tanner, 1981). The results presented in Chapter 1 suggest that this immunosuppression does not involve a defect or an impairment of the functioning of antibody-producing B cells. An attempt was made to further delineate the mechanism underlying this immunosuppression; these studies are described in this chapter.

It is well known that the majority of immunological responses are dependent on the processing and presentation of antigens (or antigen signals) by the macrophage to T-helper cells and then to B lymphocytes. It is also well-established that some antigens can stimulate B cells directly to produce specific antibodies, without a requirement for the participation of T-helper cells (Roitt, Brostoff & Male, 1985). To further clarify the nature of the defect in the lymphoid apparatus of rats infected with *T. lewisi*, the susceptibility of spleen cells to activation *in vitro* by the specific T cell mitogen Concanavalin A (ConA) and by the B cell mitogen lipopolysaccharide (LPS) was determined.

Lymphocytes stimulated with these mitogens display the same spectrum of functional characteristics as antigen-stimulated lymphocytes (Klein, 1982) since mitogen-stimulated B and T cells produce immunoglobulins, secrete lymphokines and can act as cytotoxic cells. The stimulating effect of these substances can be assessed by enlarged lymphocyte counts or by determining the

incorporation of labeled thymidine into the DNA of the stimulated cells; the latter method of assessment was used in this study. Spleen cells taken from infected animals were stimulated with the two mitogens in the presence of tritiated thymidine; the results of this study support the conclusions which were reached in the study described in Chapter 1.

MATERIALS AND METHODS

Maintenance of parasite

The maintenance of the parasite, the induction of infection with *T. lewisi* and the recovery of the parasite have been described in Chapter 1.

Mitogens

Concanavalin A (ConA) was purchased from Sigma (St. Louis, MO.). The lipopolysaccharide (LPS) was isolated by phenol-water extraction from *Escherichia coli* E 145 by the method described by Westfal, Luderitz and Biester (1952); it was a gift from Dr J.E. Tanner, Department of Microbiology, Macdonald College of McGill University. Both mitogens were found, in preliminary trials on splenocytes of infected or normal rats, to activate rat spleen lymphocytes at a concentration of 10 μg / 0,1 ml of splenocyte culture.

Spleen cell preparation

Spleens removed from normal and from infected rats at days 7, 12, 20 and 35 of the parasitaemia were passed aseptically through an 80 mesh (60 gauge) stainless steel screen with a glass pestle into sterile complete ice-cold RPMI 1640 medium (Flow Laboratories, Inc., McLean, VA.), supplemented with 10% heat-inactivated fetal bovine serum, 100 μg of streptomycin and 100 U of penicillin per ml. The spleen cell suspension was transferred into 50 ml sterile centrifuge tubes in an ice bath and left for a few minutes to allow for the sedimentation of large particles. The supernatant was then removed, the cells were resuspended in

the complete medium, and centrifuged at 350 x g for 10 minutes at 4°C to pellet the cells. Splenocytes were resuspended again in the complete culture medium and haemacytometer cell counts of the suspension of cells were performed on a 100 µl aliquot of these cells in the presence of 0,2% trypan blue; only the live cells (those that excluded the dye) were counted: 90% of the cells in the suspension were viable. Cell counts were also made after 4 days of culture; percentage of viable cells from infected and normal uninfected animals were comparable.

Culture system

Unfractionated cells were cultured in 96 well microculture plates (Linbro; No. 76-003-05, Flow Laboratories, Inc., McLean, VA) as a suspension of 5 x 10^5 cell / 0,1 ml of the complete medium in each well. Mixed cell cultures of plastic-adherent spleen cells from infected or normal animals and nonadherent cells from normal or infected rats were prepared as follows: 100 µl of 5 x 10^5 splenocytes / 0,1 ml of the complete medium were dispensed into each well of 6-well culture plates (Linbro; No. 76-058-083; Flow Laboratories, Inc., McLean, VA.) and incubated for 2 hours at 37°C in 5% CO_2 to allow for the attachment of the adherent cells to the plastic of the well. The nonadherent spleen cells of each culture from normal (or infected) animals were removed aseptically and transferred into wells coated with adherent spleen cells from infected (or normal) rats, respectively. After adherence, the splenocytes were washed once with the complete medium before the non-adherent cells were added to them.

Stimulation with specific mitogens

ConA and LPS were added to the cells in each well at a final concentration of 10 g per 100 l culture for each mitogens. Activation of the spleen cell cultures was measured by culturing the cells with either of the mitogens for 48 hours at 37°C with 5% CO_2; 1 Ci of tritiated-thymidine (^{3}H-TdR, 2 Ci/mmol, ICN Biochemicals, Montreal) was then added to each culture Cells were harvested 24 hours later on glass fiber filters using a MASH II multiple cell harvester (Microbiological Associates, Bethesda, MD) The incorporation of labeled-thymidine into the DNA of the cells was determined in triplicate by liquid scintillation spectrophotometry with an LKB 1219 Rackbeta Counter The results are expressed as Stimulation Indices (SI)

$$SI = \frac{\text{cpm of mitogen - stimulated culture}}{\text{cpm of unstimulated control culture}}$$

- mitogen-stimulated cultures ConA or LPS stimulated cultures from normal (uninfected) or infected animals
- unstimulated control culture non-stimulated cultures from normal (uninfected) animals.

Statistical analysis is performed using the Student's t test (Snedecor and Cockran, 1967)

RESULTS

The blastogenic activity, as the incorporation of tritiated-thymidine into DNA, of spleen cells recovered from infected animals on the 7^{th}, 12^{th}, 20^{th} and 25^{th} days of the infection, after stimulation with ConA and LPS was determined. The results in counts per minute (cpm) were compared to the incorporation of the tracer by similarly-stimulated control represented by splenocytes from normal uninfected rats, the data is plotted in Figure 2 1 as stimulation indices (SI) The DNA synthetic response to ConA of day 12, 20 and 35 infected spleen cells was not significantly different from that of the control cells. However, the splenocytes recovered on day 7 of the infection were significantly suppressed ($p < 0,001$) in their ability to respond to ConA, the response to LPS of these day 7 cells, as well as the day 35 cells, was comparable to the control Interestingly, a significant increase in the LPS response ($p < 0,01$ and $p < 0,05$) of spleen cells from day 12 and 20 infected rats was observed.

It was of interest to determine whether the suppressed response of day 7 splenocytes to ConA was due to a defect in the T cells of these animals, or to the possible action of a suppressor (adherent) cell population Equal numbers of spleen cells from day 7 infected rats and from normal rats were mixed in a preliminary experiment to evaluate the response of normal splenocytes in presence of the suppressed day 7 infected spleen cells. Results, shown in Table 2.1, demonstrate that splenocytes from day 7 infected rats were able to depress the response of

normal cells to ConA.

To determine the identity of the cell population responsible for this suppression, mixed spleen cell cultures of adherent and non-adherent cells were made from infected and normal rats. Adherent splenocytes (presumed to be largely macrophages) of day 7 infected animals significantly suppressed (Figure 2 2; $p < 0,001$) the response to ConA of nonadherent splenocytes (presumed to be largely lymphocytes) obtained from normal animals, suggesting the presence of suppressor macrophages in the spleen of animals on the 7^{th} day of their infection

Figure 2.1

ConA (▨) or LPS (■) stimulations of spleen cells from uninfected rats (control) and of rats infected for 7, 12, 20 and 35 days by *T lewisi* Results are expressed as stimulation indices +/- Standard Error (S E)

Stimulation index = cpm of stimulated test / cpm of unstimulated control

Statistical differences were calculated by comparison with the results given by the normal control (Student's t test).

*** $p < 0,001$

* $p < 0,05$

** $p < 0,01$

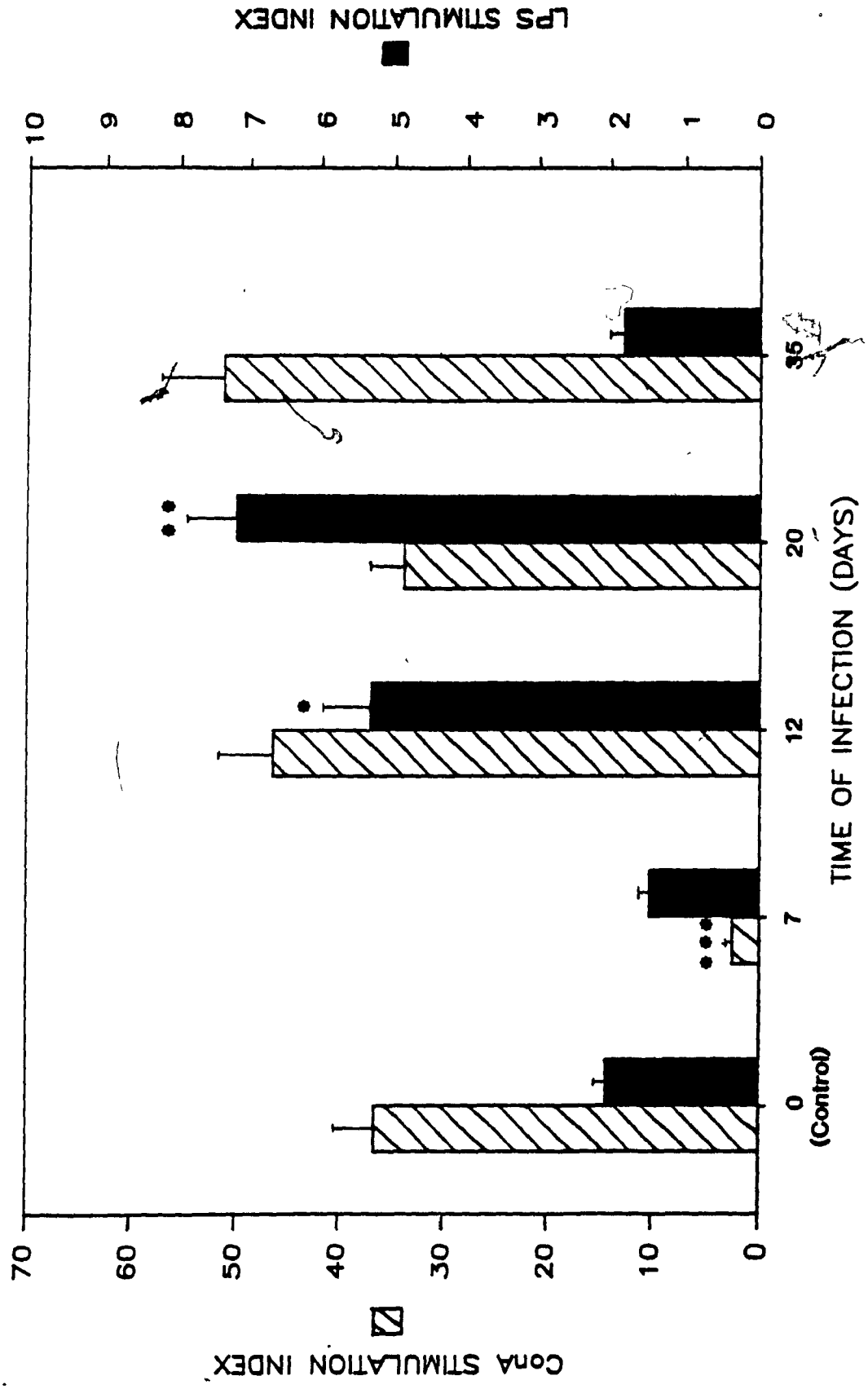

LPS STIMULATION INDEX
10
9
8
7
6
5
4
3
2
1
0
35
20
12
7
0
(Control)
TIME OF INFECTION (DAYS)
ConA STIMULATION INDEX
70
60
50
40
30
20
10
0

Table 2.1

Suppressive effect of unfractionated spleen cells obtained from *T. lewisi* - infected rats on the ConA response of normal splenocytes.

Composition of culture[a]		^{3}H-TdR Incorporation			
		ConA		LPS	
N	I	Mean cpm+/-S.E.	SI	Mean cpm+/-S.E	SI
$5x10^5$		123 610.3+/- 3 047.9	26 3	7 905.8+/- 1 982 8	1.7
	$5x10^5$	27 946.1+/- 2 808 5	5 9[b]	6 294.4+/- 2 623.2	1.3
$2.5x10^5$	$2.5x10^5$	41 040.4+/- 4 797 1	8.7[b]	5 780 8+/- 1 492 1	1.2

a. The cultures were composed of normal cells or cells from infected rats alone or together at the indicated concentrations.

- NSpC : normal spleen cells.
- ISpc : spleen cells from infected rats at day 7.

Statistical differences were calculated by comparison for the results given by the normal cells alone (Student's t test)

b. $p < 0,001$

Figure 2.2

Blastogenesis of mixed cultures of adherent and nonadherent splenocytes from normal and day 7 infected animals stimulated with ConA or LPS. Results are expressed as stimulation indices +/-Standard Error (S.E).

Nad : adherent splenocytes from normal rats

Nnad : nonadherent splenocytes from normal rats

Iad : adherent splenocytes from day 7 infected rats

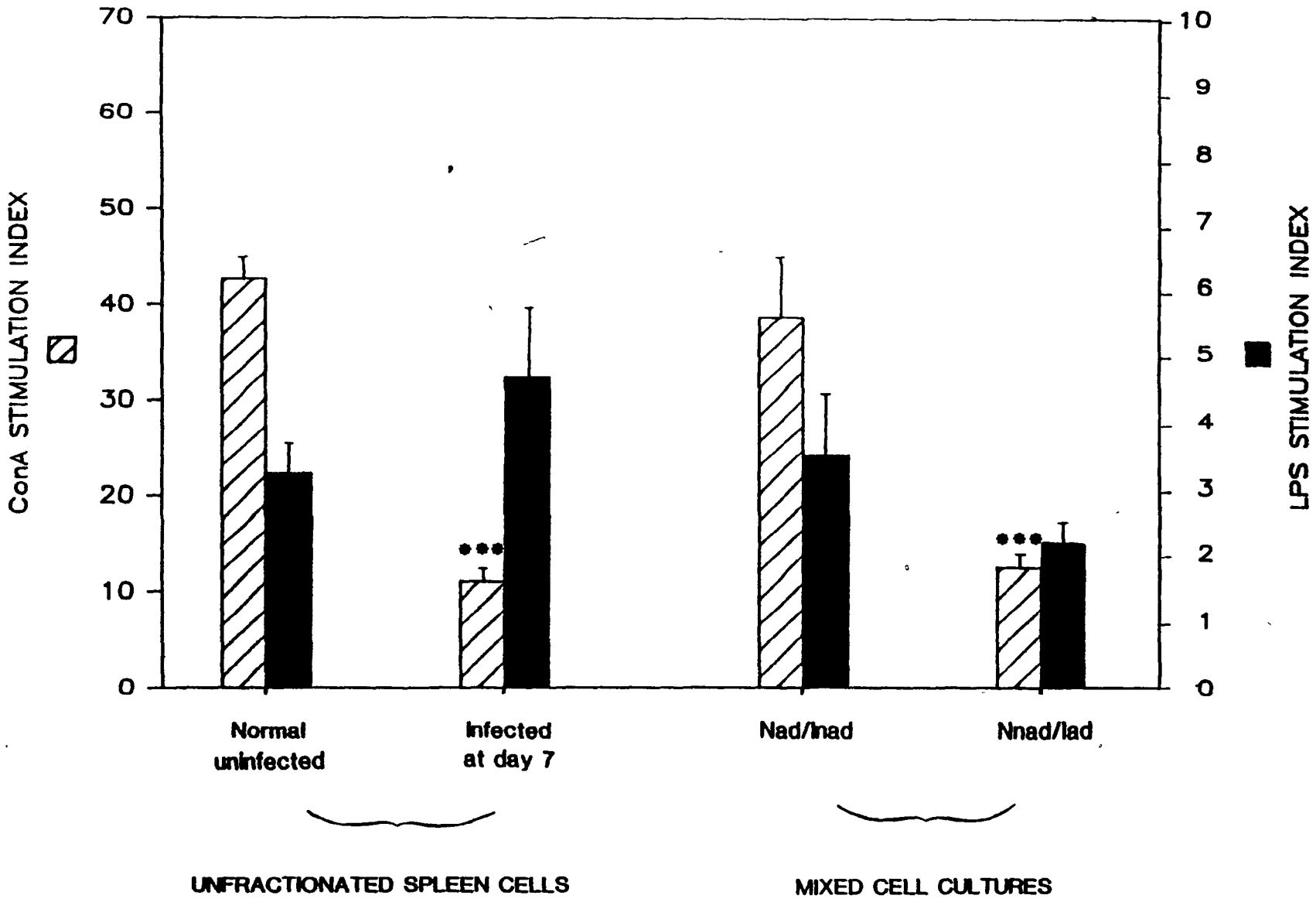
Inad : nonadherent splenocytes from day 7 infected rats.

▨ Stimulation index (SI) to ConA activation.

■ Stimulation index (SI) to LPS activation.

Statistical differences were calculated by comparison with the results given by the normal control (Student's t test)

*** $p < 0,001$


ConA STIMULATION INDEX
LPS STIMULATION INDEX
70
60
50
40
30
20
10
0
10
9
8
7
6
5
4
3
2
1
0
Normal uninfected
Infected at day 7
Nad/Inad
Nnad/Iad
UNFRACTIONATED SPLEEN CELLS
MIXED CELL CULTURES

DISCUSSION

The objective of the study described in this chapter was to further evaluate the functional abilities of spleen cells from *T. lewisi*-infected rats as an attempt to identify the reason for the immunosuppression phenomenon which is induced by these parasites. Infected spleen cells were as able as normal splenocytes to respond to stimulation by either ConA or LPS, except for those cells taken at day 7 of the infection. The data obtained in this study indicate that the T cell subset is the only population of lymphocytes affected in the immunosuppression phenomenon induced by the parasite since the response to LPS was normal if not enhanced. As a matter of a fact, it is interesting to note the rising trend of the response to LPS of the splenocytes of infected rats at days 12 and 20 This trend suggests not only that *T. lewisi* does not affect the function of B cells, as it has also been indicated in Chapter 1, but also that the parasite may actually increase the capacity of these cells to be stimulated.

The suppression of T cells at day 7 corresponds to the time of the exponential phase of the infection where mostly epimastigote forms of the parasite are found in the blood, and before the first appearance of the reproduction-inhibiting antibody ablastin and the trypanocidal antibody. A comparison of these results with those obtained from studies of the closely related trypanosome of mice, *T. musculi*, shows some differences. It was stated by Albright, Albright and Dusanic (1977) that the occurrence of suppressed splenocyte mitogenic responses to ConA, LPS and PHA

was related to splenomegaly induced by the trypanosome. Splenomegaly occurs in infections with *T. lewisi* (St-Charles, M.Sc. Thesis, 1979) but maximum enlargement of this organ occurs at the time when the parasitaemia is cleared, not in the early phases. In *T. lewisi* infection the thymus-derived lymphocytes are suppressed only during the exponential phase, in the first third of the infection. This fact suggests that suppression of T cells in *T lewisi*-infected animals is linked to the presence of a specific stage (specially the dividing epimastigote stage) of the trypanosome, rather than to functional and physiological changes of the spleen induced by the parasite

The finding of this study which also implicates an action for suppression of a suppressive adherent macrophage, or macrophage-like cell, has not been demonstrated for *T. musculi* (Albright, Albright and Dusanic, 1978; Albright and Albright, 1980; 1981). These latter authors discussed the possibility of a direct action by the parasite on B cells, or on an essential accessory cell, by a soluble substance rather than the activation of suppressor cells. Adherent macrophages in the spleen of rats infected with *T. lewisi* showed a suppressive action on the response of splenocytes from normal animals to the stimulating action of ConA on T lymphocytes.

Suppressive macrophages have also been reported in laboratory infections with the pathogenic African trypanosomes *T. gambiense* (Oka *et al*, 1984) and *T. rhodesiense* (Wellhausen and Mansfield, 1979; Charoenvit, Campbell and Tokdda, 1981; Mansfield *et al*, 1981) and in experimental Chagas' disease produced by *T. cruzi*

(Kierszenbaum, 1982; Cunningham and Kuhn, 1980). In infections with the latter parasite, it has been demonstrated that the parasite itself also suppresses the mitogenic response of normal T and B cells by interfering with the initial stages of the commitment which lymphocytes must make to proliferation (Maleckar and Kierszenbaum, 1983). Parasitic infections such as visceral leishmaniasis induced by *Leishmania donovani* stimulate the generation of suppressor macrophages to T and B cell mitogenic responses (Nickol and Bonventre, 1985) in a fashion similar to *T lewisi*, only when the parasite burden is significantly elevated.

The partition of splenocytes from infected animals into adherent and nonadherent population indicated that the suppressive activity of spleen cells was found only in the adherent fraction; this would thus eliminate the possibility of a direct action of the parasite on the T cells since both populations were contaminated with the parasite. The effect of *T lewisi* to suppress the immunological activity of the spleen of infected animals is in the generation of suppressor macrophages whose target for action are T cells, in the early stages of the infection.

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CHAPTER 3

KINETIC OF INTERLEUKIN 2 PRODUCTION DURING THE COURSE OF INFECTION BY *TRYPANOSOMA LEWISI*.*

* for submission to Immunology

Summary. Spleen cells from rats infected with *Trypanosoma lewisi* taken at different times of the infection on days 7, 12, 20 and 35 were activated *in vitro* with Concanavalin A (ConA) and the production of Interleukin 2 (Il 2) by these stimulated cells was determined Il 2 was titrated using the Il 2 dependent cell line CTL L2 The results of this study indicate that splenocytes at days 7 and 20 of the infection are defective in their capacity to produce the soluble mediator Il 2, splenocytes taken at days 12 and 35 of the infection produced amounts of Il 2 comparable to control normal uninfected spleen cells These findings correlate with those obtained in previous studies (Chapter 2) of the ConA suppression of spleen cells recovered from infected animals.

INTRODUCTION

Trypanosoma lewisi infections perturb the immune system of the host (St-Charles, Frank & Tanner, 1981; Chapter 2), suppressing the response to the thymus-dependent sheep red blood cells and the ability of T lymphocytes to respond to the specific mitogen Concanavalin A induced by the action of an adherent (macrophage) cell. The latter findings suggest that suppression may be produced by the interaction of these macrophages with T cells in the infected animal. To elucidate details of the mechanism of action of the parasite and/or suppressor cells on the T cells, the capacity of spleen T cells to produce the essential soluble mediator of immunity Interleukin 2 (Il 2) during the course of the infection was examined Interleukin 2 is produced by T lymphocytes and represents one of the key elements of the lymphokine cascade which is necessary for priming the immune and inflammatory responses (Roitt, Brostoff & Male, 1985) Its function is to potentiate the activation and proliferation of helper and effector T cells. Because of its central involvement in protecting responses, the production of Il 2 by cells recovered from infected animals, and subsequently stimulated by ConA, was determined; an impairment in the yield of Il 2 of these splenocytes could account for the T cell defect in rats infected by *Trypanosoma lewisi*.

MATERIALS AND METHODS

The maintenance of the parasite has been described in Chapter 1; the mitogens and the assessment of the activation of spleen cells have been described in Chapter 2.

Il 2 production.

Spleens from normal and from infected animals at days 7, 12, 20 and 35 of their infection by *T. lewisi* were recovered and processed to obtain cell suspensions as described in the previous chapter. The splenocytes were resuspended and adjusted to a concentration of 5 x 10^6 cells / ml in the complete tissue culture medium RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (10% FBS) and antibiotics. The cells were dispensed in 0,1 ml aliquots into 96-well culture plates (Linbro; No 76-003-05, Flow Laboratories, Inc , MacLean, VA) and then 0,1 ml of the medium containing 10 μg of ConA was also added to each well. The plates were incubated at 37°C in 5% CO_2 for 24 hours; the cultures were then recovered into small centrifuge tubes and centrifuged at 1000 x g for 10 minutes to sediment the cells; the supernatants of each stimulated cell culture were stored at 4°C until they were titrated for Il 2.

IL 2 titration.

The assay was performed as described by Harel-Bellan *et al* (1983). Briefly, CTL.L2 IL 2-dependent cells kept in our laboratory by twice-weekly serial passages, were washed twice in the complete RPMI 1640 medium and pelleted in D-MEM medium containg 5% FBS (Dulbecco's Modified Eagle Medium; Cat. No. 430-

2100EB; GIBCO Laboratories, Grand Island, N.Y.) for one hour at 37°C to allow for the binding of the Il 2 present in the supernatant of the ConA-stimulated cells onto the surface of the CTL.L2 cells. After binding of the Il 2 the CTL.L2 cells were washed and resuspended in fresh D-MEM +5% FBS and adjusted to a concentration of 2 x 10^5 cells / ml of medium. One-tenth ml of this cell suspension was then dispensed into each well of 96-well plates containing 0,1 ml volumes of halving dilutions of the Il 2-supernatants of the ConA-activated normal or infected cell cultures. As a control, the CTL L2 Il 2-dependent cells were cultured alone in D-MEM + 5% FBS. CTL-L2 cells, grown in different halving dilutions of the supernatant of cultures of the Il 2-producing cell line MLA-144 were used to construct a standard curve for the activation by CTL L2 cells by the lymphokine The plates containing the test supernatants and the controls were incubated for 40 hours at 37°C in 5% CO_2 and then 1 µCi of tritiated-thymidine (^{3}H-TdR, 2 Ci/mmol; ICN Biochemichals, Montreal) was added to each well. The cells were harvested 16 hours later on glass fiber filters using the MASH II multiple cell harvester (Microbiological Associatiates,Bathesda, MD). The incorporation of labeled-thymidine into DNA of cells was measured in triplicate by liquid scintillation spectrophotometry, as described in Chapter 2. The results are expressed in Figure 3.1 as the percentage of the radioactivity incorporated by the control (test / control x 100) and in Table 3.1, as counts per minutes (cpm; mean count of 3 cultures +/- Standard Error: S.E.). Statistical analysis is performed using the Student's t test (Snedecor and Cockran, 1967).

RESULTS

Spleen cells from normal uninfected rats and from animals at days 7, 12, 20 and 35 of infection by *T. lewisi* were stimulated with the mitogen ConA to induce the production of Il 2. The results presented in Figure 3.1 indicate that the production of the lymphokine by T cells in the spleen was suppressed at day 7 and 20 of the infection. Spleen cells from days 12 and 35 were as efficient as normal cells in their capacity to produce Il 2; the data from the suppressed spleen cells (days 7 and 20) were statistically different from each other ($p < 0,01$), from the control ($p < 0,001$) and from infected but normal producers of Il 2 (day 12 and 35; $p < 0,001$) The suppressed response of Il 2 at days 7 and 20 of the infection is comparable to the one from CTL.L2 cells alone in the D-MEM medium without Il 2 (Figure 3.1); this indicates that the suppression represents the absence of Il 2 in the supernatant from the cultures composed of spleen cells taken from these infected animals.

Table 3.1 shows the data obtained from the control cultures: responding cells without addition of the Il 2 supernatant from the MLA-144 conditioned medium (CTL cells alone); D-MEM medium alone without Il 2 or CTL.L2 cells (D-MEM alone); and CTL.L2 cells cultured in the supernatant of unstimulated spleen cells from normal or day 7, 12, 20 or 35 infected rats (CTL cell + supernatant from unstimulated cultures). No significant difference was observed between any of the values presented in Table 3.1, indicating that there was no other stimulation (from

viruses, for example) than that of ConA, which was responsible for the responses obtained from the spleen cells of both normal and infected animals in this study.

Figure 3 1

Interleukin 2 production by spleen cells recovered from *T lewisi*-infected rats at different days of the infection and stimulated with ConA The data is expressed as a percentage of the Il 2 response of the control (response of infected animal / response of normal animal x 100). The response presented is that of a 1.4 dilution of the supernatant - Statistical analysis is done by Student's t test

a. Test vs control : $p < 0,001$

b. day 7 vs day 12 : $p < 0,001$

c. day 7 vs day 20 . $p < 0,01$

d. day 7 vs day 35 : $p < 0,001$

e. day 20 vs day 12 : $p < 0,001$

f. day 20 vs day 35 : $p < 0,001$

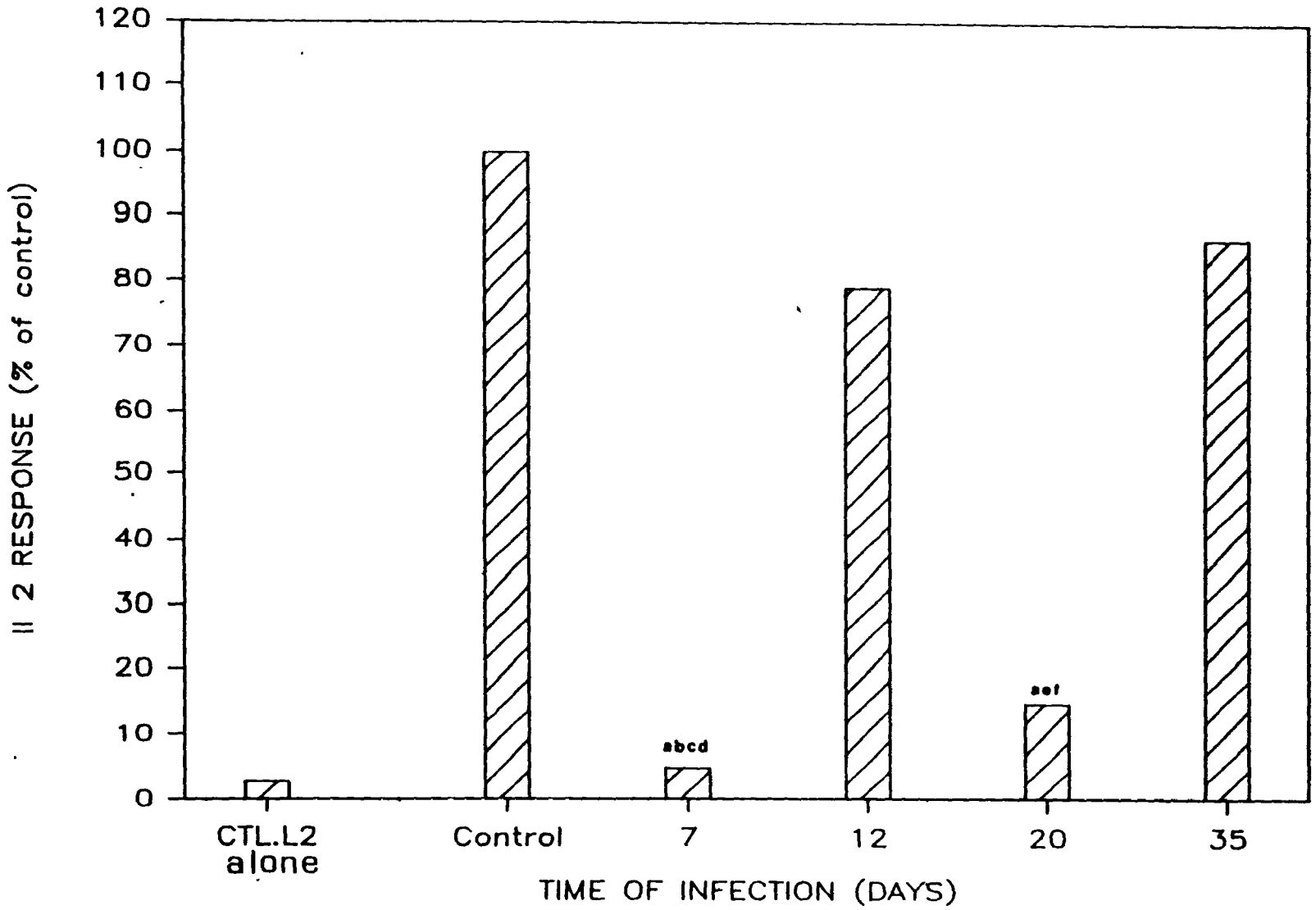
120
110
100
90
80
70
60
50
40
30
20
10
0
IL 2 RESPONSE (% of control)
abcd
aef
CTL.L2 alone
Control
7
12
20
35
TIME OF INFECTION (DAYS)

Table 3.1

Incorporation assay of labeled-thymidine from the control cultures

	D-MEM alone[1]	CTL cells alone	CTL cells + Supernatants from unstimulated cultures[2]
CPM+/- S.E.	1 719 1+/- 239 4	2 425.7+/- 391.3	3 180.4+/- 329 4

- Values are expressed as cpm +/- Standard Error of mean numbers of 3 cultures.

1- ^{3}H-thymidine was not added

2- CTL.L2 cells cultured in the supernatant of unstimulated spleen cells from normal or day 7, 12, 20 or 35 infected rats; mean number of cpm counts.

DISCUSSION

It was demonstrated earlier (Chapters 1 and 2) that T cells are the specific targets for the action of an infection by *Trypanosoma lewisi* to immunosuppress its host during the exponential phase of the infection. This suppression was found to be related to the induction of suppressor macrophages in the spleen of the infected animals. Given the fact of T cell unresponsiveness, it was of interest to analyse the defect to elucidate the mechanism on which the suppressor macrophage exerts its activity. The results of the present study indicate that spleen T cells from rats infected for 7 and 20 days are restrained in their ability to produce normal amounts of Il 2. Suppression of the production of Il 2 at day 7 of an infection by *T. lewisi* coincides with the suppressed capacity of spleen T cells from infected animals at the same time of the infection to proliferate in response to activation by the mitogen ConA (Chapter 2, Figure 2.1). Several authors have described similar immunosuppression phenomena related to a depression in the production of Il 2 in laboratory infections by *Trypanosoma cruzi* (Harel-Bellan *et al*, 1983; Tartleton & Kunh, 1984; Reed, Inverso & Roters, 1984; Choromanski & Kuhn, 1985), *T. brucei* (Alcina & Fresno, 1985; Sileghem, Hamers & de Baetselier, 1986), *T. congolense* (Mitchell, Pearson & Gauldie, 1986), *Leishmania donovani* (Reiner & Finke, 1983) and *Plasmodium falciparum* (Troye-Blomberg *et al*, 1985). Many of these authors have been able to restore susceptibility to activation by ConA (or PHA) by the

addition to cultures of exogenous Il 2 and the Il 1 soluble factor produced by macrophage which activates T cells. However, in visceral leishmaniasis, exogenous Il 2 produced only a partial recovery of the response of T cells to PHA, indicating that the defect in the cell lay in its ability to respond to Il 2, as well as in its capacity to produce the lymphokine (Reiner & Finke, 1983). Micthell *et al* (1986) have shown a defect in both Il 1 and Il 2 production, concurrent with a depression in the ability of spleen cells from *T. congolense*-infected animals to respond to ConA; they suggested that the impairment could be related to an incapacity of macrophages to produce Il 1, thus leading to a decrease in Il 2 synthesis.

It is more difficult to explain Il 2 suppression at day 20 of the infection because T cells recovered from these rats respond normally to stimulation by ConA (Chapter 2, figure 2.1). It is known that when lectins are used for the stimulation of lymphocytes, Il 2 activity appears and peaks rapidly in the supernatants, followed by a rapid decline due to the adsorption of the lymphokine by the cells proliferating in culture (Smith *et al*, 1979). However, adsorbed Il 2 is sufficient to keep cells proliferating for 6 to 7 days (Lowry *et al*, 1951). Spleen cells from rats at day 20 of infection by *T. lewisi*, fail to produce Il 2 (Figure 3.1) but proliferate normally to stimulation by ConA (Chapter 2, Figure 2.1) perhaps because of the presence of a sufficient amount of Il 2 adsorbed on their surface. This hypothesis and the fact that twice in the infection the yield of Il 2 is significantly suppressed (day 7 and 20) are yet to be elucited.

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CHAPTER 4

INFECTIVITY AND ROUTE OF PENETRATION OF BLOODSTREAM AND IN VITRO CULTURED-METACYCLIC FORMS OF *TRYPANOSOMA LEWISI* IN RATS AFTER ORAL AND INTRAPERITONEAL INOCULATIONS.*

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Abstract: Morphological changes of *Trypanosoma lewisi* cultured in the insect medium, SDM, supplemented or not with uric acid (UA), were compared to the ones cultured in a control medium (M-199). No difference in trypanosome quality and quantity was observed between SDM-UA and SDM cultures. As for the control cultures (in M-199) trypanosomes deigned to transform into the metacyclic stages and kept the trypomastigote form. The infectivity of bloodstream forms was always higher than that of SDM- or SDM-UA-cultured metatrypanosomes, whether they were inoculated orally or intraperitonealy (i.p.). No difference was observed between the infectivity of SDM- and SDM-UA-cultured metacyclic stages. Parasitaemia was highest in rats inoculated i p. with bloodstream forms but lowest when trypomastigotes were inoculated orally. The oral inoculation of rats with labeled-cultured metatrypanosomes and bloodstream forms showed that metatrypanosomes remain longer in the mouth of the animal than blood trypanosomes.

The studies which have been described above have concerned the immunobiology of the relationship between *Trypanosoma lewisi* and its specific rat host. It has been demonstrated that the establishment and the maintenance of the parasite in its host must be facilitated by the suppression of the T cells which characterizes infection by this rodent trypanosome This study of immunological relationships in trypanosomiasis stimulated a number of questions concerning the biology of *T lewisi*, one of them concerning the natural transmission of the parasite from fleas to rats A study of this colateral question has clarified the means by which this trypanosome is maintained in nature

Trypanosoma lewisi is a digenean stercorarian protozoan and a specific trypanosome of black and brown rats (*Rattus rattus* and *R. norvegicus*) (Hoare, 1972) In our laboratory, the parasite is maintained by serial intraperitoneal syringe passage from day 12 infected rats into normal animals In nature, however, the parasite is transmitted from rat to rat by the flea vector *Nosopsyllus fasciatus* or *Xenopsylla cheopis* (Hoare, 1972); Minchin and Thomson (1915) have described in detail the developmental changes of *T. lewisi* in the flea. When the vector takes a blood-meal from an infected rat, trypanosomes are transported to the lumen of the posterior alimentary tract where they undergo morphological changes, multiply and eventually transform into infective metacyclic trypomastigotes which are discharged in the faeces. The mechanism of transmission of *T.*

lewisi by the vector from rat to rat was first proposed by Rabinowitsch and Kempner (1899), but truly proved later by a number of authors (Noller, 1912 b, b; Wenyon, 1913a, c; Minchin and Thomson, 1915). Using different models, these authors showed that, when feeding upon rats, the flea released faeces containing the metacyclic trypanosomes; the rat would become infected by ingesting the faeces and/or the infected flea during grooming. This hypothesis was confirmed when it was demonstrated that rats became infected when they were passively given infected faeces by mouth (Minchin and Thomsom, 1915) The metacyclic trypanosomes are believed to pass through the oral mucous membrane into the blood of the rat in a manner similar to that of the crocodile-trypanosome, *T. grayi* (Hoare, 1929).

Recent studies on the closely-related trypanosome of mice, *T. musculi*, has indicated that, when these parasites are cultured in Schneider's Drosophilia Medium (SDM) at room temperature, the parasite transforms through multiple intermediate stages into infective metatrypanosomes in 10 days (Roger and Viens, 1986, b; Mohamed and Molyneux, 1987) which were equally infective to mice whether they were inoculated i.p. or orally; bloodstream forms, on the other hand, were unable to induce an infection when orally inoculated. The same authors have suggested (Roger and Viens, 1987) that the addition of uric acid to the SDM medium potentiated the differentiation of *T. musculi* to metacyclic trypanosomes in culture and rendered them more infective by the oral route.

The natural transmission of *T. lewisi* metacyclics from the flea

vector to the mammalian rat host by the oral route is accepted, but the mechanism of its entry into the blood of the rat is not well understood. The close phylogenetic relationship of *T. lewisi* with *T. musculi* and their similar life-cycle and course of infection suggested that it would be of interest to compare the infectivity of culture-derived metacyclics of *T lewisi* with that of bloodstream trypomastigotes This study describes the morphological changes of *T lewisi* when cultured in Schneider's Drosopholia Medium (SDM), with or without uric acid, and the infectivity of the metatrypanosomes from SDM cultures, in comparison with the one of bloodstream forms, after oral or intraperitoneal inoculations

MATERIALS AND METHODS

The maintenance of the parasite in the laboratory has been described above.

Cultivation of *T. lewisi*.

The trypanosomes were cultured in three different media to produce the insect metacyclic forms: (1) Schneider's Drosophilia Medium (SDM; Schneider, 1974; Gibco, Grand Island, NY) was supplemented with inactivated 20% fetal bovine serum (FBS; Flow Laboratories, McLean, VA) and 1% (v/v) penicillin-streptomycin; (2) the complete SDM medium above further supplemented with 2 mg / ml saturated uric acid (SDM-UA), centrifuged at 650 x g and filtered before use; and (3) the tissue-culture medium-199 (Gibco), supplemented with 20% FBS and antibiotics All three media were sterilized by filtration through a 0,22 μm Milipore filter (Millipore Corporation, Bedford, MA.) and kept at 4°C until used.

Each medium was dispensed 10 ml aliquots into sterile tissue culture flasks (Corning 25 cm), in triplicate. Ten million washed blood trypomastigotes, taken at day 10 post-infection, were dispensed into each flask and incubated at 26°C for 14 days. Every second day 100 μl volumes of each parasite culture were diluted 1:2 with 1% formalin in phosphate-buffered saline and the trypanosomes in the culture were counted under phase-contrast illumination in a Neubauer haemacytometer; methanol-fixed parasites were also stained by Giemsa, at room temperature after sedimenting 300 μl of the culture by centrifugation and the

different stages of the parasite were identified according to the criteria of Hoare (1972).

Kinetics of the penetration into the tissue by radioactively-labeled *T. lewisi*

The trypanosomes were labeled with tritium as described by Kiderlen *et al* (1986). Briefly, metacyclic trypanosomes cultured in the SDM medium for 14 days and bloodstream trypomastigotes recovered on day 10 post-infection were washed once with the SDM medium, they were then incubated for 24 hours at a density of 1 x 10^6 parasites / ml in 50 ml tissue culture flasks in medium containing 5 μCi / ml tritiated thymidine (^{3}H-TdR, 2 Ci/mmol, ICN Biochemichals, Montreal) Labeled trypanosomes were then washed twice in RPMI 1640 medium containing 10% FBS; the parasites were then incubated for at least one hour to permit the liberation of non-specifically bound ^{3}H-TdR, washed again and finally resuspended in RPMI 1640, + 10% FBS, to a concentration of 1 x 10^7 parasites / ml. One ml of radiactively-labeled cultured and bloodstream forms were inoculated orally into normal rats, using a 20 gauge blunted feeding needle. Four and 24 hours after the inoculation, the inoculated rats were killed, blood was obtained by cardiac puncture and different tissues were removed: the tongue and the salivary glands, the oesophagus, the stomach and the intestine and placed into separate 8 ml vials; 8 ml of Cytoscint (ICN Biochemicals, Montreal) was added to each vial to dissolve the tissue. Twenty-four hours later the vials were counted in an LKB Rackbeta liquid scintillation

spectrophotometer. Statistical analysis was done using Student's *t* test (Snedecor and Cockran, 1967).

RESULTS

T. lewisi in cultures.

Following the inoculation of bloodstream forms of *T. lewisi*, there was no significant difference in the number of trypanosomes per ml of any of the culture media throughout the 14-day length of incubation, irrespective of whether the organisms were in medium-199, or in Schneider's Drosophilia medium with (SDM-UA) or without uric acid (SDM). There was also no significant difference in the kinetics of development of the parasite when it was cultured in either SDM or SDM-UA. In both media, *T. lewisi* developed from the trypomastigote, through the epimastigote and intermediate stages, to metacyclic forms (Figure 4.2 a and b; the different morphological forms are presented in Figure 4.1). However, most of the trypanosomes cultured in M-199 remained in the trypomastigote form without much development in this medium into the metacyclic stage (Figure 4.2 c). As shown in Figure 4.2 a and b, a relatively high percentage (~ 40%) of the organisms in SDM were dividing as early as day 3 in the cultures; the dividing epimastigote forms gradually decreased and the proportion of the organisms that were metacyclic forms increased during the course of the 14-day culture. Although all the recognized morphological types of metacyclic trypanosomes were evident, especially in the SDM media, the predominant type was the stage in multiple division (SMD), whether uric acid was, or was not, present in the culture medium. The parasite seemed to transform more rapidly into metacyclic trypanosomes in the SDM-UA

cultures; however, no significant difference was observed in the proportion of the different types of metacyclics present in the SDM or the SDM-UA media after 14 days of culture.

Infectivity of cultured or bloodstream *T. lewisi*

Bloodstream trypomastigote forms of *T. lewisi* and the metacyclics which developed in the SDM media were inoculated into normal rats either by the intraperitoneal (i.p.) or oral routes to determine their relative capacities to induce an infection. The culture metacyclic forms were infective and showed a pattern of parasitaemia similar to that presented by Hoare (1972; Figure 4.3 a and b). It was no particular surprise that the blood trypomastigotes produced a significantly greater infection than the metacyclics from the 14-day cultures in the SDM media, when the two forms of the parasite were inoculated intraperitonealy (Figure 4.3 a), particularly in the first half of the infection from day 5 to day 15. After day 15 until the end of the infection, there was no significant difference in the parasitaemia produced by the blood trypomastigotes, or the cultured forms. There was also no significant difference at any stage of the infection between the parasitaemia in rats inoculated with the cultured forms from SDM or from SDM-UA. Oral inoculation of blood trypomastigotes induced a lower parasitaemia than when these same forms were inoculated intraperitonealy (Figure 4.3 b). The interesting fact was that they induced an infection whatsoever and that, with the exception of the results obtained on day 12, there was no real, significant difference between the parasitaemias which developed following the oral

inoculation of trypomastiogotes or metacyclics. Moreover, more rats became infected when inoculated orally with trypomastigotes than with cultured metacyclics (Figure 4.4).

The prepatent period of the parasitaemia in animals inoculated i.p. was longer (5 days) for those that received the cultured metacyclics than for those inoculated with trypomastigotes (3 days; Figure 4.3 a) When the parasites were inoculated by the oral route the prepatent period was prolonged to 7 days for animals inoculated with bloodstream forms and to 10 days for those animals that received cultured metacyclics The parasitaemia lasted for a shorther time (~ 26 days) in those animals inoculated by the oral route when compared to those inoculated i p. (32 days; Figure 4 3 a and b).

Intraperitoneal inoculations with bloodstream forms resulted in all of the rats becoming infected (100%) compared to 80 and 50% of the rats showing an infection after being inoculated with SDM- and SDM-UA-cultured forms, respectively (Figure 4.4). The percentage of infectivity after oral inoculation was somewhat lowered for all 3 forms: 80, 40 and 20% of the rats became infected after their inoculation with bloodstream trypomastigotes, SDM- and SDM-UA-cultured metacyclics, respectively.

Passage of *T. lewisi* from the mouth through the blood of normal rats.

The results of the study to determine the fate of the trypanosomes in oral inocula of blood trypomastigotes or cultured metacyclics are found in Table 4.1. The two developmental forms

of the parasite were incubated with tritiated thymidine in an attempt to label them radioactively in order to follow their progress after their inoculation into the animal.

Samples taken from animals four hours after inoculation indicated that the radioactivity in the rats that had received trypomastigotes or metacyclics was distributed mainly in the tissue of the mouth and stomach; counts in the tissue of the intestine and in the blood were equal to background levels. The distribution of the counts was changed 20 hours later: a high proportion of the counts were still found in the tissue of the stomach, but at this time many of the counts had passed into the tissue of the small intestine in animals inoculated orally with the two developmental forms of the parasite; counts in the blood, and in the tissue of the mouth from animals inoculated with blood forms were equal to background levels. A difference exists between the results of the oral inoculation with blood forms or metacyclics: the counts in the tissue of the mouth of animals that had received blood forms indicated a decrease of 82% after 20 hours from the first count (4hours) whereas the counts in the same tissue of animals inoculated with metacyclics showed a decrease of only 23%.

Figure 4.1

Trypanosoma lewisi in culture. (x 1500). Different stages observed in cultures from the three media. M-199 medium SDM medium and SDM+U.A medium.

a. Trypomastigotes

b. Epimastigotes

c. Metacyclic stages

d. Stages of multiple division

e. Spheromastigotes

(Based on drawings from Hoare, 1972).

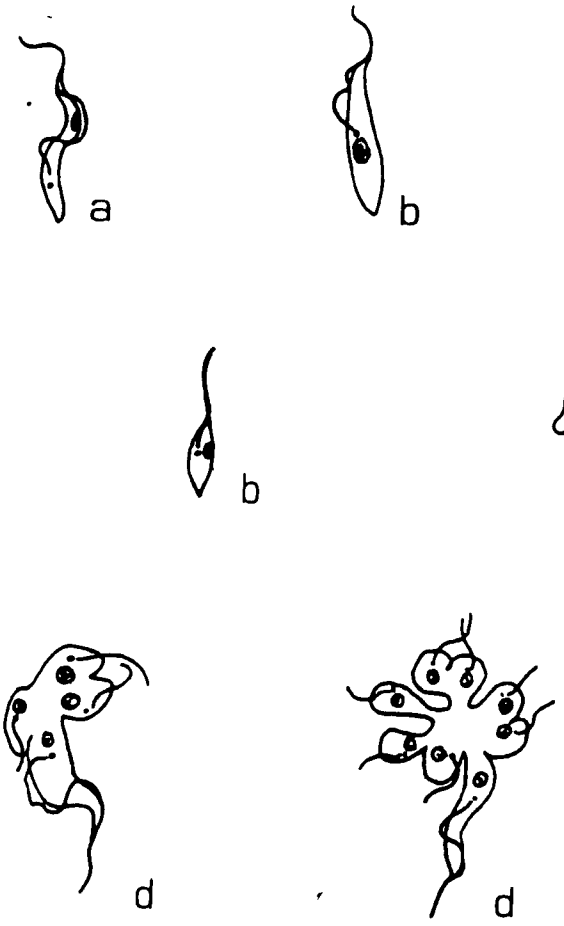

a
b
b
b
c
d
d

Figure 4.2

Percentage of 5 different stages of the parasite after the inoculation of a- SDM, b- SDM+U A. and c- M-199 media with 10^7 bloodstream forms of *Trypanosoma lewisi*.

Trypomastigotes

Epimastigotes

Metacyclic stages

Stages of multiple division

Spheromastigotes

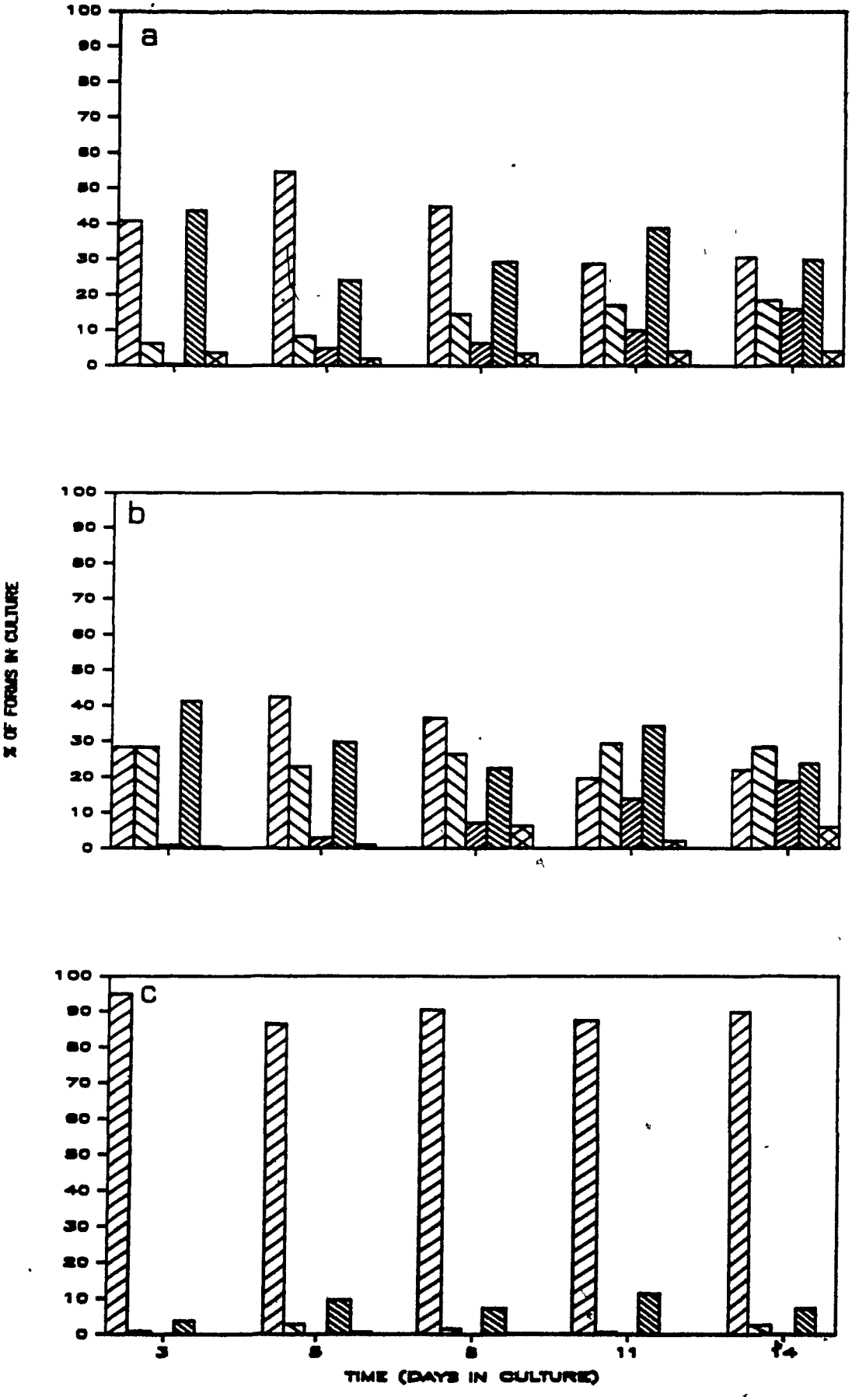

a
b
c
100
90
80
70
60
50
40
30
20
10
0
% OF FORMS IN CULTURE
3
8
11
14
TIME (DAYS IN CULTURE)

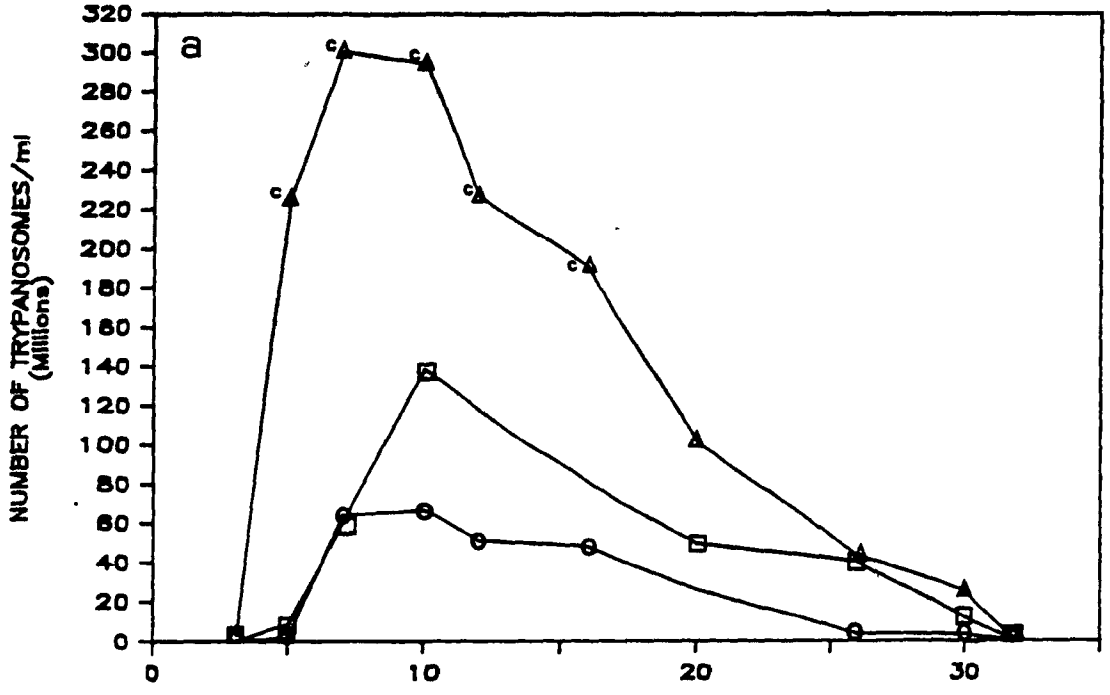
Figure 4.3

Parasitaemias obtained from intraperitoneal (a) and oral inoculations (b) of normal rats with bloodstream forms (Δ), cultured forms from day 14 SDM cultures (□) and cultured forms from day 14 SDM+U.A cultures (O)

- Statistical analysis is by the Student's t test. Stastical differences were calculated by comparision with the results given by the inoculations with cultured forms (Both SDM and SDM+U.A.)

c- $p < 0,01$

d- $p < 0,05$


a
NUMBER OF TRYPANOSOMES/ml (Millions)
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
0
10
20
30

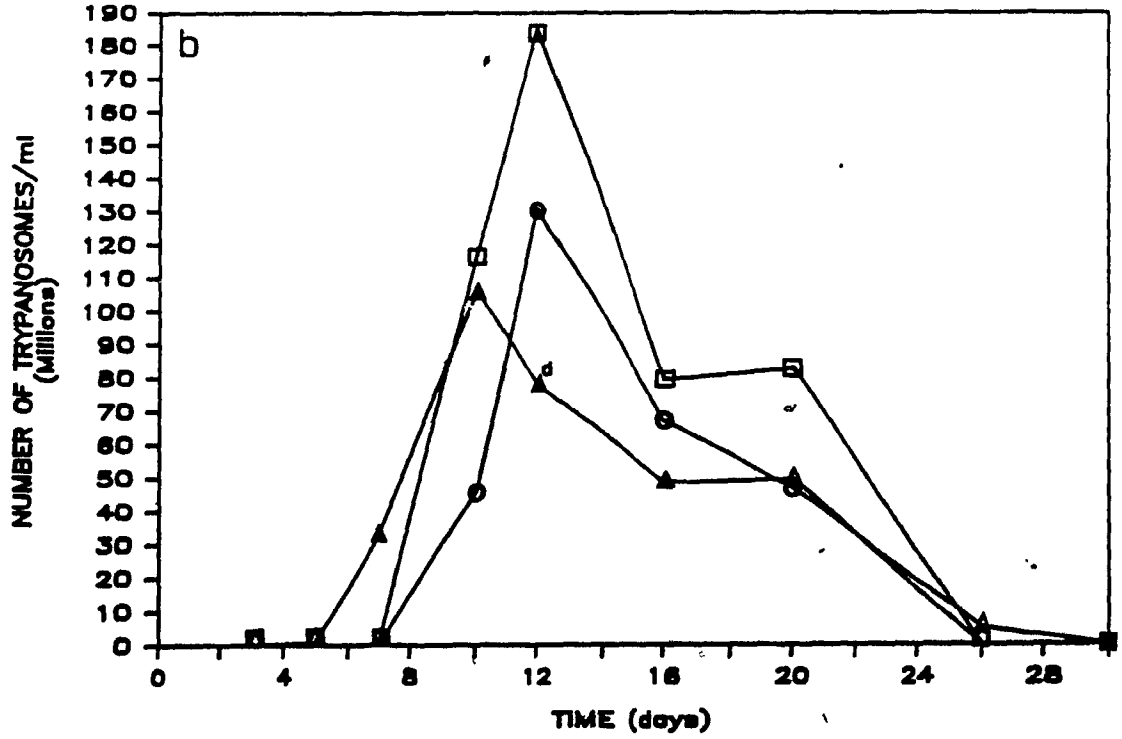

b
NUMBER OF TRYPANOSOMES/ml (Millions)
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
28
TIME (days)

Figure 4 4

Percentage of infectivity in rats inoculated by the i.p or the oral route with the bloodstream forms (▨), SDM-cultured forms (▧) and SDM+U A cultured forms (▨).

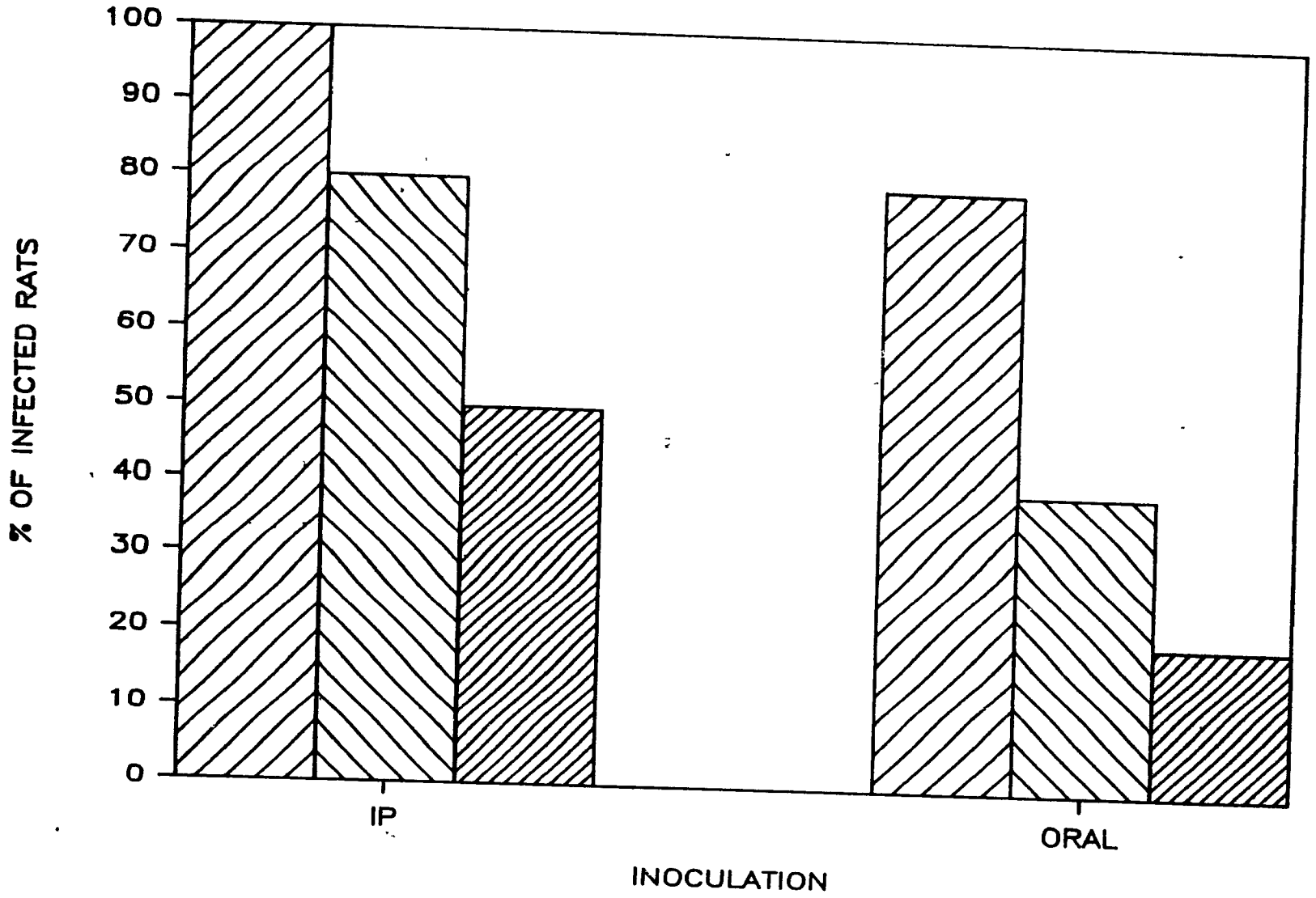

100
90
80
70
60
50
40
30
20
10
0
% OF INFECTED RATS
IP
ORAL
INOCULATION

Table 4.1

Progress of the parasite after its inoculation by the oral route into the animal.

	4 HOURS		24 HOURS	
	Blood forms (%)	Metacyclics (%)	Blood forms (%)	Metacyclics (%)
Mouth	44	43	10*[a]	31[b]
Oesophagus	11	11	13	11
Stomach	37	38	32	24
Intestine	7*	7*	42	33
Blood	1*	1*	4*	1*
	100	100	100	100

. The numbers are percent of the total counts recovered.

* The CPM numbers are equal to background levels.

a. 82% decrease in cpm counts after 20 hours from the first count (4 hours)

b. 23% decrease in cpm counts after 20 hours from the first count (4 hours)

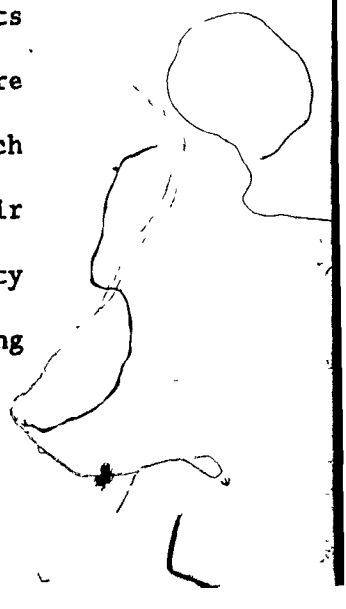
DISCUSSION

The present study has shown that bloodstream trypomastigotes and metacyclic trypanosomes are infective for normal rats whether inoculated orally or intraperitonealy (i.p.). These studies also show that the presence of uric acid in the SDM medium did not potentiate the differentiation of the parasite into metatrypanosomes, nor did it augment their infectivity. This finding does not agree with the report of Roger and Viens (1987) which indicated that uric acid increases the number of metacyclic forms of *T. musculi* in culture and enhances the infectivity of these forms when orally inoculated into their mouse hosts; it is obvious that uric acid does not act to regulate the morphogenesis of *T. lewisi*, as it appears to do for *T. musculi*.

Bloodstream trypomastigotes were infective for naive rats by the oral, as well as by the traditional laboratory intraperitoneal route of inoculation; cultured metacyclics were also infective by both routes, although they did not produce as intense a parasitaemia as intraperitonealy-inoculated trypomastigotes. The relatively lesser success of metacyclics to induce infections as intense as those produced by trypomastigotes could be due to the need of the culture forms to adapt to their "new" animal host. This adaptation might be a reason for the more prolonged prepatent period of the infections induced by the culture forms. The fact that oral inoculation by trypomastigotes induced infections in more naive rats than the metacyclics from SDM medium cultures was a surprise since the reverse was

anticipated. The reason for this unexpected result is not easy to propose, but it may have been due to a lowering of the infectivity of the parasite by passage through artificial conditions in culture which were not, it is easy to presume, as optimal as those which the parasite finds in its normal arthropod vector. However, even if this hypothesis is revealed to be true, the results suggest, nevertheless, that the transmission of the parasite does not solely depend on the interaction of a flea vector, as believed earlier.

The oral inoculation also showed that rats inoculated with bloodstream forms have lower parasitaemia than rats inoculated with metacyclics: the reason could be related to a lower number of blood trypanosomes passing into the blood of the inoculated animal, because of the hostility offered by this "unsuitable" mouth environment, thus eliciting a weaker infection. This hypothesis is supported by results of an earlier study (St-Charles, M.Sc.Thesis, 1979) where, by decreasing the number of trypanosomes in the inoculum, the parasitaemia is parallely lowered. The study of the distribution in the tissue of radioactively-labeled trypomastigotes and cultured metacyclics after oral inoculation indicated that the latter form remained longer in the mouth than the blood forms; this result suggests that, irrespective of any deficiency in the medium, the culture forms retained an affinity for the tissue through which metacyclics are believed to naturally (Hoare, 1972) infect their rat hosts. However, the appearance of a significant radioactivity in the tissue of the stomach as early as 4 hours after feeding



tritium-labeled parasites and the fact that the tissue of the small intestine was also radioactive 24 hours later suggest strongly that a route (if not preferred) of infection by metacyclics and trypomastigotes of *T. lewisi* is through the gastro-intestinal tract. It is possible that the organisms that multiply in the kidneys early during the course of an infection by *T. lewisi* (Wenyon, 1926) originate, not from a penetration of the tissues of the mouth, but from a trans-gastric or a trans-intestinal route. It would be of considerable interest to study the induction of infections by trypomastigotes inoculated directly into the stomach or into the small intestine through a catheter.

In summary, these results indicate that bloodstream forms are, at least, as capable as, or more efficient than, metacyclic trypanosomes in eliciting an infection when the parasite is inoculated orally into normal rats. This route of infection indicates that the transmission of this trypanosome in rats is different from that of *T. musculi* to mice, where the trypomastigote is not infective by the oral route (Roger and Viens, 1987). As suggested by Molyneux (1969) for the transmission by cannibalism of *T. microti*, it is possible that *T. lewisi* could be similarly transmitted, serving as a link in evolutionary adaptation of host-parasite relationship in rodent-trypanosome models.

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CONCLUSIONS

Trypanosoma lewisi is a digenetic protozoan parasite whose intermediate, vector host is a haematophagus flea; its second is a vertebrate mammal (the rat) which plays the role of the final host (Hoare, 1972). According to Minchin and Thomson (1915), *T. lewisi* undergoes several morphological and physiological changes within the midgut of the vector: the trypomastigote stage of the parasite is introduced into the alimentary tract of the flea with the blood-meal; the parasite penetrates into the epithelial cells of the stomach, to reproduce by multiple division The young adults trypomastigotes escape into the lumen of the stomach, pass through the arthropod's colon into the rectum and transform into a variety of epimastigote forms. The latter multiply repeatedly by binary fission, transforming into small, club-shaped trypomastigotes (the metatrypanosomes) which represent the final stage of development of the parasite in the flea vector. These metacyclic trypomastigotes are discharged in the faeces of the vector when it takes a second blood-meal and serve as the infective stage for naive rats.

Six days elapse from the time the flea ingests the parasite from the vertebrate to the time of the appearance of infective metatrypanosomes in the faeces of the vector. It has been demonstrated that rats take up the metacyclic trypomastigotes during grooming and it is believed that the metratrypanosomes penetrate through the oral mucous membrane into the rat's bloodstream (Minchin and Thomson, 1915). The results of the

study reported here (Chapter 4) suggest that another route of infection by metacyclic trypomastigotes is possible: through the gastro-intestinal tract of the animal. More interestingly, it has been shown here that the bloodstream form of the parasite can also infect rats by the oral route, suggesting cannibalism as an effective mean of transmission.

After transmission into the final host, the parasite is observed in the blood of infected rats following a short period of incubation of 3 to 5 days after intraperitoneal inoculation with bloodstream trypomastigotes or metatrypanosomes and of 5 to 10 days after oral inoculation (Chapter 4). The trypomastigotes in the inoculum transform very quickly into epimastigotes which reproduce by unequal multiple division until the first crisis; the number of epimastigotes may exceed 3×10^8 per ml of blood (Taliaferro, 1924; 1932). The first crisis occurs at about the tenth day of the infection and is characterized by the appearance in the serum of the rat of the specific antibody ablastin which inhibits the reproduction of the parasite (Taliaferro, 1925). A trypanocidal antibody also appears in the serum at about the same time; this antibody destroys many of the trypanosomes in the blood (Coventry, 1930; Taliaferro, 1932). However, a fairly large number of adult non-dividing trypomastigotes remain in the circulation that show, according to Coventry (1930), acquired resistance to the first trypanocidal antibody; these trypomastigotes are removed by a second trypanocidal antibody terminating the infection (Coventry, 1930). Attempts to reinfect a recovered rat are useless since acquired immunity to *T. lewisi*

appears to be lifelong (Corradetti, 1963).

During the course of the 32-day infection by *T. lewisi*, several authors have reported the occurence of anaemia (Duca, 1939; Barnes, 1951; Sherman and Ruble, 1967; Greenblatt, 1973), splenomegaly (Greenblatt, 1973), glomerulonephritis and a thrombocytopaenic syndrome (Cox, 1982) and immunosuppression (St-Charles, Frank and Tanner, 1981); all of these phenomena disappear with the recovery of the animal from the parasitaemia. The occurence of immunosuppression, which was the prime interest of this present study, was demonstrated by St-Charles *et al* as the depressed ability of spleen cells recovered from infected rats to respond immunologically to the T-dependent antigen sheep red blood cells (SRBC). Although the results of that study indicated that the parasite and its products suppressed the production of antibodies to SRBC, the mechanism(s) by which the trypanosome exerts its action remained to be defined. The results of the present study indicate clearly that B cells are probably not affected by the infection of the animal (Chapters 1 and 2). T cells, on the other hand, are suppressed in their capacity to proliferate in response to the specific T-cell mitogen Concanavalin A (Chapter 2); this inability to respond is reflected by the suppression of the ability of these lymphocytes to produce the soluble factor Interleukin 2 (Il 2; Chapter 3). The demonstration of an adherent suppressor cell in the population of suppressed-spleen cells (Chapter 2) suggests the following scenario for the immunosuppression: the parasite activates suppressor macrophages which inhibit T cells from their

normal response to proliferate by suppressing the production of the important soluble lymphokine Il 2.

However, other mechanism(s) of suppression must also occur since a non-specific immunosuppression was demonstrated to last throughout the course of infection (St-Charles *et al*, 1981). The results from the second chapter imply that suppression was not related to the direct action of the parasite since it was demonstrated in only one of the two populations of spleen cells but both were contaminated with the trypanosomes. It was demonstrated in our laboratory that normal spleen cell cultures are suppressed in their ability to respond to ConA when in the presence of relatively high concentrations of *T. lewisi* exoantigens; these cells, however, responded normally to the mitogens after the suppressing exoantigen was removed by washing. A part of that same study indicated as well that exoantigens can also suppress the susceptibility of normal cells to be activated by LPS. St-Charles *et al* (1981) have reported that the secondary plaque-forming cell response (PFC) to SRBC was suppressed by soluble products released by *T. lewisi* in culture, which are, apparently, confirmed by Ndarathi's results in our laboratory. The PFC response is a complex interaction by antigen-presenting cells, helper T cells and B lymphocytes and it is certainly possible that these complex interactions could be disrupted by the presence of large quantities of exoantigens produced by the parasite. The experiments reported here, done in the absence of these large quantities of exoantigens, since only isolated washed spleen cells were studied and the assay of the response of these

cells to specific T and B cell mitogens, revealed isolated responses of distinctive lymphocyte cell subpopulations. Under these conditions only one subpopulation (the T lymphocyte) was suppressed. . . It is not unreasonable to suggest that several potential mechanism of suppression could be brought into play to ensure the survival of the parasite. It is, furthermore, also not unreasonable to suggest that *Trypanosoma lewisi* has several mechanisms by which it could reduce the immunological capacity of the host: an exoantigen-dependent mechanism or, as reported here, a direct effect on T cells (but not B lymphocytes) which may be mediated by suppression macrophages which are induced during the course of the infection; this latter mechanism acting to reduce proliferation of the T cells by restricting the production of the lymphokine Il 2.

The potential mechanisms responsible for immunosuppression could be as numerous and as complex as the components of the immune system. This study has indicated one possible mechanism, but a clarification of the whole process that permits the establishment and maintenance of the parasite in the host is far from complete. Studies of the macrophage cell populations, such as their capacity to present the antigen of *T.lewisi* and to produce the soluble factor Il 1, and a study of the activity of T cells when in the presence of exogenous Il 2 and/or Il 1 are some experiments that should be considered in future inquires into the phenomenon of immunosuppression. This work has served well to update a present understanding of the immunobiology of *T. lewisi*; it is the role of my successors to further this investigation to

answer the many questions that still remain to be answered concerning the immunobiology of this and other trypanosome, and other parasite infections as well.

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