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IMMUNOCHEMOTHERAPY IN EXPERIMENTAL

LEISHMANIASIS

by

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A Thesis submitted to the Faculty of Graduate Studies and Research in Partial

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Canada

ABSTRACT

The proliferation dynamics *in vitro* of the obligate intracellular protozoan parasite *Leishmania donovani* was studied for 14 days in resting monolayers of peritoneal macrophages of C57BL/6 (*Lsh*^{s}) mice inoculated with 5, 50, or 500 promastigotes/cell. Irrespective of the inoculum, only 50 to 65% of the cells became infected initially; only 3 to 6 amastigotes were present in each macrophage initially, suggesting a limited number of parasite ligands on the host cell. The amastigotes did not divide in the first 3 or 4 days after infection and then they actively proliferated from day 5 to day 8; the number of parasites was then reduced. Infection with *L. donovani* down-regulates immunity and parasite clearance by macrophages, but IL-2-stimulated splenocytes activate leishmanicidal action *in vitro* in infected peritoneal macrophages and *in vivo* in C57BL/6 (Lsh^s) mice. IL-2-stimulated splenocytes were most effective when used in the non-proliferating phase of the infection, whereas the anti-leishmanial drug Pentostam was most effective when used during the proliferative phase. Immuno-chemotherapy was more effective than either treatment alone. Infection with *L. donovani* abolishes the ability of macrophages to produce the superoxide and INO microbicidal responses; curative treatment with IL-2-stimulated splenocytes restores the ability of infected macrophages to secrete inorganic nitrogen oxides, but not to produce a superoxide response. Pentostam had no effect to stimulate either microbicidal mechanism in infected cells; the drug is, therefore, probably directly toxic to the parasite. These studies have indicated, among other things, that IL-2-stimulated splenocytes rescue infected cells and

infected animal from the immunological deficit which *L. donovani* induces, allowing the

re-establishment of those mechanisms that make the macrophage an essential component

of the host's protective immune system.

ABREGE

La dynamique de la prolifération *in vitro* du parasite obligatoirement intracellulaire *Leishmania donovani* a été étudiée pendant 14 jours avec des couches unicellulaires de macrophages péritonéaux de souris C57BL/6 (*Lsh*), inoculées avec 5, 50 ou 500 promastigotes/cellule. Indépendamment de l'inoculum utilisé, seulement 50 à 65% des cellules étaient initialement infectées; de 3 à 6 amastigotes seulement étaient présents dans chaque macrophage, indiquant que la cellule hôte comprend un nombre limité de récepteurs pour le parasite. Les amastigotes ne se sont pas divisés durant les premiers 3 ou 4 jours après l'infection, mais ont proliféré activement du 5* au 8* jour; le nombre de parasites intracellulaires a ensuite décliné. Lors d'une infection par *L. donovani*, le système immunitaire et la capacité des macrophages à éradiquer le parasite sont déprimés. Cependant, les cellules spléniques stimulées par l'interleukine-2 (IL-2) activent *in vitro* l'action leishmanicide chez les macrophages péritonéaux infectés, et *in vivo* chez les souris C57BL/6 (*Lsh*). Les cellules spléniques stimulées par l'IL-2 ont été plus efficaces lorsqu'utilisées durant la phase de non-prolifération de l'infection, tandis que la drogue Pentostam (utilisée pour traiter les leishmanioses) a été plus efficace lorsqu'administrée durant la phase de prolifération. La combinaison immuno-chimiothérapie a donné des meilleurs résultats que chacun des traitements administrés individuellement. *L. donovani* élimine la capacité des macrophages de monter une réponse anti-microbienne composée de superoxydes et d'oxydes d'azote inorganiques

(INOs); le traitement par les cellules spléniques stimulées par l'IL-2 rétablit la capacité des macrophages infectés de sécréter des INOs, mais ne restaure pas leur capacité de produire des superoxydes. Le Pentostam n'a stimulé aucun des deux mécanismes microbicides chez les cellules infectées; la drogue est donc probablement directement toxique pour le parasite. Ces études indiquent, entre autres, que les cellules spléniques stimulées par l'IL-2 sauvent les cellules et animaux infectés d'une carence immunologique causée par *L. donovani*, permettant la ré-instauration des mécanismes qui font du macrophage un élément essentiel du système immunitaire.

ACKNOWLEDGEMENTS

I would like to extend my heartfelt thanks to my research supervisor, Dr. Charles E. Tanner whose direction and support made this study possible. Thank you for your valued guidance, advice, continual support and encouragement throughout the time of my studies.

I also acknowledge with many thanks the advice and help of the members of my research committee: Dr. M. Rau, Dr. K. Chadee and Dr. M. Olivier and that of the other professors of the Institute for sharing their time and knowledge with me. In particular, I would like to thank Dr. J.M. Smith, who was there whenever I needed help.

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Finally, I would like to thank my family for being there whenever I needed them and for their love and encouragement at all times. I thank my husband Ali and my adorable children Atefeh and Emad for their patience and support.

I would like to dedicate my thesis to my dear parents, who convinced me that I could do anything I decided to do.

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*LEISHMANIA DONOVANI***

CLAIMS OF ORIGINALITY

Chapter II:

1. It has been known for a long time that the proliferation of *Leishmania donovani* in C57BL/6 mice describes a growth curve that is characterized by an initial period of rapid proliferation of the parasite that reaches a peak in the 3rd or 4th week of the infection; this period of proliferation is followed by a plateau phase that lasts about 10 days, during which little or no further increase in the infection occurs and then by a decline in the number of infected macrophages. This chapter reports the first description of a similar growth curve of *L. donovani* in its host cell: a non-proliferative phase that lasts until day 3 to 4 of the infection *in vitro* is followed by a proliferative phase that terminates on day 7 or 8; the number of infected cells then decrease.

2. This chapter also describes for the first time the finding that macrophages in a population of cells are not all initially equally susceptible to infection *in vitro* and that each susceptible cell probably expresses only a limited number of ligands (4 or 5) to attach the parasite to the host cell prior to internalization.

Chapters III & IV :

1. It is known that the number of macrophages infected by *L. donovani* can be reduced effectively by treatment with soluble lymphokines. These chapters report for the first time that experimental visceral leishmaniasis can be more effectively and significantly reduced *in vivo* and *in vitro* by treating infected macrophages or infected mice with splenic T cells stimulated by the lymphokine IL-2 (LAK cells). The effectiveness of treatment with LAK cells *in vitro* was not impaired when the effector cells were maintained in cell-impermeable Millipore chambers, indicating that the activation of infected macrophages to reduce their burden of parasites was done by lymphokines produced by the LAK cells.

a. Monoclonal anti-IFN-γ interfered only partially with the curative ability of the LAK cells, indicating that IFN-γ was not the principal effector lymphokine produced by the cells.

b. It was also demonstrated for the first time in these chapters that the effectiveness of a curative strategy depends on the *Lsh* phenotype of the treated cells: *Lsh*^{r} macrophages were more readily cured of their parasite infection than *Lsh*^{s} cells.

c. LAK cells prepared from the spleen of infected mice were more curative *in vivo* and *in vitro* than were LAK cells prepared from normal splenic T cells; the cure of experimental kala azar by LAK cell

immunotherapy *in vivo* was significantly enhanced by subsequent intra-peritoneal treatment with exogenous IL-2.

Chapter V:

1. It is well-recognized that the antimonial drug Pentostam is very effective in the treatment of experimental and clinical visceral leishmaniasis. It is reported in this chapter for the first time that this drug is significantly more effective, however, when the parasite is in the phase of active proliferation. It is also reported in this chapter for the first time that immunotherapy with LAK cells is most effective in the initial non-proliferative phase of the infection, when the parasite is, presumably, adapting to its new intracellular habitat. LAK immunotherapy in the non-proliferative phase and Pentostam chemotherapy in the proliferative phase of *L. donovani* is more effective in curing the infection that either treatment alone.

Chapter VI:

1. Normal neutrophils and macrophages are known to destroy internalized organisms by activating the synthesis of microbicidal oxygen and nitrogen metabolic intermediates, but macrophages infected by *L. donovani* do not activate either of these microbicidal pathways. It is reported for the first time in this chapter that the down regulation of the

reactive oxygen pathway in infected macrophages is not reactivated by curative treatment with LAK cells. LAK cells do, however, reactivate the down regulated microbicidal nitrogen intermediates, suggesting that the reduction of the infection following immunotherapy is effected by the latter response.

2. It is also reported for the first time in this chapter that anti-IFN-γ was able to reduce by 50% the ability of LAK cells to reactivate the microbicidal nitrogen intermediates pathway. This latter result supports the finding presented in this thesis that IFN-γ is only one of the LAK lymphokines that are responsible for activating parasiticidal action

CONTRIBUTIONS OF OTHERS

Chapter II:

I designed this experiment and collected all the data.

Chapter III:

This study was done in collaboration with Dr. Martin Olivier of the University Laval. I designed and performed that part of the experiments which were done with rIL-2-stimulated splenocytes, the other part of this study done with the MLA-144 cell line was done by Dr. Olivier. I am responsible for the analysis and the interpretation of the results obtained.

Chapter IV:

This study was also done in collaboration with Dr. Olivier. I did the life cycle of the parasite and Dr. Olivier performed the other studies. I analyzed the results and drew the graphs.

Chapter V:

The experimental design, the maintenance of the cell cultures and data collection were done by me. Denis Gaucher helped me in that portion of the study that investigated the toxicity of Pentostam.

Chapter VI:

The experiments described in this chapter were designed by me and I performed the study by myself.

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A GLOSSARY OF SOME OF THE ABBREVIATIONS

1. **anti-Thy 1.2** - antibodies against the alloantigen of C57BL/6 thymocytes.

2. **Con A** - Concanavalin A is a lectin derived from the jack bean

   (*Canavalia ensiformis*) that induces cell division in T cells.

3. **DTH** - Delayed Type Hypersensitivity. A hypersensitivity of late onset that is

   associated with the activation of cell-mediated immunity.

4. **GM-CSF** - Granulocyte-macrophage activating factor. It is a T cell-derived

   cytokine that acts on committed bone marrow progenitor cells to

   induce their differentiation into granulocytes or/and mononuclear

   phagocytes.

5. **IL** - Interleukin: Cytokines produced by leukocytes that activate other

   leukocytes. IL-1 (LAF) is produced by activated macrophages and acts to

   enhance the proliferation of CD4$^+$ and the growth and differentiation of B cells.

   IL-2 (TCGF) is produced by activated CD4$^+$ cells and is the cytokine principally

   responsible for the progression of T lymphocytes from the G1 to the S phase of

   the cell cycle.

6. **IFN-γ** - Interferon-gamma (MAF) is produced by IL-2-secreting CD4+ helper

   T cells and by nearly all CD8+ T cells. IFN-γ is the principal MAF

   and provides the means by which T cells activate macrophages.

7. **INO** - Inorganic nitrogen intermediates.

8. **LAK** - Lymphokine-activated killer cells. Produced by the stimulating

   action of IL-2 on T cells; they are produced *in vitro*.

9. **LPG** - Lipophosphoglycan is the major cell surface glycoconjugate of *Leishmania*

   promastigotes and is an essential virulence determinant. It inhibits PKC activity

   in macrophages.

10. **LPS** - Lipopolysaccharide. It is a component of the cell wall of Gram-

    negative bacteria; it binds polyclonally to B cells without using T helper cells

    as intermediaries and it is, thus, a "T-independent" T1 antigen.

11. **MIF** - Migration-inhibition factor. MIF has not been identified with a

    particular cytokine, but describes an inhibition of the migration of

    leukocytes following cell-mediated immune reactions. This

    phenomenon may be controlled by an enhanced expression of intracellular

    matrix molecules, such as the integrins.

12. **MLA 144 cells** - A tumor cell line that is available from the American Type

Culture Collection (ATCC TIB 201) that constitutively

elaborates IL-2 into culture medium.

13. **PBS** - Phosphate-buffered saline, usually made to pH 7.2, is a common

laboratory buffer for cell-mediated reactions.

14. **PHA** - Phytohaemagglutinin. A lectin extracted from the kidney bean (*Phaseolus*

vulgaris); it is a mitogen for T cells.

15. **PKADL** - Post-kala azar dermal leishmaniasis. A condition that sometimes

develops following clinical infection by *Leishmania donovani*; it is

characterized by lightly-pigmented areas on the skin. Although PKADL

is associated with visceral leishmaniasis, its aetiology is uncertain,

especially since the parasite can not be recovered in any great numbers

from "affected" areas.

16. **ROI** - Reactive oxygen intermediates. An intermediate in the activation chain

for the generation of intracellular microbicidal H_2O_2 in neutrophils and

macrophages.

17. **RNI** - Reactive nitrogen intermediates. As for ROI, an intermediate in the

activation chain for the generation of microbicidal NO_2^- in

neutrophils and macrophages.

17. **R4-6A2** - A cell line available from the American Type Culture

Collection (ATCC HB 170) that elaborates a monoclonal rat IgG_1

anti-mouse IFN-γ.

18. **S4B2** - A cell line (ATCC HB 8794) that produces a monoclonal antibody

that reacts with mouse IL-2. This cell line was obtained from

Dr. Trevor Owens of the Department of Microbiology and Immunology,

McGill University, with permission of the DYNAX Research

Institute, Palo Alto, California, the holders of the patent for this cell line.

19. **SCID** - Severe combined immunodeficiency. A term used to describe the congenital

absence of functional B- and T-cell-mediated immunity. The syndrome is

caused by a series of autosomal recessive and X-linked genetics defects

produced by a lack of differentiation of mature, functional T cells in the

periphery, leading to the down regulation of the expression of MHC genes,

the production of IL-2 and the IL-2 receptor, a down regulation of

responsiveness to IL-1 and in signal trasduction.

20. **TGF-ß** - Transforming growth factor-beta. Cytokine produced by T cells and

other leukocytes that induces the proliferation of T cells and other leukocytes,

inducing them to progress from the G1 to the S phase in the cell cycle.

21. **Th2 cells** - A subpopulation of T helper cells that produces IL-4, 5, 10 and 13 and is involved in the production of an antibody response in *L. major* cutaneous leishmaniasis. This subpopulation of T helper cells, in contrast to the IFN-γ, IL-2-producing Th1 cells, is essential for the generation of a cell-mediated immunity and protection. Stimulation of a Th1 cell response reduces Th2-dependent cell-mediated immunity and, thus, enhances *L. major* leishmaniasis.

INTRODUCTION

Leishmania are obligate intracellular protozoan parasites in the mammalian host, transmitted by the bite of infected Phlebotomid sand-flies from non-human reservoirs. Depending upon the *Leishmania* species inoculated by the arthropod, infection can result in cutaneous, mucocutaneous, or, when caused by *Leishmania donovani*, produces the fatal visceral disease, kala azar. An incidence of 400,000 new cases per year has been reported and the world-wide prevalence of leishmaniasis is estimated to be 12 million cases (Modabber, 1987). The last epidemic of visceral leishmaniasis that occurred in India in 1977-78 caused an estimated 20,000 deaths (Thakur, 1984) and a more recent epidemic in the Sudan killed at least 40,000 individuals (TDR news, NO.46, November 1994). Most forms of kala azar are essentially zoonotic, and humans are infected only secondarily. Diagnosis is established by the identification of the parasite in, or isolated from, infected tissue or by serological or skin-tests. The mainstay of chemotherapy at present is based on pentavalent antimonials. These drugs must, however, be given in daily intramuscular doses for several weeks, but they are not, nevertheless, invariably effective and have unpleasant side effects. Amphotericin B and pentamidine are used in cases which are resistant to antimonials. No vaccines are available and vector control can be exceedingly difficult. Thus, active research is underway in many laboratories to find alternative effective preventive and therapeutic treatments.

1

In the review that follows, a summary will be made of the general features of the biology of *L. donovani* and of the recent advances which have been made in the study of the humoral, cellular and macrophage aspects of the host-parasite relationships in visceral leishmaniasis. The morphology of the amastigotes and the promastigotes, the nature of the host cell and the vector of the three complexes of the pathogenic *Leishmania* are so similar that there has been a general tendency to discuss the members of this genus as if they shared the same biology. Nothing, however, could probably be farther from the truth: the pathology, immunology and genetic control of the *Leishmania* in the three complexes are so peculiar that it is unlikely that they share a common biology. The review of the literature will deal principally with the immuno-biology of *Leishmania donovani* and visceral leishmaniasis, drawing brief analogies, where appropriate, from members of the three other complexes in this genus.

Chapter I

LITERATURE REVIEW

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THE PARASITE

Leishmania donovani , as the other member of the genus *Leishmania*, is classified

as follows (Cox, 1991):

Kingdom	Protista
Phylum	Kinetoplasta
Order	Trypanosomatida
Genus	*Leishmania*

Like other trypanosomatids, the leishmanias are heteroxenous because part of their

life cycle is spent in the gut of one of several species of the sand-fly *Phlebotomus*, where

they assume the form of a promastigote; the remainder of their life cycle is completed

as obligate parasites in the macrophages of vertebrate hosts where only the amastigote

form is found. The genus *Leishmania* includes the several species which are summarized

in Table 1.

Table 1: Agents of human leishmaniasis

ORGANISM	CLINICAL DISEASE
Leishmania tropica	Ulcer, oriental sore, long incubation and duration, leishmania recidiva
Leishmania major	Ulcer, "acute" cutaneous leishmaniasis, shorter incubation and duration
Leishmania aethiopica	Ulcer, oriental sore, diffuse cutaneous leishmaniasis (DCL)
Leishmania mexicana mexicana	"Chicleros ulcer", usually single or few lesions ear infection often involved, lesion may persist, no nasopharyngeal lesions
Leishmania mexicana amazonesis	Ulcer, DCL association
Leishmania mexicana pifanai	Rare, DCL association
Leishmania braziliensis brazilensis	Ulcer, single or few lesions, persistent and disfigurating, metastases (mucocutaneous leishmaniasis)
Leishmania braziliensis guyanensis	Ulcer (pain bois), metastases
Leishmania braziliensis panamensis	Ulcer, single or multiple , persistent
Leishmania peruviana	Ulcer, single or few lesions, self-resolving
Leishmania donovani	Visceral leishmaniasis (kala azar), post-kala azar dermal leishmaniasis, also self limiting dermatoid form
Leishmania infantum	Visceral leishmaniasis (kala azar) of childern
Leishmania chagasi	Visceral leishmaniasis of the new world

a. Morphology and Ultrastructure

Leishmanial amastigotes reside in the vertebrate host only in macrophages and are readily recognized in the cell by light microscopy. They are rounded, measure 2 to 5 μm in diameter and, with Giemsa staining, their nuclei stain purple, their cytoplasm azure, and their kinetoplast an intense purple. The body and flagellum of the insect promastigote form are each approximately 20μm long, and their organelles have staining characteristics similar to those found in amastigotes. The microtubular system of the organism functions as a cytoskeleton, imparting shape and flexibility to the parasite and perhaps also serves to anchor intrinsic plasma membrane proteins. The flagellar pocket, although not a true cytosome, has coated vesicles which appear to be involved in pinocytosis. The kinetoplast is a specialized mitochondrion which contains supercoiled DNA (kDNA), existing as a network of catenated minicircles and maxicircles; the kDNA divides before nuclear DNA.

b. Life Cycle

With the rare exception of cases of leishmaniasis transmitted by blood transfusion, human infection is exclusively initiated by the bite of infected female sand-flies; no other vector has ever been identified (Figure l). When the sand-fly takes a blood meal from an infected host, minute amounts of blood, lymph and infected macrophages are ingested. Amastigotes exit from the macrophages in the blood meal and undergo at least one division before they transform into promastigotes. These latter enter the midgut and are surrounded by a peritrophic membrane created by material secreted by the insect's gut

Fig. 1: Leishmanial life cycle in sandfly and in mammalian hosts. (Chang *et al* 1985)

1. Delivery of promastigotes (proboscis form) into human skin by the bite of the sandfly vector; 2. attachment and engulfment by phagocytosis of promastigotes by a macrophage; 3. fusion of phagosome containing a promastigote with lysosome in a macrophage; 4. differentiation of promastigote into amastigote in the phagolysosome of the infected macrophage; 5. multiplication of an amastigote in a parasite-containing or parasitophorous vacuole; 6. formation of large parasitophorous vacuole and continuing replication of intravacuolar amastigotes; 7. rupture of heavily parasitized macrophage and release of amastigotes; 8. phagocytosis of released amastigotes by a macrophage; 9. ingestion of parasitized macrophage by sandfly after a blood meal taken from infected person or reservoir animal; 10. rupture of the ingested macrophage and release of amastigotes in the gut of sandfly; 11. replication of amastigotes and their differentiation into promastigotes; 12. replication of promastigotes (termed neptomonads for *L. mexicana* group) in the abdominal midgut and insertion of their flagella into microvilli of the gut epithelial cells; 13. replication of *L. braziliensis* group in the pylorus and ileum of the sandfly hindgut as promastigotes with broadened flagella attached to the chitinous gut wall via hemi-desmosomes; 14. forward movement of promastigotes to thoracic midgut as haptomonads with boad flagella attached to the chitinous gut wall; 15. sessile promastigotes with broad flagella attached to the chitinous wall of stomodeal valve, pharynx and bucal cavity (cibarium); 16. actively motile promastigotes found in the proboscis or mouth parts of the sandfly. (Chang *et al* 1985)
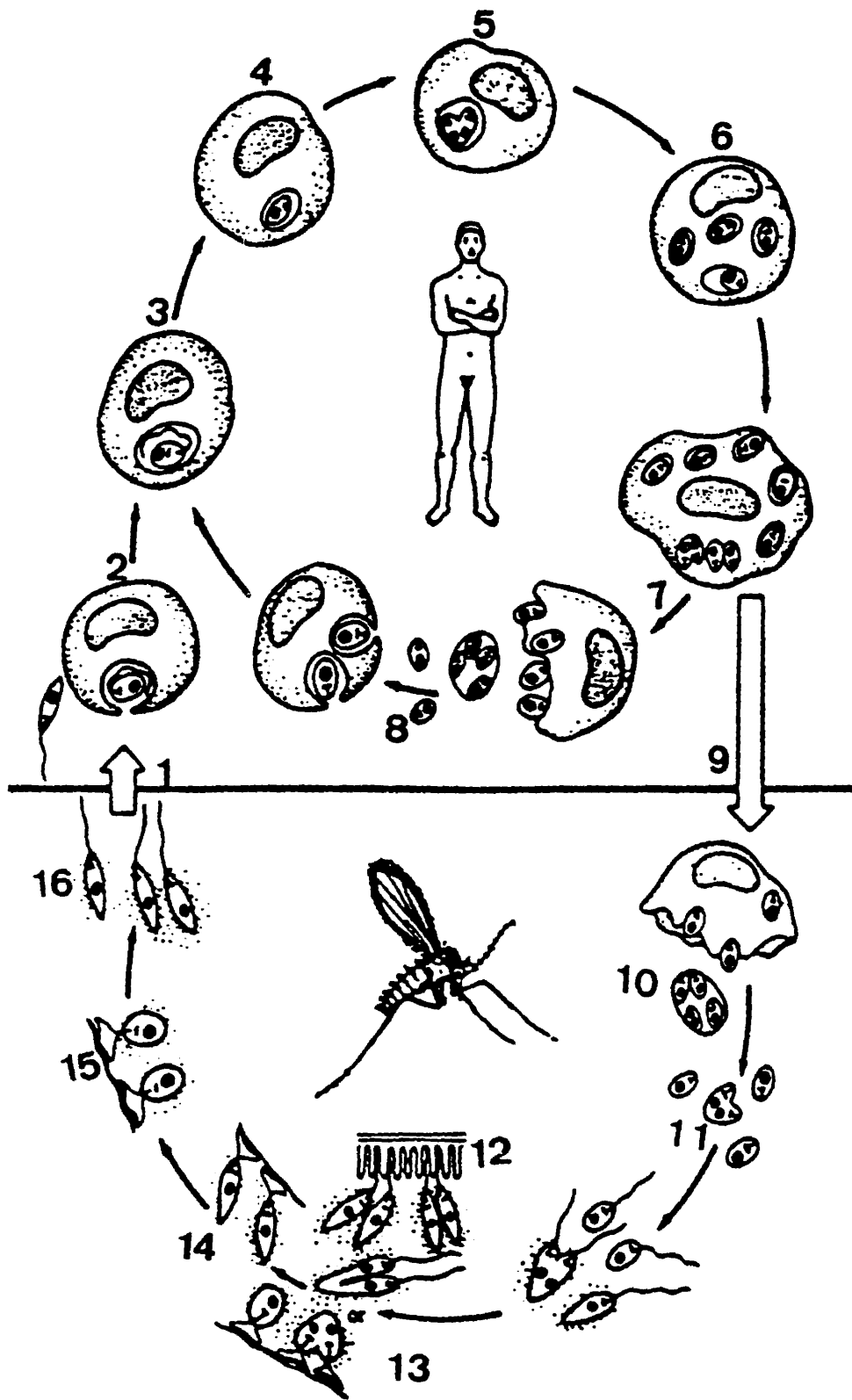
epithelium. The promastigotes replicate initially within, and later exit through, the peritrophic membrane. Further development and replication occur in the midgut of the fly, except for *L. brasiliensis* which develops and replicates in the hindgut. The midgut promastigotes interdigitate their flagella between the microvilli on the surface of the gut epithelial cells; however, no electron-dense tight junctions are formed. Following replication, promastigotes migrate anteriorly and become attached to chitinous parts of the stomodeal valve near the foregut and the parasite spreads to the oesophagus and the pharynx. Here the promastigotes become "stumpy", less motile and cease dividing. A subpopulation of the parasite finally moves to the proboscis where the organisms remain viable for a short period of time and it is from this site that they are injected at the time of subsequent feeding by the insect. Rarely are more than 10 promastigotes found in the proboscis at any one time, and it is believed that there is a continual attrition from and a repopulation of the proboscis by promastigotes from the pharynx and the oesophagus. Reproduction of the parasite is believed to occur by binary fission; no sexual stage has yet been identified.

VISCERAL LEISHMANIASIS

Clinical Studies

Visceral leishmaniasis presents a clinical picture much different from that of the cutaneous infection. It generally begins with a primary undetectable nonulcerating skin lesion and, following an incubation period which may last as long as several months, the parasites may be found in the reticuloendothelial cells of the liver, spleen, bone marrow

and lymph nodes. Parasite multiplication leads to splenomegaly, hepatomegaly and lymphadenopathy. Early stages of the infection can be characterized by fever, chills, and general malaise; advanced cases may display degenerative changes in every major organ of the viscera and anaemia, leucopaenia and eventual monocytosis may be observed. The symptoms may subside following treatment, but in some cases post-kala azar dermal lesions (PKADL) develop.

Unlike cutaneous leishmaniasis, untreated cases of visceral leishmaniasis are often fatal (Modabber, 1987). When cure occurs, cell-mediated immune responses may be detected (Turk and Bryceson, 1971; Preston and Dumonde, 1976) and resistance to reinfection has been reported (Manson-Bahr, 1964), but it is not always complete (Guirges, 1971), as indicated by the development of post-kala azar dermal lesions. Some patients with active kala azar often show no detectable delayed-type hypersensitivity (DTH) in response to the intradermal injection of leishmanin (Maekelt, 1972), although Manson-Bahr (1961) noted positive immediate skin reactions during active infections.

Antibody titers rise sharply during visceral leishmaniasis but the antibody is not protective (Bray and Lainson, 1967) and not parasite specific (Clinton, *et al.* 1969). The response may have some diagnostic value (Sen Gupta, 1969), although it does not often develop significantly until after the acute phase of the infection is well under way. Successful chemotherapy is, however, often followed by decreasing antibody levels and the development of DTH, corresponding to an initiation of resistance to reinfection (Manson-Bahr, 1961). Verss (1977) and Rezai *et al* (1978) reported extensive fibrosis of thymus-dependent areas of the spleen and lymph nodes in cases of human visceral

leishmaniasis, a depletion of T lymphocytes, and plasma cell hyperplasia; these observations explain the lack of DTH, or of cell mediated immunity during visceral leishmaniasis. It is not yet clear whether the lack of DTH to leishmanin during infection is the result of a general or of a specific immune suppression phenomenon, although it has been shown that mice acutely infected with a reticulotropic strain of *Trypanosoma cruzi* had suppressed cell-mediated responses to heterologous antigens (Reed *et al*. 1977).

The factors that lead to disseminated infection with *L. donovani* are not well understood. It is likely that host factors and the parasite strain and the inoculum size are important in determining the course of infection. It has been recognized that a mild dermal form of *L. donovani* infection exists and that it is apparently quite common in some parts of the world (Hoogstraal and Heyneman, 1969b): such infections are thought to be often asymptomatic and they may represent a successful host response prior to the internal dissemination of the infection (Garnham and Humphrey, 1969).

LEISHMANIA-MACROPHAGE INTERACTION

a. Phagocytosis

Serum from many animal species displays a potent lytic activity against *Leishmania* promastigotes, due to the activation of the alternate pathway of the complement cascade (Hoover, *et al*. 1984). It may be that survival of the parasite within the animal host depends on its capacity to become intracellular. Interestingly, however, no specific organelle that facilitates cell invasion has ever been detected, and *Leishmania* appear to rely entirely on the phagocytic activity of macrophages to reach a safe intracellular

location. Studies of the phagocytosis of *L. donovani* by hamster macrophages has revealed no preferential orientation (tail first, head first) of the parasite during internalization (Chang, 1979). Attachment to and internalization of *Leishmania* parasites by their macrophage hosts is a complement receptor-mediated event (Klemper *et al.* 1983). Following the activation and fixation of C3 to the surface of promastigotes, the uptake of the parasite is increased dramatically (Puentes *et al.* 1988), presumably by the attachment to complement receptors CR1 and CR3 (Wozencraft *et al.* 1989) which can occur in the absence of a respiratory burst (Wright and Silverstein, 1983. *L. major* metacyclic promastigotes fix C3b on their surface by the classical complement pathway and binding to the macrophage surface occurs through CR1 receptors (Da Silva *et al.* 1989). In contrast, *L. donovani* promastigotes bind iC3b following activation of the alternate pathway and uptake of this parasite occurs through the CR3 receptors (Mosser *et al.* 1992). LPG and gp63, the two best characterized molecules on the surface of *Leishmania* promastigotes (Russel and Wilhelm, 1986; Moody, 1993) are both acceptors for C3 deposition and are thus involved in the ingestion of promastigotes by macrophages (Handman and Goding, 1985).

Despite this evidence, several studies have claimed that the internalization of promastigotes through CR3 can occur in the absence of serum (Wozencraft *et al.* 1989) because the phagocytosis of LPG-deficient mutants is similar to that of wild type promastigotes and imply that there is an LPG-, serum-independent pathway for internalization (McNeally and Turco, 1990). The ingestion of *Leishmania* promastigotes by macrophages has been shown to stimulate a potentially-lethal respiratory burst

(Pearson *et al.* 1983b), suggesting that CR1 and CR3 may not be the exclusive parasite receptors. The fucose-manose receptor (MFR) of macrophages presents an alternative candidate for parasite uptake, although phagocytosis by this pathway is also known to stimulate a respiratory burst (Berton and Gordon, 1983). The MFR has, nevertheless, been identified as the receptor for the attachment and ingestion of *Leishmania* promastigotes by mouse and human macrophages (Channon and Blackwell, 1985). Blocking of the MFR on human macrophages inhibited by 40% the attachment of *L. donovani* promastigotes, whereas also blocking the CR3 receptor inhibited parasite ingestion by 81% (Wilson and Pearson, 1990).

Receptors involved in the uptake of amastigotes into macrophages are not known in as great detail as those involved in the internalization of promastigotes, but it is likely that each form may have evolved a separate mechanism of entry. *Leishmania* amastigotes are known to be deficient in LPG (McConville and Blackwell, 1991), or to express an altered form of this molecule; unlike promastigotes, amastigotes do not appear to utilize the MFR to enter the macrophage (Guy and Belosevic, 1993). The CR3 complement receptor and the Fc receptor have also been implicated as primary receptors for the attachment of amastigotes, as they are known to be involved in the uptake of opsonised promastigotes (Chang, 1981).

Once a *Leishmania* is attached to its receptor on the macrophage, internalization is achieved through phagocytosis and the formation of a phagosome. Secondary lysosomes then fuse with the parasitophorous phagosome vacuole to form a phagolysosome in which the parasite multiplies as amastigotes (Berman *et al.* 1979). This behaviour is in contrast

to that of other intramacrophage parasites, such as *Trypanosoma cruzi*, that leave the phagolysosome shortly after fusion with the lysosome (Nogueria and Cohn, 1976) and multiply in the cytoplasm of the host cell. Other intramacrophage parasites such as *Mycobacterium tuberculosis* (Armstrong and D'Arcyhart, 1971) and *Toxoplasma gondii* (Jones *et al.* 1972) avoid destruction by preventing the fusion of lysosomes with the phagosome.

The principal target cell population that expresses the susceptibility/resistant phenotype of the host animal is the Kupffer cell of the liver (Olivier and Tanner, 1987). Peritoneal, pulmonary and spleen macrophages of resistant (C57L) and susceptible (C57BL/6) mice are equally susceptible to infection in *vitro* by *L. donovani*. It is very well-known that macrophages are key elements in the induction and in the effector functions of the immune response; infection by *L. donovani* interferes with both activities. Macrophages infected with the parasite *in vitro* have a reduced ability to produce IL-1 in response to stimulation with silica (Olivier and Tanner, 1989) and a soluble product produced *in vitro* by macrophages infected by *L.donovani* reduces the ability of normal macrophages to process antigen. Macrophages infected with this parasite express reduced levels of both class I and II MHC epitopes (Reiner, 1987), a phenomenon which is also induced by the suppressor factor secreted *in vitro* by infected macrophages (Fielding and Tanner, submitted).

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b. Intracellular Survival

Intracellular survival implies that all the nutritional requirements of the parasite are satisfied by it's host cell, and that the metabolism of L. donovani has become adapted to the normally hydrolytic environment within the phagolysosome; survival of L. donovani in this habitat indicates that measures have been taken by the parasite either to avoid the action or to suppress the production of lysosomal hydrolases and the toxic oxygen and nitrogen metabolites which can be produced by the macrophage. The observed fusion of the secondary lysosome with the parasitophorous vacuole suggests that intracellular Leishmania may have access to the extracellular environment via the vacuolar apparatus of the host cell (Chang, 1979). L. donovani is unable to synthesize purines and requires the host cell to provide it with preformed bases in order to synthesize DNA and RNA (Bhattachargo and Janovy, 1975).

Amastigotes proliferate in the acidic environment of the host cell's phagolysosomes (Ohkuma and Poole, 1978), carrying out respiration, catabolism of energy substrates, and the incorporation of precursors into macromolecules optimally at pH 4.0 to 5.5 (Mukkada et al 1985) and the presence of the parasite seems to have little impact on lysosomal pH (Chang, 1979). The parasite membrane contains a proton-translocating ATPase (Zilberstein and Dwyer, 1988) which may help to maintain pH homeostasis in the parasite; the proton gradient thus established drives the active transport of nutrients required for parasite growth (Zilberstein and Dwyer, 1985). That such a transport system is critical to the parasite's survival has been clearly demonstrated by the observation that tricyclic antidepressants block the parasite's plasma membrane ATPase, leading to

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reduced membrane potentials, inhibition of active transport, disruption of pH homeostasis, and rapid death of the organism (Zilberstien and Dwyer, 1984).

Transition of the insect-adapted promastigote to the mammal-adapted amastigote entails major metabolic changes. The respiration rate and glucose catabolism (Hart and Coombs, 1982) and the activity of several glycolytic enzymes (Coombs *et al.* 1982) are significantly lower in amastigotes. The temperature shift experienced by the parasite during the transition is sufficient to trigger the synthesis of several heat-shock proteins (HSP) (Lawrence and Robert-Gerro, 1985) and has been correlated with increases in the infectivity of the organism (Smejkal *et al.* 1988). It remains, however, to be established whether HSP synthesis is related to the acquisition of an ability for intracellular life.

Phagocytosis by normal macrophages and neutrophils is usually accompanied by a respiratory burst, where oxygen is reduced to water with the production of the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical (OH-) and singlet oxygen (Andrew *et al.* 1985). These events are involved in one of the principal physiological effector functions of macrophages: the elimination of invading pathogens (Andrew *et al.* 1985). Upon phagocytosis by macrophages, *L. donovani* promastigotes can elicit a stronger respiratory burst than when amastigotes are internalized (Haidaris and Bonventre, 1982) and products of the oxidative burst, particularly hydrogen peroxide (H_2O_2), are deleterious to *Leishmania* promastigotes (Pearson *et al.* 1983a). A deficiency in both catalase and glutathione peroxidase, which are both scavengers of hydrogen peroxide, can explain the high susceptibility of promastigotes to H_2O_2 (Murray, 1981). Amastigotes of *L. donovani* do not activate the macrophage respiratory burst during

phagocytosis and are, in addition, more resistant than promastigotes to the toxic effects of H_2O_2 (Murray, 1982; Pearson *et al.* 1983b) since they contain high levels of glutathione peroxidase (Murray, 1982); amastigotes also display a superior ability to scavenge H_2O_2 (Channon and Blackwell, 1985) by producing catalase. The novel H_2O_2 scavenger trypanothione may have replaced the normal glutathione system in *Leishmania* and may contribute to the protection of the parasite against the action of oxygen metabolites (Fairlamb *et al.* 1985).

The binding of LPG on the promastigote to iC3B on the macrophage surface prior to entry of the parasite into the cell does not stimulate a respiratory burst (Wright and Silverstien, 1983; Puentes *et al.* 1988). It has, in addition been shown (McNeely and Turco, 1987; Chan *et al.* 1989; Frankenburg *et al.* 1990) that LPG can inhibit the respiratory burst induced in phorbol myristate acetate- or zymosan-stimulated macrophages *in vitro* and is capable of directly scavenging oxygen radicals. In resident peritoneal macrophages, no quantitative differences have, however, been observed in the respiratory burst elicited by the uptake of *Salmonella typhimurium* (Blumenstock and Jann, 1981) or of *L. donovani* (Blackwell *et al.* 1985a) by macrophages obtained from resistant or susceptible mice. Paradoxically, the antiparasite effect of genetically-resistant macrophages against *L. donovani* is most pronounced in Kupffer cells (Crocker *et al.* 1984; Crocker *et al.* 1987), which are oxidatively inactive in response to *Leishmania* spp (Blackwell *et al.* 1985a; Lepay *et al.* 1985) and to other stimuli (Ding and Nathan, 1988).

The presence of the parasite within macrophages depresses, however, the response

of the cells to agents that can normally stimulate the respiratory burst (Buchmuller-Rouiller and Mauel, 1987). This phenomenon is similar to the blockage of the superoxide anion in neutrophils by acid phosphatases (Chang and Dwyer, 1976); in addition, the parasite's LPG inhibits protein kinase C (PKC), a modulator of macrophage oxidative metabolism (McNeely and Turco, 1987). *Leishmania* amastigotes also inhibit lysosomal hydrolases by producing polyanionic substances capable of complexing to positively-charged hydrolases, or of binding calcium ions (Eliam *et al.* 1985). There is, therefore, a defective regulation of Ca^{2+} and calcium-dependent signalling in *Leishmania*-infected monocytes which provides a basis for understanding abnormalities in responses that involve signalling through the Ca^{2+}-regulated pathway (Olivier *et al.* 1992). The ability of PKC by activating the macrophage's oxidative metabolism to respond to its natural activator diacylglycerol is also reduced in infected macrophages, despite a large increase in the intracellular concentration of Ca^{2+} in the infected cell (Olivier, 1996).

There has also been considerable interest in the functional role of the L-arginine-dependent production of inorganic nitrogen oxides (INO) as a potent leishmanicidal pathway in macrophages (Liew and Cox, 1991). In this system (Figure 2), L-arginine is oxidized by the inducible NADPH-dependent enzyme NO synthase to yield the highly reactive nitric oxide (NO) as an intermediate (Hibbs *et al.* 1987; Martella *et al.* 1988) which can mediate tumour cytolysis, possibly via a nitrosylation reaction (Drapier *et al.* 1988). INO has been demonstrated to play a role in the cytostatic or cytocidal activities of macrophages against the intracellular pathogens *T. gondii* (Adams *et al.* 1990) and *Leishmania major* (Green *et al.* 1990; Liew *et al.* 1990). It has also been shown (Roach

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Fig 2. Physiological and toxic actions of NO. (Kolb and Klob-Bachofen, 1992)
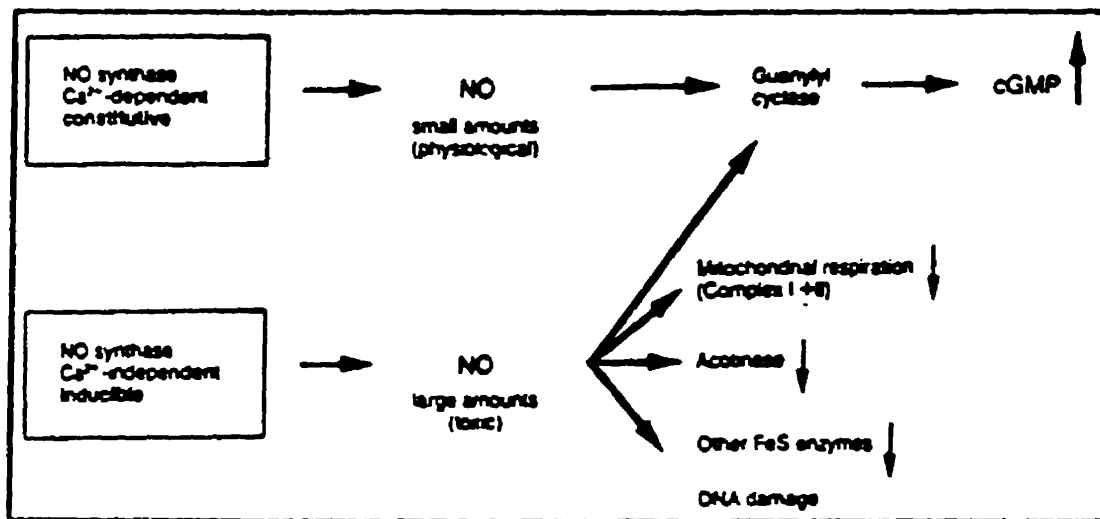
et al. 1991) that, as for *L.major*, an IFN-γ and TNF-α-stimulated production of INO by murine macrophages provides a potent effector leishmanicidal pathway against *L.donovani*. The enhanced respiratory burst observed after the priming or activation of normal *Lsh*[r] peritoneal and splenic macrophages appears, *a priori*, to be a good marker of a change in the physiological status of the cell, but this action may not be, however, involved in the final effector pathway of *L. donovani* antiparasite activity.

Cytokines such as interferon and TNF-α and IL-1 and bacteria are able to induce NO synthesis by many cell types (Green and Nacy, 1993; Nathan and Xie, 1994a; Nathan and Xie, 1994b). In murine macrophages stimulated by IFN-γ and LPS, the induction of NOS depends on the activation of PKC, tyrosine kinase and endogenous ADP ribosylation factors (Paul *et al.* 1995). In addition, the induction of iNOS following stimulation by IFN-γ and LPS is blocked by actinomycin D, an inhibitor of RNA synthesis, demonstrating that iNOS is activated at the transcriptional level (Xie *et al.* 1992). The iNOS promoter contains the consensus sequences for the binding of transcriptional factors that are involved in activation by LPS, such as the binding sites for NF-kappa B, AP-1 and the response elements for IFN-γ and TNF-α, the IFN-stimulated response elements and the regulatory factor binding element for IFN (Xie and Nathan 1994). The iNOS promoter can be induced by LPS alone, or by IFN-γ (Ding *et al.* 1988; Salvemine *et al.* 1993); IFN-γ synergises with LPS to enhance the transcription rate of iNOS (Lorsbach *et al.* 1993) and to stabilize iNOS mRNA (Waisz *et al.* 1994) to permit the accumulation of iNOS mRNA. TNF-α is an important cytokine involved in the regulation of the production of NO (Langerman *et al 1992).*

TNF-α produced by activated macrophages acts in an autocrine fashion to enhance the expression of iNOS mRNA (Deng *et al.* 1993) and, in parasite infections, endogenous TNF-α plays an essential role to induce the production of NO and to kill *L. major* (Green *et al.* 1990; Green *et al.* 1992).

Cytokines such as IL-4 and IL-10 produced by CD4$^+$ Th2 cells and TGF-ß from activated macrophages also suppress NO synthesis (Oswald *et al.* 1992b; Bogdan *et al.* 1994). In addition to down-regulating the Th1 response, IL-10 is also capable of inhibiting cytokine-induced NO production and nitric oxide activity by macrophages (Cunha *et al.* 1992; Oswald *et al.* 1992a; Wu *et al.* 1993). Incubation of infected macrophages with rMIF and IL-10 inhibits the killing of *L. donovani* and the production of NO by human macrophages stimulated by MIF (Wu *et al.* 1993); successful chemotherapy of acute infection by *L. donovani* abolishes the production of IL-10 (Ghalib *et al.* 1993).

Because exogenously added FeSO₄ inhibits macrophage-mediated antileishmanial activity (Mauel *et al.* 1991), the proposed targets of NO in antileishmanial activity may be enzymes with labile iron prosthetic groups required for the replication of DNA or mitochondria or in the citric acid cycle. *L. donovani* has superoxide dismutase (SOD) as an additional target enzyme which would be susceptible to NO inactivation. Because, unlike the SOD of mammals and bacteria, the SOD of *Leishmania* contains an iron group that renders it susceptible to NO; inactivation of this enzyme would markedly increase the vulnerability of the parasite to INO (Meshnick and Eaton, 1991).

Infected macrophages are significantly affected by *L. donovani* in a number of

other different ways: among the more curious effects of the infection is to inhibit the PKC-mediated expression of the c-fos gene (Descoteaux *et al.* 1991). *Leishmania* infection also inhibited apoptosis of bone marrow macrophages (Moore and Matlashewski, 1994) thus, the parasite enhances the longevity of the infected cells, ensuring for itself a long host parasite association.

MACROPHAGES , THE IMMUNE RESPONSE AND IMMUNOTHERAPY

Macrophages are involved in the induction and in the effector phases of the immune response, but infection by *Leishmania* interferes with both activities. Macrophages from patients infected by *L. braziliensis* or *L. m. amazonensis,* or mouse macrophages infected with *L. donovani in vitro,* have a reduced capacity to produce Interleukin-1 (Olivier and Tanner, 1989). Similarly, the susceptibility of T-cells to mitogenic stimulation and the production of IL-2 by spleen cells from *L. major*-infected mice are depressed, perhaps because of an excessive prostaglandin synthesis (Cillari *et al.* 1986). Of particular interest is that infection *in vitro* of macrophages from BALB/c mice with *L. donovani* suppressed expression of both class I and class II major histocompatibility complex gene products, as induced by interferon-gamma (Reiner and McMaster, 1987). Perhaps as a result of this latter phenomenon, macrophages from infected animals, or macrophages cultured in the supernatant of infected cells, are down-regulated in their ability to present antigen (Rodriguez *et al.* 1992). Such phenomena contribute to a reduced ability of macrophages

to function as effector cells for the intracellular killing of the parasite.

The location of the parasite within phagolysosomes raises the question of the mechanisms by which parasite antigens could be presented by host macrophages to the immune system. Parasite constituents are released from infected macrophages as indicated by the presence of antigenic material in the supernatant of short-term cultures of hamster organs (Schnur *et al.* 1972). *Leishmania* immune complexes in the serum of patients with kala azar (Casali and Lambert, 1979) and the presence of parasite antigens in the urine of patients (Kohanteb *et al.* 1987) adds to this evidence. Membrane-bound *Leishmania* antigens have also been demonstrated on infected macrophages *in vitro* (Berman and Dwyer, 1981), suggesting that they could have a role in both the induction and in the effector phases of the immune response.

The result of immunization experiments suggest that, when anchored in the membrane of infected cells, the LPG major surface molecule of *L. donovani* induces a protective T-cell response, whereas the delipidated glycoconjugate stimulates proliferation of non-protective T-cells (Mitchell and Handman, 1985). Alternatively, it has been suggested that antigens presented by macrophages harboring dead parasites might promote the development of T-cells clones that fail to activate those macrophages which contain live microorganisms (Muller *et al.* 1989). It has also been postulated (Mitchell and Handman, 1985) that, depending on the nature of the molecular fragments that are presented by parasitized macrophages, different subsets of T-cells might be stimulated. It has been shown that the fate of *L. major* cutaneous leishmaniasis in mice is associated with the generation of Th1 and Th2 T-cell subsets which appear well before skin lesion

become apparent. Resistance of mice to cutaneous leishmaniasis involves the secretion IFN-γ by Th1 lymphocytes, whereas susceptibility to the parasite is associated with the production of IL-4 and IL-5 (or IL-3) by the Th2 subset (Scott, 1991).

a. Cell-Mediated Immune Responses

Clinical observations and histopathology tend to point to a central role of cellular immunity in the control of *Leishmania* infection. In general, mice infected with *L. major* and made T-cell deficient by thymectomy and irradiation fail to heal cutaneous ulcers spontaneously (Scott *et al.* 1988). T cells obtained from the spleen, peritoneal exudate, or lymph nodes of mice healed of cutaneous or visceral leishmaniasis have been shown to protect syngeneic recipients; but the serum of healed mice is not protective, although it can act synergistically with lymphoid cells administered concurrently (Scott *et al.* 1988).

Many different cell types could be involved during the development of an effective anti-leishmanial cellular immunity. For example, Pham and Mauel (1987) showed that the *in vitro* destruction of infected macrophages by cytotoxic T cells was possible, but Bray and Bryceson (1968) could find no evidence that this occurred *in vivo*. It has, however, been reported (Kirkpatrick *et al.* 1985) that natural killer (NK) cells have a role to play in destroying the parasite during the phase of acquired resistance to *L. donovani* in mice.

The late "cure" in BALB/c mice chronically infected with *L. donovani* is determined in part by genes at the H-2 locus (Blackwell *et al.* 1985d) and the spleens from such

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mice contain T-cells that respond to leishmanial antigen by proliferating and producing

IFN-γ (Murray *et al.* 1983). An Ly^{1+2}, L^{3-}T^{4+} T-cell line has been isolated from a late -

curing BALB/c mouse that produced IFN-γ in response to *L. donovani* antigens, when

co-cultured with *L. donovani*-infected BALB/c macrophages; this T cell was able to

activate macrophages to kill intracellular amastigotes. After adoptive transfer of this cell

line, naive BALB/c mice challenged with amastigotes had a markedly reduced parasite

burden (Murray *et al.* 1983). The same authors showed that another

Leishmania-dependent T-cell line (also Ly^{1+2}, L^{3+}T^{4+}) which produced no IFN-γ failed

to activate macrophages against *L. donovani in vitro*, or *in vivo*.

b. Lymphokines

Important insights into immunobiology and into the basic principles of cellular

immunology have emerged from studies of lymphocyte-macrophage interactions in

leishmaniasis. Murray *et al.* (1982) demonstrated that, in response to *Leishmania* antigen,

T lymphocytes produce lymphokines which can induce infected macrophages to kill the

parasite *in vivo*. They also reported that recovering mice produce macrophage-activating

lymphokines and that recovery and a capacity of spleen cells to respond to mitogenic

stimulation are correlated.

IFN-γ is a principal activating factor for macrophages in leishmaniasis and

antibodies against IFN-γ abrogate this effect (Murray *et al.* 1983). IFN-γ is not,

however, the only lymphokine which can activate macrophages and it has been suggested

(Heinzel *et al.* 1989) that GM-CSF and IL-4 may also have a role to play in the control

of kala azar in patients. Many T cell functions are, however, impaired during experimental infections with *L. donovani:* for example, the capacity of C57BL/6 spleen cells to produce IL-2 following stimulation with the mitogen PHA is suppressed (Olivier and Tanner, 1989). The production of IFN-γ is also suppressed by the infection and restoration of the secretion of IL-2 and IFN-γ in response to *Leishmania* antigen coincides with the onset of a control of the parasite (Murray *et al.* 1987). Treatment of infected mice with human rIL-2, however, had no effect to control the infection, whereas murine rIFN-γ completely stopped multiplication of parasite. IL-2 production by human leukocytes is also defective in visceral clinical leishmaniasis (Carvalho *et al.* 1989) and Barral-Netto *et al.* (1991) have shown that the impairment of IL-2 production can be mediated by the serum of animals infected by *Leishmania*. Progress of the infection, therefore, may depend on the ability of the parasite to suppress the secretion of mediator cytokines which stimulate important cellular mechanisms.

IFN-γ, administered to susceptible mice after inoculation of the parasite, did not modify the progress of the infection, suggesting that factors other than IFN-γ may influence cell expansion in cutaneous leishmaniasis (Sadick *et al.* 1990; Scott, 1991). One such factor could be the T cell growth factor IL-12 which (Brunda, 1994) induces IFN-γ production (Nakamura *et al.* 1989; D'Andrea *et al*, 1992; Perussia *et al.* 1992), and plays an important role in the initiation a of Th1 response for resolving of experimental cutaneous leishmaniasis (Heinzel *et al.* 1993; Sypek *et al.* 1993). IL-12 is a pluripotent cytokine that interacts with NK and T cells to play a role in the initiation and maintenance of Th1 responses and IFN-γ production in infections with *L. major* (Ghalib

et al. 1995). These authors also reported that this lymphokine may also play an important role in the regulation of cellular immune responses in human visceral leishmaniasis.

Treatment of susceptible BALB/c mice with rIL-12 during the first week of infection with *L. major* resulted in the clearance of the parasite; this reversal of the susceptible phenotype was attributed to a shift in the T cell response from Th2 to Th1. IL-12 treatment also resulted in a marked depression of IL-4 production and in an increase in IFN-γ. A role for IL-12 in determining the outcome of a cutaneous infection has been proposed because treatment of resistant C57BL/6 mice with anti-IL-12 resulted in exacerbating *L. major* disease, shifting the responding T cells to Th2 response (Sypek *et al.* 1993). Treatment of macrophages from resistant mice with LPS produced a 40 fold higher IL-12 level than in similarly-treated macrophages of susceptible mice (Heinzel *et al.* 1993), implying that IL-12 plays a principal role in initiating the Th1 cell response and in resolving experimental cutaneous leishmaniasis. IL-12, by promoting the development of leishmanial specific Th1 cells, is an effective adjuvant for protective cell mediated immunity against *L. major* infection (Afonso *et al.* 1994). The participation of different CD4$^+$ T cell subclasses in a protective response in leishmaniasis has been eloquently demonstrated using SCID mice in *L. major* infections (Holaday et al. 1991). In these mice, that congenitally lack CD8$^+$ T and B cells, the transplantation of a Th1-like cell line resulted in the healing of cutaneous lesions after the inoculation of the parasite. On the other hand, transplantation of a Th2-like cell line resulted in the exacerbation of the lesion after *L. major* challenge; in each case, the donor cells were found at the site of the infection and maintained their cytokine phenotype. The

production of a Th1 *vs.* a Th2 response may depend, however, on the site of inoculation of the parasite challenge. Nabors et al. (1995b) report that in the intermediate susceptibility mouse phenotype (BALB/c X C57BL/6 F1) a protective Th1 response was produced when these animals were inoculated into the footpad, whereas an infection-potentiating Th2 response was obtained when these animals were inoculated in the dorsal skin. These authors reported further that IL-4 and IFN-γ production by these animals was the same regardless of the site of inoculation of the parasite challenge, suggesting that the production of these lymphokines might be influenced by factors other than the immune response generated.

The resolution of infection by *L. donovani* correlates closely with the capacity of splenic T cells to secrete macrophage-activating lymphokines (Murray, 1982). This fact is relevant to an anti-leishmanial host defense mechanism; however, *in vitro* and *in vivo* activation by stimulated lymphocytes is required before mouse peritoneal macrophages or human mononuclear phagocytes gain an ability to kill, or to inhibit replication of intracellular amastigotes (Haidaris and Bonventre, 1981; Hoover *et al.* 1985). Depletion of IFN-γ or IL-2 exacerbates systemic infection with *L. donovani* (Squires *et al.* 1989) and Murray *et al.* (1987) have shown that susceptible BALB/c mice acquire a resistance to *L. donovani* after treatment with IFN-γ; IFN-γ appears to be particularly important (Sadik *et al.* 1986) in some (Murray *et al.* 1984) but not all (Sypek *et al.* 1984) models of experimental leishmaniasis. Greater tissue parasite burdens should be anticipated, however, in infected patients in whom lymphokine production is impaired or absent because of iatrogenic immunosuppression, or disorders such as AIDS (Male *et al.* 1985).

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To determine, nevertheless, if IFN-γ could enhance the effect of conventional chemotherapy against *L. donovani*, Murray, *et al.* (1988) treated infected human macrophages *in vitro* with rIFN-γ and Pentostam, finding the drug's action was significantly enhanced by the immunological treatment. These authors have suggested that treatment with this lymphokine may be one method by which drug's efficacy can be enhanced and its toxicity reduced. It has been shown that combination therapy using the immunopotentiator compound 86/450 with sodium stibogluconate yielded better results than when drug treatment was administered alone (Zehra *et al.* 1995). Other studies (Murray *et al.* 1991) have shown that the treatment of *L. donovani*-infected mice with monoclonal antibodies to IFN-γ and IL-2 did not diminish a curative effect of Pentostam, suggesting that these lymphokines may not participate in the curative action of the drug. In *L. major* experimental cutaneous leishmaniasis, Nabors et al. (1995) have reported that treatment with IL-12, in combination with Pentostam, switched the T helper cell response of infected mice to a protecting Th1 response from a Th2 response. This change led to a healing of the infection, suggesting that the administration of IL-12 may be important in inducing a cure in nonhealing forms of human leishmaniasis.

c. Humoral Factors

The promastigotes of many *Leishmania* species are susceptible to being killed by the complement membrane attack complex (C5b-C9) generated by antibodies (Wozencraft *et al.* 1989). The lethal effects of complement on the amastigote vary with the *Leishmania* species: *L. donovani* amastigotes are relatively resistant to killing by

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complement, whereas *L. major* amastigotes are killed rapidly after activation of the alternate pathway. The mechanisms by which complement is activated have been extensively reviewed by Wozencraft *et al* (1994) and the general idea is that all *Leishmania* species activate complement through the alternate pathway. Stationary growth-phase promastigotes are ,however, more resistant to complement-mediated killing than those in the logarithmic-phase. Fixation of complement on the parasite surface results either in the death of the organism, if the membrane attack complex is functional, or in the opsonization of the protozoan by C3b and C3bi, if it is not functional. Complement opsonization produces the chemotactic attraction of the macrophage to the parasite and facilitates internalization of the infecting promastigote into the cell. However, if complement-opsonised promastigotes are ingested by polymorphonuclear leucocytes, the internalized parasites are killed by the cell. The relative resistance of *L. donovani* amastigotes to the action of complement probably contributes to spreading the organism throughout the reticuloendothelial system, whereas the susceptibility of *L. major* may prevent or limit dissemination (Wozencraft *et al.* 1988).

Markedly elevated immunoglobulins levels result from the polyclonal activation of B-cells and occur in humans and animals with kala azar; some of the antibodies produced in visceral leishmaniasis are, however, specific to the parasite, permitting the serological diagnosis of the infection (Allain and Kagan, 1975). Antileishmanial antibodies are also present in the various forms of cutaneous leishmaniasis, but their titers are not nearly as elevated as they are in kala azar (Wilson and Pearson, 1990). Attempts to provide protection with immune serum have failed in several animal systems (Liew *et al.* 1985);

the finding (Olivier and Tanner, 1989) that infected resistant mice produce less antibody than infected susceptible animals adds support to a belief that immunoglobulins play no role to control kala azar.

d. Activation of Macrophages

Human and murine macrophages can be activated to kill the *Leishmania* parasite *in vitro* by exposure to lymphokine-rich preparations, such as the supernatants of mitogen or antigen-stimulated T cell cultures. Interferon-gamma may be one of the activating principles (Murray *et al*, 1985) in these supernatants and the *in vivo* effect of this stimulus can be enhanced by trace amounts of bacterial lipopolysaccharide (Mauel and Buchmuler-Rouiller, 1987). In mice, depending on their genetic constitution, infection by *L. major* can result either in a self-resolving disease or in a progressive infection, and it has been suggested that the T-cell (CD4$^+$; Th1 and Th2) subsets produced in each instance are different (Liew, 1989). These two subsets of Th do not, however, play any role in the control of visceral leishmaniasis (Kaye *et al*, 1991).

Lymphokines distinct from IFN-γ have also been reported to activate macrophages for leishmania killing, GM-CSF being active in this respect (Hoover *et al*. 1986; Weiser *et al 1987*). GM-CSF induces three effects which are potentially beneficial in visceral leishmaniasis: blood monocyte mobilization, macrophage activation and amelioration of granulocytopenia. It was also shown by Murray *et al*. (1995) that GM-CSF has a role in the initial host defense response to *L. donovani* and they have indicated that GM-CSF can

be used as an antileishmanial treatment.

Parasitized mouse macrophages can, furthermore, be activated *in vitro* by lymphoid cells from recovering animals (Pham and Mauel, 1987; Pham *et al.* 1988); this activation is presumed to be induced by the interaction of lymphocytes with the parasite epitope presented on the surface of infected macrophages, although the ability of the infected macrophage to present antigen is significantly down-regulated (Rodriguez *et al.* 1992). A novel mechanism of activation has also been described that is independent of soluble mediators, but requires an intimate contact between lymphocytes and macrophages (Wyler *et al.* 1987). In addition, macrophage precursor cells devoid of phagocytic activity have been shown to kill *Leishmania* promastigotes and extracellular amastigotes by a mechanism that remains to be established (Baccarini *et al.* 1988). It has been reported that infection of macrophages both *in vivo* and *in vitro* by *L. donovani* fails to trigger expression of the costimulatory molecules B7-1 and the heat-stable antigen on APC, inhibiting the macrophage response to normal regulatory signals, such as LPS (Kaye *et al.* 1994).

GENETIC CONTROL OF INFECTION BY L. DONOVANI

Stauber (1958), in quantitive studies of the changes in the amastigote burden in the liver of outbred mice after the intravenous inoculation of *L.donovani*, found a rise in parasite numbers, followed by a fall and then a prolonged plateau phase. Subsequent studies have shown inter-strain variation in mice, but variance within any one inbred mouse strain is slight (Bradley and Kirkley, 1977). Mice of seven inbred strains studied

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by Bradley *et al.* (1979) clearly fell into two types of innate phases on the basis of the numbers of parasites in the liver at the end of the 1st or 2nd week following infection. Among those mouse strains in which the parasite proliferated rapidly during the innate phase, the subsequent course of the infection varied: some animals recovered completely and others had a continually-increasing amastigote load resembling that which occurs in hamsters (Bradley and Kirkley, 1977). In the second, subsequent chronic, or recovery phase some mice exhibited the disease for 2 years (Bradley *et al.* 1973), while some mice died of the infection earlier (Smrkovski *et al.* 1974).

Inbred strains of mice are now extensively used for studies of experimental visceral leishmaniasis, especially since Bradley (1977) demonstrated that the number of infected macrophages in the liver at day 15 post-infection clearly separated these animals into innately-resistant and innately-susceptible strains. Innate resistance against *L. donovani* and *L. tropica* in inbred mice are both determined genetically, but the genes and the mechanisms regulating the resistance are different for each of these two species of parasite (Cox, 1981). Two stages characterize the mechanism of genetic resistance in kala azar: the primary, or innate phase of susceptibility is related to the capacity of macrophages to support or inhibit infection by the parasite (Bradley *et al.* 1979), and a chronic phase which is controlled by immune responses in the mice against the parasite (Mauel and Behin, 1982).

Innate susceptibility to *L. donovani* is determined by the *Lsh* gene (Bradley, 1977; Bradley and Kirkley, 1977; Blackwell, 1983) on mouse chromosome 1. The *Lsh* gene is identical to the gene which also controls innate resistance to *S. typhimurium* (Plant *et al.*

1982), *M. bovis* (Skamene *et al.* 1982) and *M.lepraemurium* (Skamene *et al.* 1984).

Surprisingly, however, this gene does not influence innate resistance to *L. major*, or to *L. mexicana*. Control of the immunological effector arm of resistance to *L. donovani* in the chronic phase of the infection, however, appears to map at positions within, or closely adjacent to the histocompatibility locus H-2 (Crocker *et al.* 1984a; Blackwell, 1982), as well as to non-H-2 (Blackwell *et al.* 1983) genes. Interestingly, however, an H-ll-linked gene has also been suggested as exercising parallel effects on immune resistance both to *L. major* and *L.donovani* (Blackwell *et al.* 1985a).

The product of *Lsh=Bcg=Ity* gene has been designated *Nramp* (Vidal *et al.* 1993) and the $Lsh^r = Bcg^r$ product of the gene is distinguished from the $Lsh^s=Bcg^s$ product by the single non-conservative amino acid substitution of glycine for asparagine in position 169 (Vidal *et al.* 1995). In cutaneous leishmaniasis, the distinction between innate and acquired resistance is less clear, but the susceptibility of inbred mice to infection by *L. major* is controlled by the SCL-1, SCL-2 loci on chromosome 8 (Blackwell *et al.* 1984) and by H-ll-linked genes (Blackwell *et al.* 1985c); the involvement of H-2-linked genes is relatively minor in this infection. The product of the *Lsh* gene in visceral leishmaniasis determines the ability of Kupffer cells to eliminate intracellular *L. donovani* (Crocker *et al.* 1987). and, in addition, the macrophages of the resistant strain are able to respond better by reducing their intracellular burden of the parasite following activation by Con-A (Olivier *et al.* 1989). Susceptibility/resistance to *L. donovani* can be adoptively transferred by bone marrow cells in H-2-histocompatible radiation chimeras (Olivier and Tanner, 1987); the susceptibility or resistance phenotype of macrophages to infection by

this parasite is only expressed by Kupffer cells (Olivier and Tanner, 1987).

In certain susceptible strains of mice, the visceral infection is prolonged, whereas in others the infection is reduced progressively; such late healing appears to be controlled by a locus close to the major histocompatibility (H-2) complex (Blackwell *et al*. 1980), and by genes associated with the minor histocompatibility loci H-ll and Ir-2 (Detolla *et al*. 1980). Depending on their H-2 haplotype, different strains of mice can be separated into 3 distinct phenotypic types (Blackwell *et al*. 1980; Blackwell, 1983): non-cure (H-2d, H-2q and H-2f), slow-cure (H-2b) and early-cure (H-2s, H-2r).

The mechanism by which resistance is produced against infection by *L. donovani* is not yet well defined. Crocker *et al*. (1984) and Olivier and Tanner (1987) have clearly demonstrated the importance of liver macrophages in the control of the innate phase of visceral leishmaniasis, being the only population of macrophages that expresses it's innate phenotype when infected *in vitro* by promastigotes. Crocker *et al*. (1987) corroborated this finding using amastigotes to infect different macrophage populations and were able to demonstrate expression of the *Lsh* phenotype also by bone marrow and lung macrophages. Innate resistance to *L. donovani* and *S. typhimurium* (Blackwell *et al*. 1983; Crocker *et al*. 1984b) can be transferred to susceptible mice by bone marrow cells; the "adoptive" phenotype, however, will only be expressed by macrophages in the liver (Olivier and Tanner, 1987).

TREATMENT IN VISCERAL LEISHMANIASIS

Some of the evidence presented in this review of the literature shows that exogenous lymphokines can cure macrophages infected by *L. donovani in vitro* (Chang and Chiao, 1981; Haidaris and Bonventre, 1981; Nacy *et al*. 1981; Olivier *et al*. 1989) and that soluble immune mediators can reduce infections *in vivo* (Reed *et al*. 1984; Barral-Netto *et al*. 1991). The use of IL-2 for the treatment of intracellular parasites has also given encouraging results in controlling experimental American trypanosomiasis and toxoplasmosis (Choromanski and Kuhn, 1984; Tarlerton and Kuhn, 1984; Sharma *et al*. 1985) and new approaches for adoptive immunotherapy have also clearly demonstrated their effectiveness in curing some tumours (Male *et al*. 1985; Rosenberg *et al*. 1986).

Clinical visceral leishmaniasis is treated effectively by drugs, especially if the therapy is begun early in the course of the infection. There are four drugs which are currently used against *L. donovani* in humans: first-line compounds are the two pentavalent antimonials sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime); if these drugs are not effective, a second line of treatment by pentamidine or stilbamidine and amphotericin B are used. Conventional chemotherapy of visceral leishmaniasis with the pentavalent antimonials is usually highly effective, but unpleasant side effects such as coughing, severe headache and vomiting, sometimes makes the therapy difficult for the patient to support.

The largest number of *in vivo* studies of the effectiveness of chemotherapy in leishmaniasis have been carried out in infections by *L. donovani*. Originally, the

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recommended route of inoculation of the parasite for drug studies was intrasplenic or intraperitoneal, but the intracardiac route pioneered by Stauber and his colleagues (1967) has been used in subsequent investigations in mice (Handson et al. 1977). The fact that Bradley and his colleagues (Bradley, 1977) have shown that inbred mouse strains differ in their susceptibility to infection following the inoculation of either promastigotes or amastigotes has underscored the value of these animals for chemotherapeutic studies (Black et al. 1977; Trotter et al. 1980). Promastigotes, since they can be cultured in vitro are the most convenient form of the parasite for producing an infection for laboratory study. These forms are, however, most infective only up to the 4th or 5th serial passage in culture (Wonde and Honigberg, 1971), and only at the end of the promastigote's logarithmic growth phase (Keithley and Bienen, 1981) of each passage, infectivity being correlated with the development of metacyclic promastigotes (Sacks and Perkins, 1984). The toxic and curative effects of sodium stibogluconate (Pentostam) have been assessed in hamsters infected with L. donovani (Al-Khateeb and Molan, 1981), but no correlation has been found between the intensity of the infection and the treatment regimen; it is (Al-Khateeb and Molan, 1981) well known, however, that some leishmanial infections are resistant to chemotherapy. Murray et al. (1989) have shown, furthermore, that the in vivo efficacy of chemotherapy with pentavalent antimonials may be T-cell dependent since reconstitution of T cells readily restored responsiveness to Pentostam in nude mice; responsiveness could also be restored in part by the simultaneous adminstration of the drug and IL-2 or IFN-γ. These results might suggest why immunosuppressed, or T-cell - deficient patients with visceral leishmaniasis sometimes fail to respond, or relapse

quickly, despite ordinarily effective treatment with Pentostam (Fernandez-Guerrero *et al.* 1987; Berenguer *et al.* 1987; Alvar *et al.* 1989; Montalban *et al.* 1989). The question of the fate of amastigotes following treatment has also not been adequately resolved: in an *in vitro* model, Mauel *et al.* (1987) have reported that the death of amastigotes in immunologically-activated macrophages was not followed by their immediate disappearance from the cytoplasm of the cell.

Despite the large advances that have been made in understanding the factors that regulate the host-parasite association in leishmaniasis, many of the details of this association remain unclear; the application of the knowledge that has been obtained over the years to the serious problem of controlling the visceral disease also still requires considerable study. The investigation that will be described in this thesis will reveal some new insights into the details of the intimate relationship between *L. donovani* and its macrophage host in experimental visceral leishmaniasis. This thesis also describes how, despite the profound down regulation that *L. donovani* induces in its host, leishmanicidal activity can be reactivated in the infected cell by immuno- and/or chemotherapy. It is hoped that the results of this investigation will shorten the time required to bring the scourge of visceral leishmaniasis under effective control.

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RATIONALE FOR THE RESEARCH

1. The entrance of *Leishmania donovani* into macrophages occurs after the attachment of the flagellated promastigote to the cell membrane of its host and the subsequent internalization of the organism. The aflagellated promastigote that develops in the macrophage proliferates in the phagolysosome, apparently unaffected by the harsh physiological conditions in the organelle. This parasite induces anergy in the immunological functions of the infected cell, in infected laboratory animals and in humans.

2. Most investigators have studied the proliferation of *L. donovani* only from the 3rd or 4th day after the parasite has entered its host cell and, thus, the dynamics of the very early phase of the macrophage-parasite interaction was known only incompletely. It was, therefore, of interest to determine in the early stages of this study the relationship between the size of an inoculum of promastigotes and the progression of the intensity of infection in by amastigotes in individual cells and in the proportion of infected resting peritoneal macrophages. These and subsequent studies were done in macrophage monolayers *in vitro* to reduce any regulating effects which might be contributed by immunological reactions *in vivo*.

3. Infected macrophages can be activated *in vitro* to kill their intracellular parasite burden of *L. donovani* and rescued from parasite-induced anergy by treatment

with native lymphokines, or with splenic leukocytes previously stimulated *in vitro* by the lymphokine IL-2. It was of interest to study the nature of the kinetics of activation of the intracellular leishmanicidal activity produced in the infected macrophages following activation by the lymphokine-stimulated cells and to determine if a favoured time exists during the course of the infection when the parasite would be more susceptible to the curative action of the IL-2-stimulated splenocytes.

4. Pentostam is the traditional drug of choice for the therapy of clinical visceral leishmaniasis and it was of interest to compare the drug's effectiveness with that of immunotherapy to reduce experimental visceral leishmaniasis *in vitro*. It was also an interest of this study to determine, not only when during the course of an infection *in vitro* would Pentostam be most effective against the intracellular parasite but also if a combined drug and immunotherapy treatment would enhance a curative action in experimental visceral leishmaniasis.

5. The fact that *L. donovani* proliferates in macrophages clearly indicates that the well-known intracellular microbicidal pathways of these phagocytic cells are not activated during the course of the infection. It was of interest to establish that the success of the parasite in colonizing macrophages depends on the fact that leishmanicidal oxygen and nitrogen radicals are not produced in macrophages infected by *L. donovani*. It was also of great interest to determine if a reactivated production of these microbicidal inorganic radicals following immuno- and/or chemotherapy could account for the cure obtained in

infected macrophages following treatment.

6. It was hoped that this study would clarify the immunobiology of the host-parasite relationship in experimental visceral leishmaniasis and suggest effective strategies for the control of infections by this obligate intramacrophage parasite.

Chapter II

TIME COURSE AND INTENSITY OF INFECTION *IN VITRO* IN THE RESIDENT PERITONEAL MACROPHAGES OF RESISTANT AND SUSCEPTIBLE MICE EXPOSED TO DIFFERENT DOSES OF *LEISHMANIA DONOVANI*

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Running Title: Experimental kala azar *in vitro* in peritoneal macrophages

Abstract - Eslami, Z. and Tanner C.E. 1994. Time course and intensity of infection *in vitro* in the resident peritoneal macrophages of resistant and susceptible mice exposed to different doses of *Leishmania donovani* promastigotes. *International Journal for Parasitology* 24:743-747. A study was made of the proliferation dynamics *in vitro* of *Leishmania donovani* amastigotes in the resting peritoneal macrophages of C57BL/6 (*Lsh^r*) and C57L/J (*Lsh^s*) mice. Monolayers were inoculated with 5, 50 or 500 promastigotes per macrophage and the number of infected cells and the number of parasites per cell were determined 1, 3, 5, 7 and 14 days following inoculation. Results indicate that, irrespective of the phenotype of the donor mouse and of the inoculum, only 50 to 65% of the cells became infected initially. Expansion of the infection proceded more rapidly in monolayers of *Lsh^s* cells and may have involved the "recruitment" of non-susceptible macrophages, perhaps by the action of a soluble factor. Also irrespective of the inoculum and phenotype, only 3 to 6 amastigotes were present in each macrophage initially, suggesting a limited number of ligands for the attachment of the parasite to the cell. Amastigotes did not proliferate for 3 to 4 days and then divided actively until day 7 when more parasites were present in the macrophages of the susceptible phenotype. Differential expansion of the infection and the proliferation of amastigotes *in vitro* suggest that resting peritoneal macrophages may, indeed, express the *Lsh* gene.

Index Key Words: *Leishmania donovani*; *Lsh* phenotype; proliferation *in vitro*; "recruitment"; ligands for attachment.

INTRODUCTION

Leishmania donovani, the agent of disseminated visceral leishmaniasis, is a well-known vector-transmitted obligate intramacrophage protozoan parasite of vertebrates. Infection *in vivo* by this parasite is characterized by two distinct phases: an innate phase where the intensity of the parasite burden depends entirely on the expression of the *Lsh* gene by the host macrophage (Skamene, Gros, Forget, Kongshavn, St-Charles & Taylor, 1982) and a proliferative phase which appears (Blackwell, Freeman & Bradley, 1980) to be under the influence of the immune response of the infected animal. However, the mechanism by which this control is excercised on the development of the infection in humans (Manson-Bahr, 1961) and in laboratory mice (Olivier & Tanner, 1989) is difficult to fully understand because of the paradoxical fact that individuals parasitised by *L. donovani* are profoundly immunosuppressed and their macrophages are unable to activate the protective respiratory burst (Buchmuller-Rouiller & Mauel, 1987). As a further example of down-regulation, although antibodies are generally elevated (Bray & Lainson, 1967) in experimental kala azar, the ability of infected macrophages to express class II MHC determinants is suppressed (Reiner, Ng & McMaster, 1987). These cells don't produce IL-1 (Olivier & Tanner, 1989) and the lymphocytes of infected animals, which are never infected by the organism, do not produce normal quantities of IL-2 (Reiner & Finke, 1983).

The entrance of the organism into its macrophage habitat depends on the attachment of the promastigote to receptors on the surface of the cell and its subsequent internalization by phagocytosis (Chang, 1979). The amastigotes then proliferate in the

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phagolysosome of the macrophage, apparently unaffected by the harsh conditions in the organelle (Mukkaka, Meade, Glaser & Bonventre, 1985). Crocker, Davis & Blackwell (1987) have studied the progress of the infection *in vitro* in amastigote-inoculated monolayers of macrophages of *Lsh* and *Lsh'* mice isolated from different tissues and have confirmed (Olivier & Tanner, 1987) that the *Lsh* gene may be expressed only by liver macrophages. It was of interest to re-examine the relationship *in vitro* between *L. donovani* and resident peritoneal macrophages to determine if the resistant and susceptible phenotype of the host cell is expressed equally by cells exposed to different inoculating doses of the parasite. Monolayers of resident peritoneal macrophages of resistant and susceptible mice were exposed to different inocula of the parasite; the percentage of parasitised macrophages and the numbers of amastigotes within each cell were determined for 14 days in culture. The results of this study confirm that not all resident peritoneal macrophages are susceptible initially to infection, that macrophages may have a limited number of receptors for the attachment of the parasite and that amastigotes begin to proliferate only after an initial resting stage of 3 to 4 days.

MATERIALS AND METHODS

Animals: Two to 4 week-old female resistant C57L/J (*Lsh'*) mice were obtained from The Jackson Laboratory (Bar Harbor, Maine), or from the breeding facility of the Institute of Parasitology. Two to 4 week-old female susceptible C57BL/6 (*Lsh'*) mice and Syrian hamsters (Lak:Lvc) used for the maintenance of the parasite were purchased from the Charles River Laboratories (St-Constant, Quebec).

Parasite: The Ethiopian LV9 strain of *Leishmania donovani* was maintained in the laboratory by the periodic passage of 10^7 amastigotes in hamsters. The amastigotes, obtained from the liver of infected animals using a sterile Ten-Broeck homogenizer and counted using a haemacytometer, were transformed into promastigotes in RPMI 1640 (ICN Biomedicals, Mississauga, Ontario), supplemented with 10% foetal bovine serum (ICN) and 100 ug streptomycin (Sigma, St. Louis, Missouri) and 100 U penicillin (Sigma) per ml; the complete, supplemented medium will be referred to hereafter as RPMI. The promastigotes were kept at room temperature in RPMI and transfered weekly; those used for the inoculation of macrophages were from day 8 cultures of the second passage in culture (stationary phase metacyclic forms). The promastigotes to be used for inoculation were centrifuged from the culture medium at 650 x g for 10 min at 4°C, suspended in fresh medium, counted using a haemacytometer and adjusted to the appropriate concentration in RPMI.

Isolation of macrophages: The peritoneal cavity of 15 unstimulated susceptible C57BL/6 or 15 unstimulated resistant C57L/J mice was each lavaged with 5 ml of ice-cold RPMI and the lavage fluid from each strain was pooled. The resident peritoneal cells in each pool were washed 2x by centrifugation at 250 x g for 10 min at 4°C, counted with a haemacytometer in the presence of trypan blue and made up to 10^7 viable cells/ml in the RPMI medium; 100 ul of this cell suspension, placed on a sterile 18 mm glass cover slip yielded 1 x 10^4 adhered macrophages after 24 hrs incubation at 37°C in humidified 5% CO_2. Twenty-five cover slips, each bearing 10^4 adhered peritoneal macrophages of the C57BL/6 (*Lsh*^s) or the C57L/J (*Lsh*^r) strains, were inoculated in

separate chambers of 6-well Linbro culture plates (ICN) with 5 x 10⁴ promastigotes (5 parasites/macrophage); another 25 cover slips of each mouse strain were inoculated with 5 x 10⁵ promastigotes (50 parasites/macrophage) and another 25 cover slips of each strain were inoculated with 5 x 10⁶ promastigotes (500 parasites/macrophage). The cover slips were then incubated for 2 hrs at 37°C in humidified 5% CO_2 to permit infection of the cells; each cover slip was then washed carefully 2x with fresh RPMI to remove the non-adherent cells and the non-internalized parasites. After the addition of 100 μl of fresh RPMI to each cover slip, they were returned to the incubator. The percentage of infected macrophages and the numbers of amastigotes per macrophage were determined on five cover slips for each strain of mouse and for each dose of promastigotes on days 1, 3, 5, 7 and 14 following inoculation of the parasite. After harvesting, the cover slips were fixed in methanol and stained with Giemsa; the numbers of infected macrophages and the number of amastigotes in each infected cell were determined.

RESULTS

Time course of the infection in vitro of resident peritoneal macrophages inoculated with different doses of promastigotes.

The results of this investigation, illustrated in Fig.1, indicate that, irrespective of the dose of promastigotes used for inoculation and also irrespective of the *Lsh* phenotype of the donors of the cells, only 50 to 65% of the macrophages in the monolayer were infected in the first 24 hrs after exposure of the cells to the parasite. It is also notable that expansion of the infection to uninfected macrophages appeared to

proceed more slowly in the macrophages of the resistant than of the susceptible mouse strain; The percentage of infected cells declined in the first 48 hrs of the infection in the monolayers inoculated with 5 promastigotes/macrophage, especially in those containing cells from the resistant strain; expansion of the infection to uninfected macrophages appeared to proceed more slowly in the cells of the resistant than of the susceptible mouse strain. The macrophages in the monolayers exposed to the larger doses (50 or 500/macrophage) became infected more rapidly, especially those of the susceptible C57BL/6 strain; all the cells of this phenotype were probably infected by day 8, whereas the cells of the resistant C57L/J animals did not all become infected until later. It should also be noted that, especially in the monolayers of C57L/J (*Lsh*) macrophages and in those cultures inoculated with the lower dose of promastigotes, there was a trend which suggests a decline in the number of infected cells in the first two or three days which followed the inoculation.

Proliferative cycle of amastigotes in C57BL/6 and C57L/J resting peritoneal macrophages inoculated with different doses of promastigotes.

The results presented in Fig.2 indicate clearly that the initial density of the infection in each macrophage, irrespective of its *Lsh* phenotype and the size of the inoculum, was 3 to 6 amastigotes; the numbers of amastigotes per macrophage only began to increase approximately 4 days after inoculation, reaching a density of about 12 parasites on day 7; there was a trend thereafter for a decrease in the number of amastigotes in the macrophages. These results also indicate that there was a trend for

Figure 1: Change over 14 days in the percentage of infected cells in C57BL/6 (*Lsh^r*) or C57L/J (*Lsh^s*) resting peritoneal macrophage monolayers inoculated with 5 (●), 50 (○) or 500 (▼) promastigotes per cell.
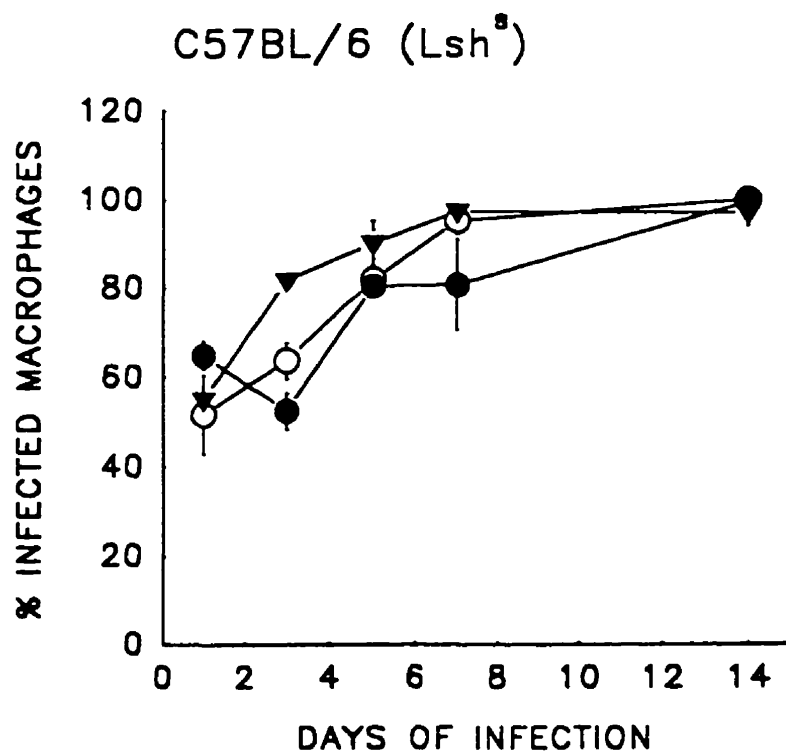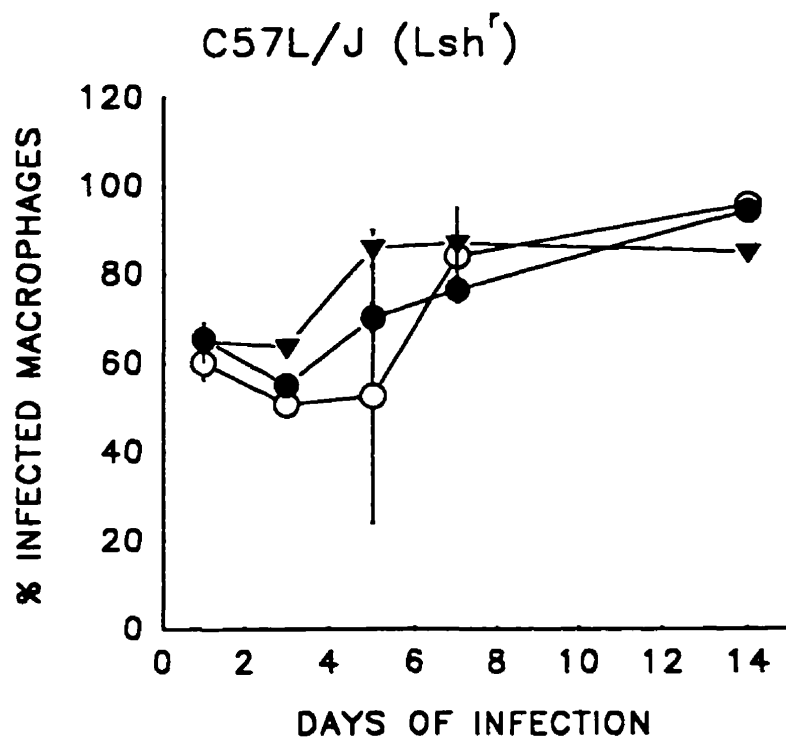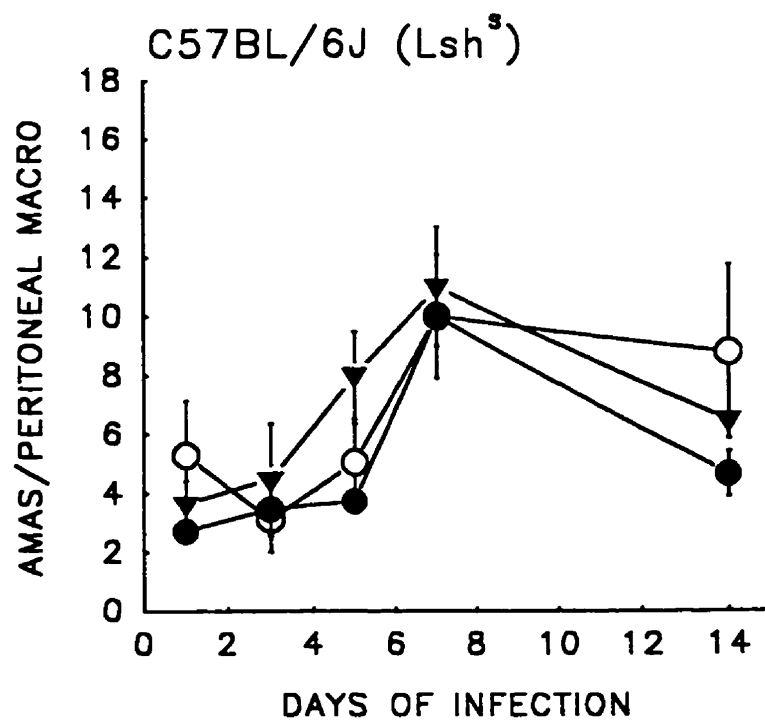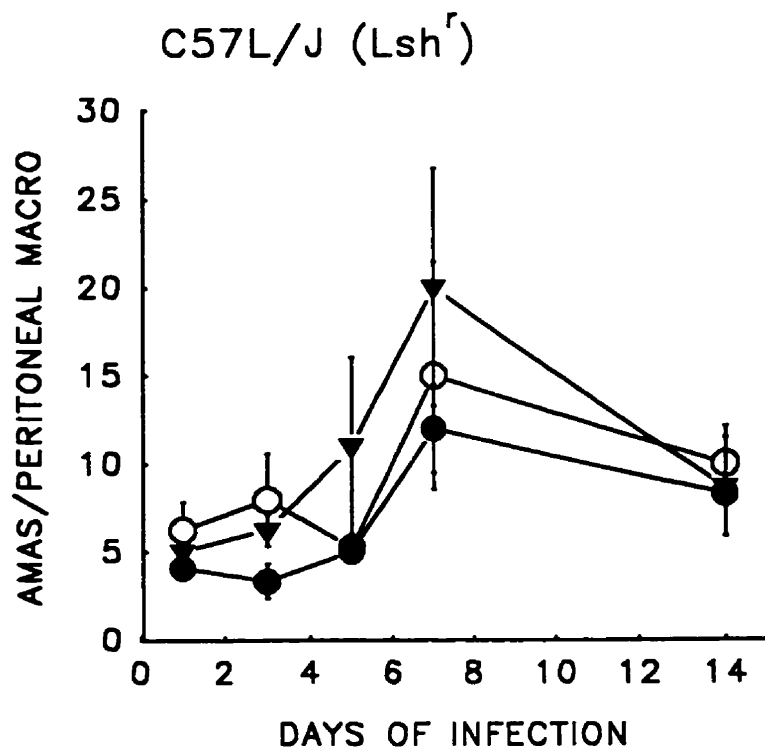
C57L/J (Lsh^r)

% INFECTED MACROPHAGES

DAYS OF INFECTION

C57BL/6 (Lsh^s)

% INFECTED MACROPHAGES

DAYS OF INFECTION

Figure 2: Change over 14 days in the number of amastigotes per macrophage in C57BL/6 (*Lsh*^r) or C57L/J (*Lsh*^s) resting peritoneal macrophage monolayers inoculated with 5 (●), 50 (O) or 500 (▼) promastigotes per cell.

C57L/J (Lsh^r)

C57BL/6J (Lsh^s)

a lower intensity of infection in the cells of the genetically-resistant C57L/J mice. There was no significant decrease in the total number of cells on the cover slips in the first 7 days of culture; on day 14, however, only 20-25% of the initially-adhered cells remained on the cover slips (data not shown).

DISCUSSION

The invasion of macrophages by *L. donovani* requires that the parasite binds to a receptor on the surface of the cell; the nature of this receptor is uncertain, although several candidate molecules have been suggested (Russel & Wilhelm, 1986; Handman & Goding, 1985; (Blackwell, Ezekowitz, Roberts, Channon, Sim & Gordon, 1985; Handman & Goding, 1985; Russel & Wilhelm, 1986). Whatever the nature of this receptor, the evidence that we present that only 55 to 65% of the macrophages in the monolayers of resident peritoneal cells were invaded by the parasite confirm (Crocker *et al.*, 1987) that not all macrophages in the peritoneal cavity are susceptible to infection *in vitro*, suggesting that the appropriate receptor for the parasite may not be expressed initially by all macrophages from this site. Our result that only 3 to 6 promastigotes penetrated into the peritoneal macrophages of C57BL/6 and C57L/J mice, even when the doses of inoculation were in large excess, supports the report of Haidaris & Bonventre (1982) and suggests that the receptor for the parasite is expressed only in a limited number (3 to 6) of copies. Progression of the infection to initially uninvaded cells suggests, in addition, that the infection of previously unpenetrated cells involved a "recruitment" into the susceptible pool of cells, perhaps due to the action of the soluble

factor which is produced by *in vivo* (Evans, Smith & Pearson, 1990) and *in vitro* by infected macrophages (Moore, Turco & Matlashewski, 1994; Fielding and Tanner, unpublished). The evidence that we present here also confirms for mouse peritoneal cells the report of Pearson, Harcus, Roberts & Donowitz (1983) that, after the penetration of human macrophages *in vitro*, *L. donovani* amastigotes do not proliferate (in their study) for 3 days. In this present study the numbers of intracellular parasites remained unchanged for the first 4 to 5 days of the infection. Rapid proliferation occurred thereafter *in vitro* for 2 to 3 days, reaching a peak of about 12 amastigotes per macrophage on the 7th day after inoculation.

In experimental visceral leishmaniasis it is generally-accepted (Olivier & Tanner, 1987; Crocker *et al.*, 1987) that the *Lsh* gene is not expressed by resident peritoneal cells; the results presented here confirm this fact in the sense that there was no significant difference in the numbers of amastigotes in resistant or susceptible peritoneal macrophages 24 hrs after exposure to any of the promastigote inocula. The intensity of the activation of the intramacrophage proliferative phase demonstrates, however, a trend which suggests that the cells of the susceptible strain harboured more parasites on day 7 than the peritoneal cells of the resistant animals. This observation, coupled with the fact that monolayers from susceptible animals became infected more rapidly, confirms the fact that other aspects of the host-parasite association are also reflections of the susceptibility/resistant phenotype of the donor animal. For example, infected resting peritoneal macrophages of resistant mice are more easily cured by Con A-stimulated splenocytes than infected cells of susceptible animals (Olivier, Bertrand & Tanner, 1989).

It is also significant, however, that a proliferative cycle of *L. donovani* occurred *in vitro* in macrophage monolayers, in isolation from specific immunological and non-specific inflammatory controls which are (or should be) abundantly available to regulate the proliferation of the parasite *in vivo*. This identification of the dynamics of the proliferative cycle of *Leishmania donovani* in its macrophage host indicates when and where an attack should be mounted to effect a control *in vitro* of infections with this parasite.

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CONNECTING STATEMENT I

We have found that the course of the intracellular infection of macrophages by *L. donovani* describes a cycle *in vitro* which is characterized by an initial non-proliferative phase, followed by a phase of rapid division. It was of interest to determine if IL-2-stimulated lymphocytes would activate infected macrophages to reduce their burden of *Leishmania donovani*.

Chapter III

IMMUNOTHERAPY WITH IL-2-STIMULATED SPLENOCYTES REDUCES *IN VITRO* THE LEVEL OF *LEISHMANIA DONOVANI* INFECTION INTRAPERITONEAL MACROPHAGES

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Running Title: Immunotherapy in Visceral Leishmaniasis

Abstract - Eslami Z. Olivier M. and Tanner C.E. 1995 **Immunotherapy with IL-2-stimulated splenocytes significantly reduces** *in vitro* **the number of peritoneal macrophages infected by** *Leishmania donovani*. *International Journal for Parasitology* **25**: 975-981. A study was done *in vitro* to determine if IL-2-stimulated lymphocytes (LAK cells) would activate infected macrophages to reduce their burden of *Leishmania donovani*. Macrophage-depleted splenocytes from normal or infected C57BL/6 (H-2^b;Lsh^s) mice, stimulated *in vitro* by the IL-2-containing supernatant of the MLA 144 cell line or by rIL-2, significantly reduced the number of syngeneic resting peritoneal macrophages infected by *L. donovani*; LAK cells from infected animals were significantly more effective in reducing the numbers of infected cells. Supernatants of MLA 144-stimulated spleen cells and rIL-2-stimulated splenocytes isolated in Millipore chambers also induced a significant reduction of the infection *in vitro*. Anti-Thy 1.2 eliminated the ability of the supernatant of MLA 144 to induce an activating function in C57BL/6 splenocytes; monoclonal anti-IL-2 abolished the ability of rIL-2 and of the MLA 144 supernatant to stimulate the splenocytes. Infected resting peritoneal macrophages of C57L (H-2^b;Lsh^s) mice were more responsive to activation than those of the C57BL/6 animals, irrespective of the phenotype of the stimulating LAK cells. Lymphokine stimulation reverses the immunological anergy induced in T lymphocytes by *Leishmania donovani*; IL-2-stimulated LAK splenocytes are highly effective in reducing the intensity of experimental visceral leishmaniasis in vitro in peritoneal macrophages.

INDEX KEY WORDS: Immunotherapy, visceral leishmaniasis, IL-2-stimulated lymphocytes, LAK cells

INTRODUCTION

Leishmania donovani is well-known as an obligate intracellular protozoan parasite of macrophages. To survive within the potentially-harsh environment of the cell, and to cause a persisting infection, the parasite has developed the ability to interfere with various macrophage functions that are crucial to the integrity of the immune system. Important cell defensive functions, such as the production of oxygen radicals, have been shown (Pearson, Wheeler, Harrison & Kay, 1983; Lepay, Nathan, Steinman, Murray & Cohn, 1985) not to occur in infected macrophages; this latter effect is produced by an inhibition of the stimulus-response coupling through calcium- and PKC-dependent pathways (Olivier, Baimbridge & Reiner, 1992; Olivier, Brownsey & Reiner, 1992). The ability of phagocytes to express MHC determinants (Reiner, Ng & McMaster, 1987) and to produce the cytokine IL-1 (Reiner, 1987; Olivier & Tanner, 1989) are significantly reduced in infected macrophages. Despite the fact that they are never colonized by the parasite, lymphocytes are also significantly down-regulated *in vivo* (Reiner & Finke, 1983; Carvalho, Badoro, Reed, Jones & Johnson, 1985; Olivier & Tanner, 1989), producing sub-normal quantities of IL-2; the presence of suppressor T cells (Blackwell & Ulczak, 1984) and of suppressor macrophages (Nickol & Bonventre, 1985) also characterize infection by *L. donovani*.

Notwithstanding this significantly compromised state, it has been shown that leishmanicidal activity can be induced *in vitro* in either resident peritoneal or hepatic

macrophages by treatment with native lymphokines either before or after infection with *L. donovani* (see Chang & Chiao, 1981; Haidaris & Bonventre, 1981; Murray, Masur & Keithley, 1982; Lepay *et al.*, 1985; Murray, Stern, Weltke, Rubin, Carreiro & Nathan, 1987; Olivier, Bertrand & Tanner, 1989). The effectiveness of cytokines in overcoming the anergic state in experimental visceral leishmaniasis suggested that IL-2-stimulated splenic leucocytes (LAK cells) might be effective in reducing the number of infected macrophages in cultures *in vitro*. A similar strategy using LAK cells has been applied to the study of tumor immunotherapy by Mule, Shu & Rosenberg (1985) and by Rosenberg, Speiss & Lafreniere (1986). The present study examined the capacity of macrophage-depleted spleen lymphocytes from infected or from normal C57BL/6 or C57L mice, stimulated *in vitro* by rIL-2, or by the IL-2-containing supernatant of MLA 144 lymphocytes, to reduce *in vitro* the number of peritoneal macrophages infected by *L. donovani*. The data presented here indicates that IL-2-stimulated T cells from infected or normal mice are capable of activating infected macrophages to significantly reduce the intensity of experimental visceral leishmaniasis *in vitro*.

MATERIALS AND METHODS

Animals and the parasite: C57BL/6 (H-2^k;*Lsh^s*) mice 6 to 8 weeks of age and Syrian hamsters (Lak:LVG) were purchased from the Charles River Laboratories (St-Constant, Quebec); 6 to 8 week-old C57L (H-2^k; *Lsh^r*) mice were obtained from the breeding colony of the Institute of Parasitology. The Ethiopian LV9 strain of *Leishmania donovani* was maintained in the laboratory by intraperitoneal passage in hamsters; amastigotes were obtained from the spleen of these animals and promastigotes were prepared from these amastigotes as previously described (Eslami & Tanner, 1994).

Splenocytes: Splenocytes from normal mice, or from C57BL/6 or C57L animals infected intravenously or intracardially 2 weeks previously with 10^7 amastigotes were suspended in RPMI 1640 medium (Flow Laboratories, McLean, VA), supplemented by 10% heat-inactivated foetal bovine serum (Flow), 100 μg/ml streptomycin (Sigma, St. Louis, MO) and 1000 U/ml penicillin (Sigma); the complete, supplemented RPMI 1640 medium will be indicated hereafter as RPMI. Splenocytes were obtained by passing the spleen aseptically through a sterile stainless-steel screen (60 mesh, 80 gauge) into ice-cold RPMI with a glass pestle; erythrocytes in this cell suspension were lysed with Tris-buffered ammonium chloride, or with Gey's solution (Mishel & Shiigi, 1980). Before lymphokine stimulation, all splenocyte preparations were depleted of macrophages in petri dishes by adherence for 2 hrs (or overnight) at 37°C in humidified 5% CO_2 in air. The incubation of all cell preparations in this study was done at 37°C in humidified 5% CO_2 in air. Non-adherent spleen cells for stimulation by the lymphokine were recovered from the adhered cell carpet by washing with warm RPMI; the depleted spleen cell

suspensions were then washed twice with cold RPMI, counted in a haemacytometer and adjusted to 5 x 10^6 cells/ml. The viability of all cell preparations was at least 95% by trypan-blue exclusion.

Stimulation of cells with IL-2: One ml of RPMI containing 2 x 10^6 macrophage-depleted splenocytes from normal or infected mice was seeded into each well of 3.5 cm dia x 1 cm deep 6-well Linbro culture plates (Gibco, Grand Island, NY). One ml of a 1:5 dilution in RPMI of the supernatant of MLA 144 T cells (ATCC TIB 201), which constitutively elaborate IL-2 (Henderson, Hewelson, Hopkins, Sowder, Newbauer & Rabin, 1983), was then added to each well. Alternatively, the same number of macrophage-depleted splenocytes was stimulated under the same conditions with 1 ml RPMI containing 250 U/ml rIL-2 (Cedarlane, Hornby, Ontario). Unstimulated cells were cultured without the supernatant, or the rIL-2; some splenocytes were stimulated by the MLA 144 lymphokine in the presence of 100 μg/ml (by the Lowry assay) of *Leishmania* promastigote soluble antigen obtained by extracting the organism by ultrasounds. The cells were harvested 72 hrs after stimulation with the crude or recombinant lymphokine, washed 3x in RPMI and adjusted to 5 x 10^6 viable cells/ml. To confirm that the activating action of the rIL-2 or of the supernatant of the MLA 144 cell line was due indeed to IL-2, 100 μl of a 1:4 dilution of the monoclonal anti-IL-2 from S4B6 cells (ATCC HB 8794) was added to some splenocyte cultures to neutralize the lymphokine which was added subsequently. It was also of interest to determine if IFNγ was the agent produced by the lymphokine-stimulated splenocytes which activated parasiticidal activity in the infected macrophages. Some stimulated splenocytes were

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suspended in a 1:4 dilution in RPMI of the monoclonal anti-IFNγ from the R4-6A2 cell line (ATCC HB 170) before adding these effector cells to infected target macrophages; this concentration of the monoclonal anti-IFNγ reduced by 50-60% the ability of 10 U/ml of recombinant IFNγ (Genzyme, Boston, MA) to stimulate the production of NO by normal macrophages (Ding, Nathan & Stuehr, 1988).

Depletion of T cells: To reduce the T phenotype in the spleen cell populations, 10^7 macrophage-depleted normal splenocytes were incubated at 4°C for 60 min in RPMI with 1 ml of a 1:10 dilution of anti-Thy 1.2 (Cedarlane). The cells were then washed with RPMI and treated for 60 min at 37°C with 1 ml of a 1:5 dilution of guinea-pig complement (Cedarlane); dead cells were removed by density gradient centrifugation using Lympholyte-M (Cedarlane). The depleted splenocytes were washed twice with RPMI and adjusted to 5 x 10^6 viable cells/ml.

Isolation and infection of macrophages: Resident cells were harvested from the peritoneal cavity of mice by lavage with 5 ml of ice-cold RPMI. Cell numbers were adjusted to contain 1 x 10^7 cells/ml and 100 μl of this cell suspension was plated onto sterile 18 mm glass cover-slips in 5 cm petri dishes, or in 6-well Linbro culture plates (Gibco); the macrophages in the cell suspension on each cover-slip were allowed to adhere for 2 hrs. The cells in each cover-slip culture were then exposed for 4 hrs to 1 x 10^6 promastigotes; non-adherent cells and uninternalised promastigotes were then removed from the cover-slip cultures by washing with warm RPMI.

Immunotherapy: The washed cover-slip monolayers of *L. donovani*-infected adherent cells were cultured in 2 ml of RPMI alone, or were treated on day 3 of the

infection *in vitro* with 2 ml of fresh RPMI containing 5×10^5 macrophage-depleted splenocytes from normal or from *Leishmania*-infected mice stimulated or not stimulated by rIL-2, or were treated on day 1 of the infection with the same number of splenocytes stimulated with the MLA 144 supernatant. Infected macrophage monolayers were also treated with 5×10^5 splenocytes depleted of T cells by anti-Thy 1.2 before lymphokine stimulation (please see above). To determine if the supernatant of MLA 144 stimulated the secretion of macrophage-activating mediators of leishmanicidal activity from normal splenocytes, infected cover-slip monolayers were also treated with 2 ml of the supernatant collected from 5×10^5 normal spleen cells which had been stimulated with MLA 144 in the presence or absence of the promastigote antigen. In addition, some infected macrophage monolayers were treated with 5×10^5 rIL-2-stimulated splenocytes contained within 0.45 μm Millicell-HO culture chambers (Millipore, Mississauga, Ontario). Forty-eight hours after these treatments, the cover-slips were washed, air-dried, fixed in methanol and stained with Giemsa. The percentage of infected cells was assessed in 300-500 macrophages.

Statistical analysis: Statistical analysis of all the results of this study was done by the analysis of variance (ANOVA); the level of significance was taken as $P < 0.05$. Unless otherwise indicated, each of the experiments was replicated at least 5 times.

RESULTS

Reduction of the parasite burden in infected macrophages induced by LAK splenocytes stimulated by MLA 144 supernatants, or by rIL-2 and the effect of the presence of antigen and the depletion of T cells on the stimulation of the spleen cells.

The results shown in Fig. 1A indicate that, when infected resident C57BL/6 peritoneal macrophage monolayers were treated *in vitro* with MLA 144-stimulated LAK splenocytes from normal or from infected syngeneic mice, the percentage of parasitised macrophages in culture declined significantly, especially in those monolayers treated with stimulated spleen cells obtained from infected mice. Macrophage-depleted, rIL-2-stimulated LAK splenocytes from infected C57BL/6 mice also had an identical effect to significantly reduce the number of syngeneic infected macrophages in culture (Fig. 1B). Unstimulated splenocytes from infected or non-infected mice had no effect to decrease the percentage of infected macrophages in culture. The parasiticidal activity induced in the monolayers of infected peritoneal macrophages by spleen cells stimulated by the MLA 144 supernatant was significantly enhanced (Fig. 1A) by the presence of promastigote antigens during stimulation by the lymphokine. MLA 144-stimulated splenocytes from mice which had been inoculated 2 wks earlier with 5×10^6 CFU of *Mycobacterium bovis* BCG also activated infected macrophages to kill the parasite, but no more so than IL-2-stimulated spleen cells from normal C57BL/6 mice (results not shown). Lymphokine-stimulated normal peripheral blood leucocytes were as effective as stimulated normal splenic lymphocytes in reducing the number of infected syngeneic macrophages (results also not shown). Figure 2 shows that, when stimulation of

Fig. 1A: The percent of infected C57BL/6 peritoneal macrophages in monolayers

inoculated with 1 x 10^6 promastigotes 48 hrs after treatment with 5 x 10^6

syngeneic spleen cells from normal mice or from infected mice inoculated

2 weeks previously with 10^7 amastigotes, and stimulated or not with the

IL-2-containing supernatant from the MLA 144 cell line. The infected

macrophages were treated with

    ☐   unstimulated normal splenocytes

    ▨   unstimulated infected splenocytes

    ▨   stimulated normal splenocytes

    ⊞   stimulated infected splenocytes

    ▤   normal splenocytes stimulated in the presence of 100 µg/ml

           promastigote antigen

    ▨   normal splenocytes stimulated in the presence of monoclonal

           anti-IL-2

Columns labeled by the same letter are not significantly (P<0.05)

different from each other, by ANOVA.
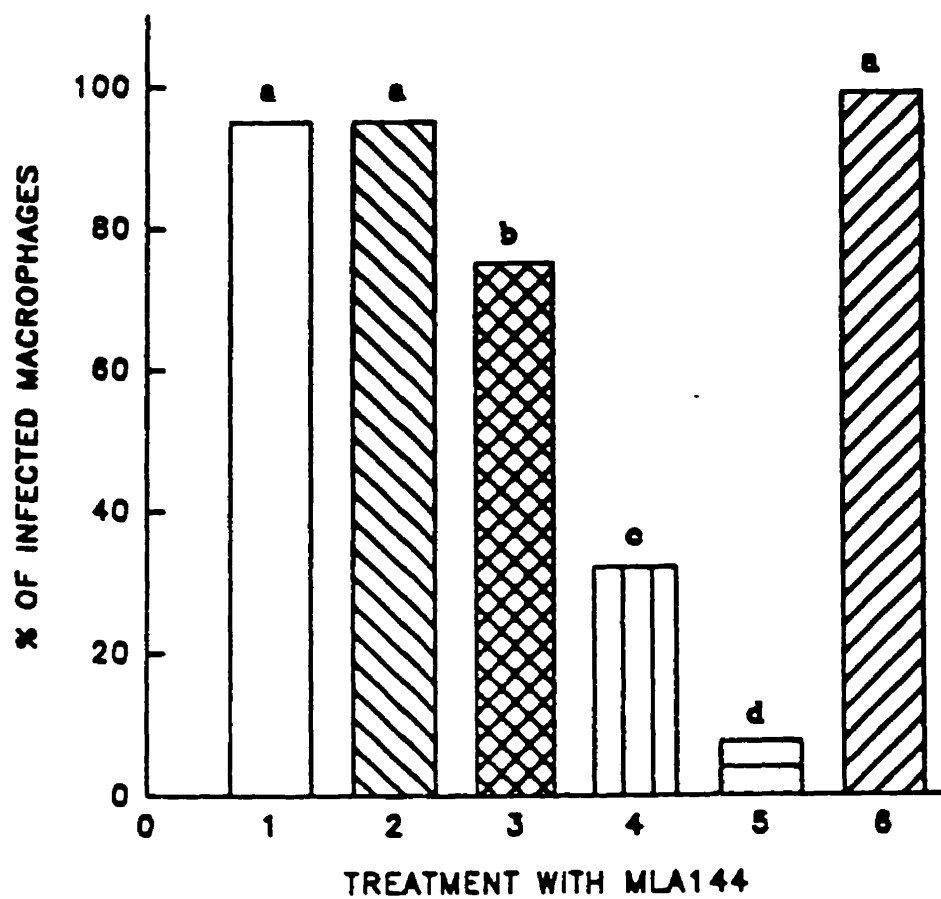
splenocytes by MLA 144 followed the depletion of T cells by anti-Thy 1.2 and complement, these cells did not activate leishmanicidal action in infected macrophages. When exposed to the anti-IL-2 monoclonal S4B6, the supernatant of MLA 144 completely lost its ability to stimulate spleen cells to reduce the number of infected macrophages; the same monoclonal also abolished the lymphocyte-stimulating activity of rIL-2 for splenocytes (Fig. 1B). Figure 2 also shows that the anti-IFNγ monoclonal R4-6A2 did not inhibit the macrophage-activating property of MLA 144-stimulated normal splenocytes, suggesting that IFNγ might not have been the signal from these stimulated splenocytes which induced a decrease in the number of infected macrophages.

The effect of cell-free supernatants of lymphokine-stimulated splenocytes to reduce infection in vitro and the influence of the phenotype of the infected macrophage target and of the lymphokine-stimulated effector lymphocytre in reducing the number of infected cells.

It was of interest to confirm that it was soluble mediators released by lymphokine-stimulated splenocytes which were responsible for the decrease in the number of infected macrophages. The cell-free supernatant of spleen cells stimulated by MLA 144 in the presence or absence of the parasite's antigen did indeed induce a significant reduction in the number of macrophages infected by *L. donovani* amastigotes (Fig. 3). An identical curative effect was obtained in a similar experiment (Fig. 1B) where macrophage-depleted, rIL-2-stimulated splenocytes contained in Millipore chambers were placed in infected macrophage

Fig. 1B: The percent of infected C57BL/6 peritoneal macrophages in monolayers inoculated with 1×10^6 promastigotes 48 hrs after treatment with 5×10^6 syngeneic spleen cells from mice inoculated 2 weeks previously with 10^7 amastigotes, and stimulated or not with 250 U/ml rIL-2. The infected macrophages were treated with

    ▨▨▨▨ unstimulated infected splenocytes

    ▭▭▭▭ rIL-2-stimulated infected splenocytes

    ▮▮▮▮ rIL-2-stimulated splenocytes contained in Millipore chambers

    ▧▧▧▧ infected splenocytes cultured with rIL-2 in the presence of monoclonal anti-IL-2

Columns labeled with the same letter are not significantly (P<0.05) different from each other, by ANOVA.

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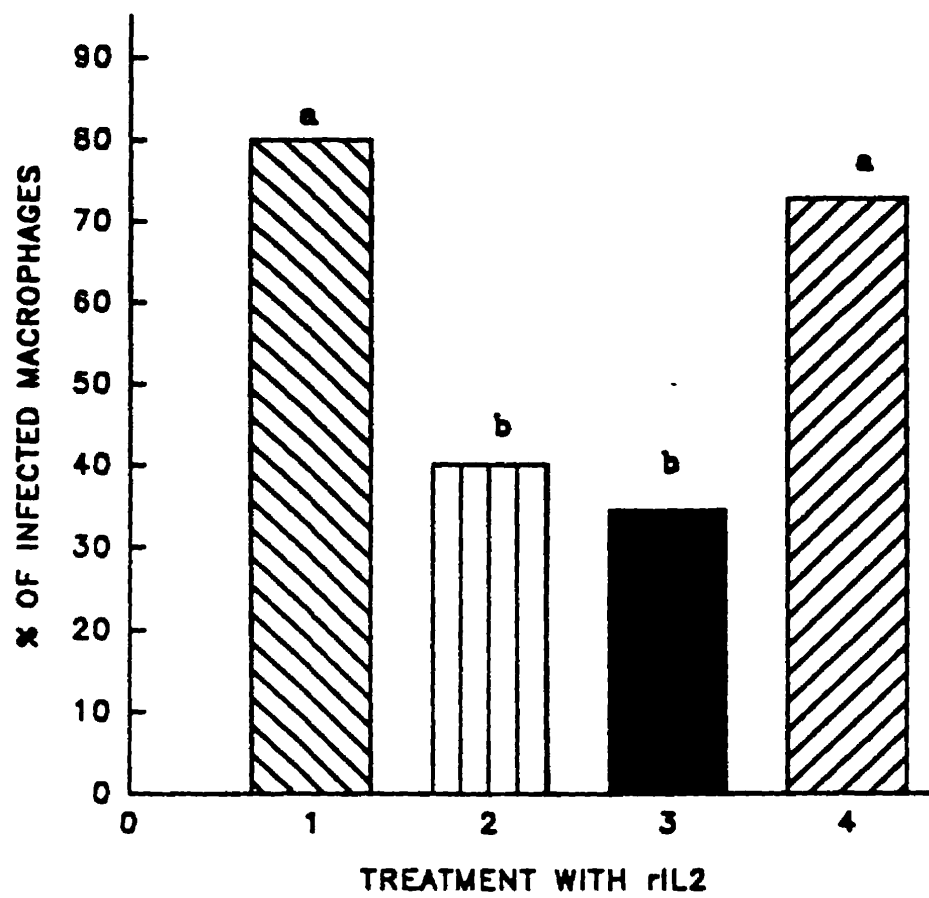
Figure 2: Effect of the depletion of spleen T cells and the effect of monoclonal anti-IFNγ on the percent of infected C57BL/6 peritoneal macrophages in monolayers inoculated with 1 x 10^6 promastigote 48 hrs after treatment with 5 x 10^6 syngeneic spleen cells from normal mice or from mice inoculated 2 weeks previously with 10^7 amastigotes, and stimulated or not with the IL-2-containing supernatant of the MLA 144 cell line. The infected macrophages were treated with

☐ unstimulated normal splenocytes

▨ unstimulated infected splenocytes

▧ stimulated normal splenocytes

▨ infected splenocytes stimulated in the presence of 100 µg/ml promastigote antigen

☐ splenocytes depleted by anti-Thy 1.2 before stimulation

⊞ stimulated normal splenocytes to which monoclonal anti-IFNγ had been added before being used to treat the infected macrophages

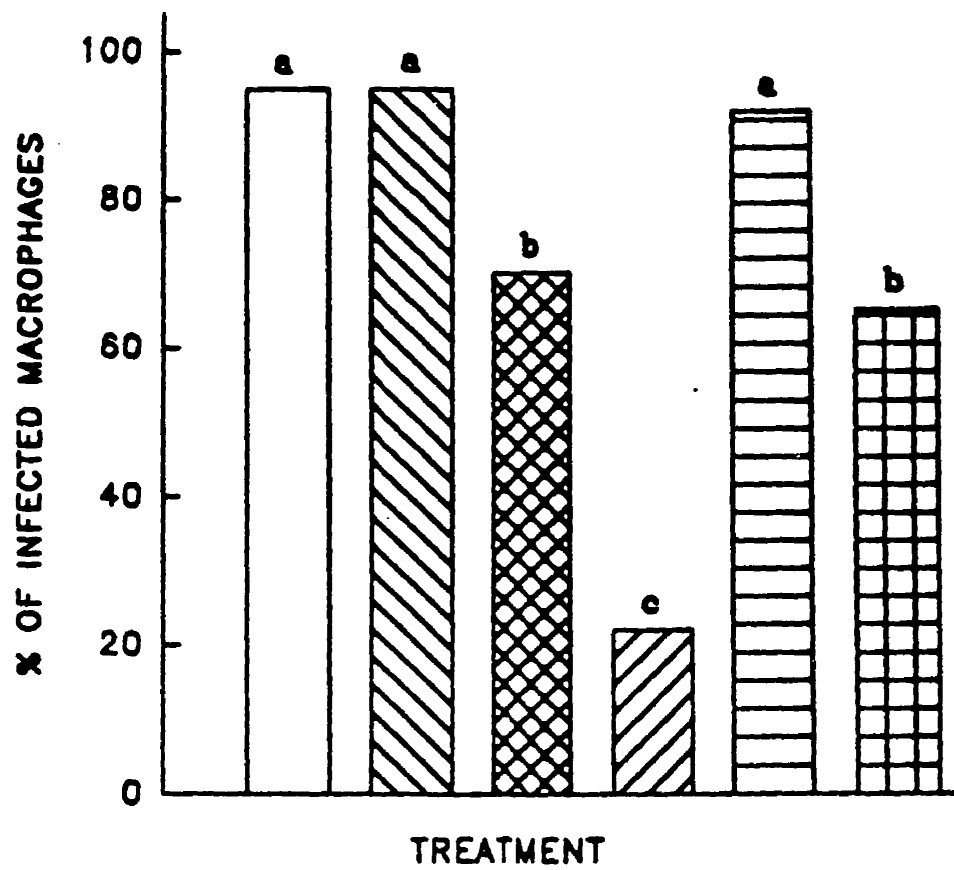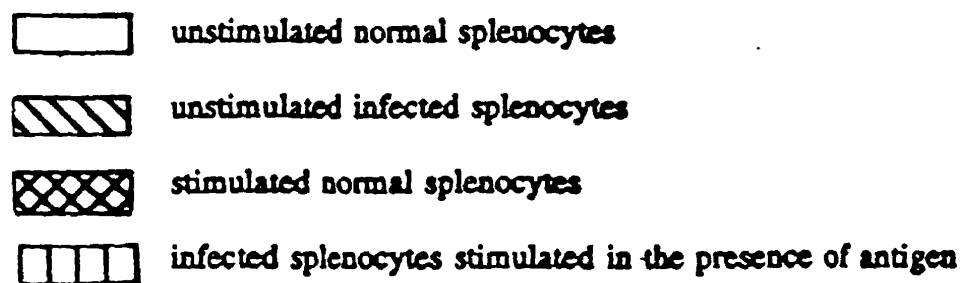Columns labeled by the same letter are not significantly (P<0.05) different from each other, by ANOVA.

Fig. 3: The curative ability for infected macrophages of 2 ml of the soluble, cell-free supernatant produced by 5×10^5 normal or infected C57BL/6 splenocytes stimulated by the supernatant of MLA 144 in the presence or absence of 100 µg/ml promastigote antigen. The supernatant was obtained from

☐ unstimulated normal splenocytes

▧ unstimulated infected splenocytes

▨ stimulated normal splenocytes

⊞ infected splenocytes stimulated in the presence of antigen

Columns labeled by the same letter are not significantly ($P<0.05$) different from each other, by ANOVA.
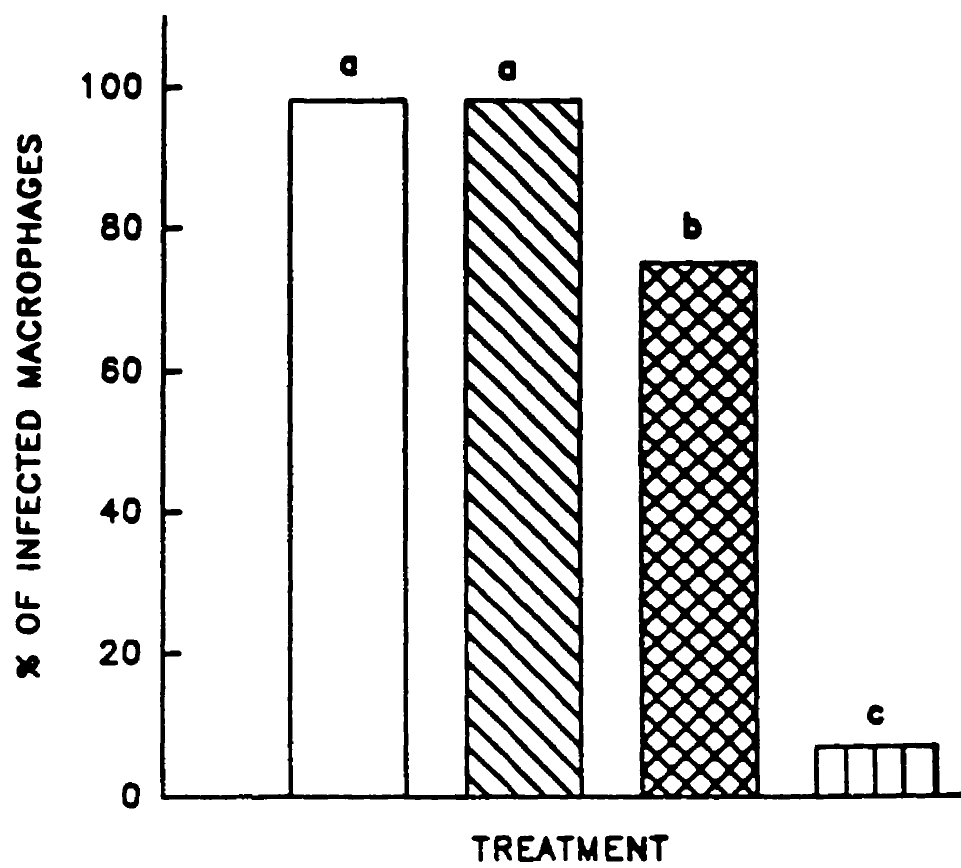
cultures.

The availability in the laboratory of resistant C57L (H-2ᵇ; Lshʳ) mice suggested a study to determine if the activation of leishmanicidal activity in infected cells depends on the phenotype of the stimulated splenocyte, or on the phenotype of the infected macrophage. Splenocytes of resistant C57L mice, stimulated with the MLA 144 supernatant, were, therefore, seeded onto infected macrophages of the susceptible histocompatible C57BL/6 strain and, conversely, stimulated spleen cells of susceptible mice were seeded on to infected macrophages of resistant animals. The results of the study clearly show (Fig. 4) that the leishmanicidal activity induced in infected macrophages was independent of the susceptibility/resistant phenotype of the activating splenocytes. These results also indicate that infected peritoneal macrophages of the resistant mice were significantly more responsive to activation than were those of the susceptible strain, irrespective of the phenotype of the activating lymphocytes.

DISCUSSION

The results we report here demonstrate that macrophage-depleted spleen cells from normal or from *L. donovani*-infected mice, stimulated *in vitro* by the supernatant of the IL-2-producing MLA 144 cell line or by rIL-2, were able to activate significant leishmanicidal activity in infected macrophages. Furthermore, we also show that resident peritoneal macrophages of resistant mice

Fig. 4: The capacity of MLA 144 IL-2-stimulated spleen cells from infected resistant (C57L; H-2^b; Lsh^s) or susceptible (C57BL/6; H-2^b; Lsh^s) mice to activate leishmanicidal action in infected macrophages of resistant or susceptible animals.

■   infected C57L or C57/6 macrophages treated with unstimulated syngeneic splenocytes

☐   infected C57L macrophages treated with stimulated C57L splenocytes

◩   infected C57L macrophages treated with stimulated C57BL/6 splenocytes

▨   infected C57BL/6 macrophages treated with stimulated C57L splenocytes

⊞   infected C57BL/6 macrophages treated with stimulated C57BL/6 splenocytes

Columns labeled with the same letter are not significantly ($P<0.05$) different from each other, by ANOVA.
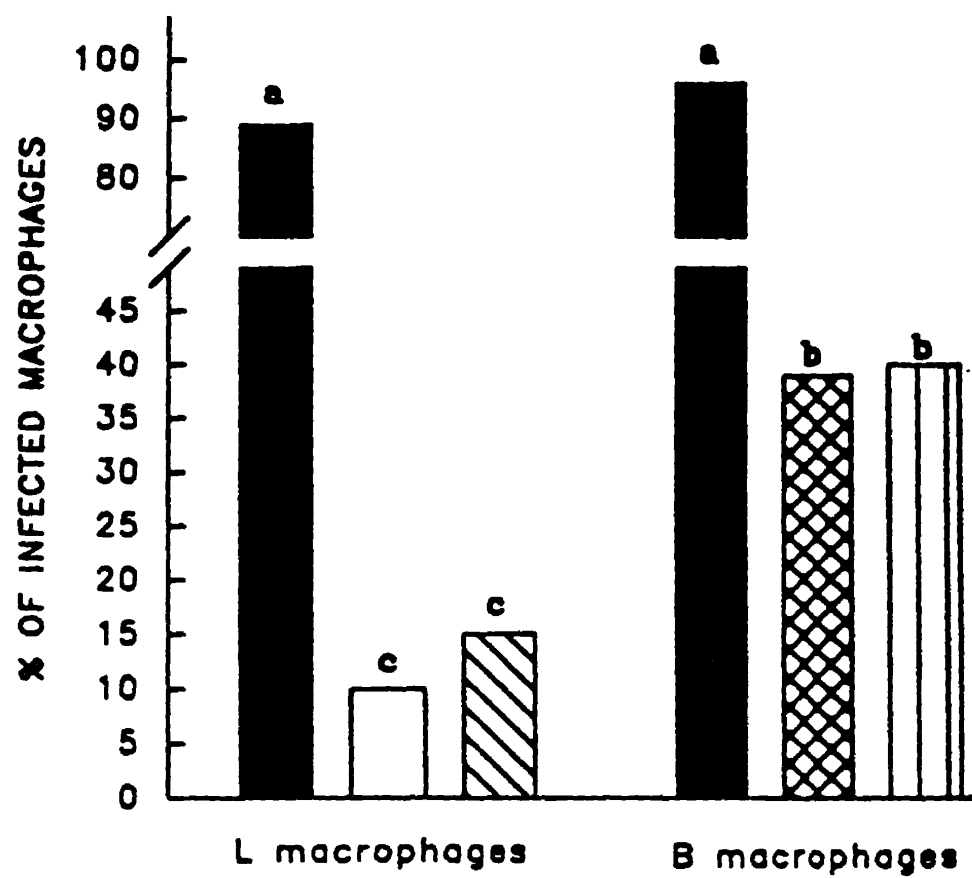
were significantly more responsive to activation by lymphokine-stimulated splenocytes than the peritoneal macrophages of susceptible animals. This latter finding is consistent with our previous report (Olivier *et al.*, 1989) which indicated that leishmanicidal activity is more readily induced by Con A-stimulated lymphokines in the infected peritoneal and hepatic macrophages of resistant than of susceptible mice. This present study adds the important finding that reduction in the number of infected cells is a property of the resistance/susceptibility phenotype of the macrophage and does not depend on the relative ability of lymphocytes to produce activating cytokines. Reduction of the parasite burden clearly involves, therefore, an innate genetic capacity of the infected macrophage to receive the T cell activating signal, and to be activated.

A significant feature in clinical and experimental visceral leishmaniasis is the unresponsiveness induced by infections with *L. donovani* and this present study confirms this in the fact that unstimulated splenocytes from infected animals were no more able to reduce the number of infected macrophages *in vitro* than were unstimulated cells from normal mice. It has been reported (Murray *et al.*, 1987) that the T cells of infected mice have a markedly-depressed proliferative response to stimulation by *Leishmania* antigen, or by Con A. In our experience IL-2 did not stimulate blastogenesis, nor did it induce splenic T cells from infected mice to respond to activation by Con A, or leishmanial antigen (results not shown); Sadick, Locksley, Tubbs & Raff (1986) have similarly reported that the capacity of splenocytes to respond to antigen is not restored by IL-2 in experimental cutaneous leishmaniasis. Despite this unresponsive state, however, we demonstrate here the paradoxical fact that anti-leishmanial activity can be induced in

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infected cells *in vitro* by IL-2-stimulated macrophage-depleted LAK splenocytes obtained from normal or from infected mice. The finding that IL-2-stimulated splenocytes of BCG-infected animals were not as effective as cells taken from mice with kala azar suggests that the activation of leishmanicidal activity in macrophages by lymphokine-stimulated splenocytes may have a specific component.

The cells which stimulated a reduction of the infection *in vitro* were clearly T lymphocytes since depletion studies with anti-Thy 1.2 abolished the ability of splenocytes to be stimulated by IL-2. The result that the anti-IL-2 S4B2 monoclonal blocked the stimulating action of both the rIL-2 and the MLA 144 supernatant indicates that IL-2 was indeed the lymphokine that stimulated the splenic T cells. A participation by cytotoxic lymphocytes in the curative action of the stimulated splenocytes requires a demonstration that IL-2 can up-regulate the reduced expression in leishmaniasis of H-2 antigens (Reiner *et al.*, 1987) on T cells, enabling them to interact with parasite epitopes expressed on the surface of the infected macrophage (Handman, Ceredig & Mitchell, 1979). We demonstrate here that it was indeed soluble lymphokines produced by the stimulated spleen T cells which activated macrophages to reduce the infection, as Pham & Mauel (1987) have reported for *L. major*; activation of leishmanicidal activity in macrophages did not require physical contact between the infected cell and the activating lymphocyte, since the supernatant of IL-2-stimulated cells and the stimulated cells themselves, isolated in cell-impermeable Millipore chambers, were capable of inducing a reduction of the infection. The inability of the R4-6A2 anti-IFNγ monoclonal to suppress IL-2-stimulated splenic T cells from activating leishmanicidal activity in infected macrophages suggests

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the intriguing possibility that another lymphokine(s) may well have been involved in reducing the intensity of infection. We have found (Eslami & Tanner, 1994) that the course of the intracellular infection of macrophages by *L. donovani* describe: a cycle *in vitro* which is characterized by an initial non-proliferative phase, followed by a phase of rapid division of the parasite. It would be intriguing to determine if IL-2-stimulated T cells are more or less effective when used at different phases of the intramacrophage development of the parasite.

Although the exact nature of the signal from lymphokine-stimulated splenocytes which activates leishmanicidal activity in infected macrophages is not known, the stimulus to lymphocytes can be provided effectively by IL-2. It is very well known that the T cells of mice and humans are immunologically depressed in clinical and experimental kala azar; this present study demonstrates that these depressed T cells nevertheless retain a potentially-stimulatable, intrinsic capacity for effector activity which, when provided with the stimulus of exogenous IL-2, can elicit parasiticidal action in infected macrophages. T cell anergy in experimental visceral leishmaniasis can be, therefore, readily reversed.

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CONNECTING STATEMENT II

The results from Chapter III demonstrated that splenocytes from normal or infected mice, following stimulation by rIL-2 or by the IL-2-containing supernatant of the MLA 144 cell line, are able to activate infected macrophages *in vitro* to reduce their parasite burden. It was only reasonable to investigate whether such lymphokine-stimulated cells would also reduce experimental visceral leishmaniasis *in vivo*.

Chapter IV

IL-2 STIMULATED SPLENOCYTES REDUCE INFECTIONS BY

LEISHMANIA DONOVANI IN VIVO

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ABSTRACT: The traditional treatment of infections produced by the obligate intramacrophage protozoan *Leishmania donovani* involves the use of antimonial drugs. Since these drugs may have toxic side effects (and are sometimes ineffective), the potential efficacy of alternative therapy with lymphokine-stimulated leucocytes was assessed. Macrophage-depleted C57BL/6 splenocytes from mice inoculated 2 wk earlier with *L. donovani* were stimulated in vitro with the IL-2-containing supernatant of MLA 144 cells and transferred intravenously into syngeneic infected mice. Compared to infected mice that had received unstimulated normal or infected spleen cells, animals treated with lymphokine-stimulated splenocytes had significantly-reduced parasite loads. Efficacy was further enhanced significantly by supplementary intraperitoneal injections of the MLA 144 supernatant; the effector function of the stimulated splenocytes was dose-dependent. The rescue of animals infected by *L. donovani* from parasite-induced down-regulation of immunity could be an important part of a strategy for the effective treatment of kala azar; lymphokine-stimulated cells are potential candidate agents to restore curative immune responses of experimental visceral leishmaniasis.

Leishmania donovani is the obligate intracellular parasite of macrophages that causes visceral leishmaniasis in humans; the prognosis of this infection is grave unless treatment with pentavalent antimonials, or other chemotherapeutic regimens is successful. Because therapy with antimonials can be toxic and may be affected by the drug-resistance of the infecting strain (Berman et al., 1982), an alternative immunotherapy regimen was investigated. This latter treatment was investigated since lymphokine-stimulated leukocytes (Chang and Chiao, 1981; Nacy et al., 1981; Olivier et al., 1989) or the crude or recombinant lymphokines (Murray et al., 1993; Sharma et al., 1985; Reed et al., 1984) can activate macrophages to destroy intracellular protozoa; IL-2-stimulated cells can, furthermore, induce the regression of tumors (Mule et al., 1985; Rosenberg et al., 1986). We (Eslami et al., 1995) have recently reported that splenocytes from normal or infected mice, following stimulation by IL-2, activate infected macrophages in vitro to reduce their parasite burden. We show here that macrophage-depleted syngeneic IL-2-stimulated splenocytes of animals infected by *L. donovani* are also capable of significantly reducing experimental visceral leishmaniasis in vivo following the adoptive transfer of these cells to infected recipient C57BL/6 mice.

MATERIALS AND METHODS

Infections with the LV9 strain of *L. donovani* were established in 6-to 8-week-old male C57BL/6 (*Lsh^s*) mice inoculated with 10^7 amastigotes obtained (Olivier and Tanner, 1987) from stock infections in CO_2-euthanised female Lak:Lvg Syrian hamsters (Charles River Laboratories, St-Constant, Quebec, Canada). The growth curve of these infections,

enumerated as Leishman-Donovan Units (LDU; Bradley and Kirkley, 1977) illustrate

(Fig. 1) a typical increase in the parasite burden of *L. donovani* in these mice in the first

15 days following inoculation of the parasite; the intensity of the infection remained

virtually unchanged in the next 15 days and then began to decrease until approximately

the 45th day, following which a low level of parasitism was maintained.

Seventeen days after the intravenous inoculation of 10^7 LV9 *L. donovani*

amastigotes and at the peak of infection in vivo, groups of 5 C57BL/6 mice were injected

once intravenously (i.v.) with 10^6 viable macrophage-depleted unstimulated splenocytes,

or with MLA 144-stimulated splenocytes obtained from normal or from infected

syngeneic mice. Gibbon ape leukemia virus-transformed MLA 144 T cells (ATCC TIB

201) constitutively elaborate IL-2 in culture (Henderson et al., 1983). The supernatant

of these cells is equally as effective as rIL-2 in stimulating splenocytes to induce

leishmanicidal activity in vitro by infected macrophages; the stimulating activity of the

MLA 144 supernatants and of rIL-2 for splenocytes is equally abolished by monoclonal

anti-IL-2 antisera (Eslami et al., 1995). Unstimulated cells were cultured in medium not

conditioned by the MLA 144 cells. All cells were cultured or suspended in RPMI 1640

medium (Flow Laboratories, McLean, Virginia), supplemented by 10% heat-inactivated

fetal bovine serum (Flow), 100 μg/ml streptomycin (Sigma, St. Louis, Missouri) and 100

U/ml penicillin (Sigma); the splenocytes were depleted of erythrocytes with Tris-buffered

ammonium chloride (Mishell and Shiigi, 1980) and of macrophages by adherence for 2

hr at 37° C in humidified 5% CO_2 in air; the latter depletion was done to reduce

suppressor macrophages (Nickol and Bonventre, 1985). The non-adherent splenocytes, recovered by washing the adhered spleen macrophage cell carpet with warm ($37°$ C) medium, were adjusted to 5×10^6 trypan-blue-viable cells/ml. Two $\times 10^6$ of these splenocytes obtained from syngeneic normal mice, or from mice inoculated 2 wk earlier with 10^7 *L. donovani* amastigotes were stimulated in 6-well Linbro culture plates (Gibco, Grand Island, New York) by 1 ml of a 1:5 dilution of the culture supernatant of MLA 144 T cells. Seventy-two hr after stimulation, these splenocytes were washed 3X and adjusted to 5×10^6 viable cells/ml ; 10^6 of these cells were injected i.v. once into C57BL/6 mice on the 7th day of their infection.

RESULTS AND DISCUSSION

Unstimulated splenocytes from normal or from infected syngeneic mice, adoptively-transferred i.v. into recipient animals at the peak of their infection on day 17, failed to reduce the burden of *L. donovani* in liver macrophages by day 7 after the treatment (Fig. 2). The parasite burden in mice was, however, significantly ($P < 0.05$) reduced by treatment at the same stage of the infection with the IL-2-containing MLA 144 supernatant alone, or with IL-2-stimulated splenocytes from normal animals. The reduction was significantly further enhanced when the cells used for immunotherapy were prepared from splenocytes obtained from infected donors, whether 100 μg/ml metacyclic promastigote antigen extracted at $0°$ C by 3 $\times$ 30 sec pulses of 1.5 Kc ultrasounds had been present or not during stimulation by IL-2. Supplementary intraperitoneal (i.p.) treatment with 1 ml of the MLA 144 supernatant on each of the 7 days following adoptive transfer further enhanced the significant ($P < 0.05$) reduction of the parasite

Figure 1: Course of infection by <u>Leishmania donovani</u> in C57BL/6 (Lsh^s)

mice inoculated with 10^7 amastigotes intravenously. The intensity of

the infection was determined on different days following the

inoculation in Giemsa-stained liver impression smears as LDU ($\pm$SE)

(LDU = number of amastigote/100 nuclei x weight of the liver in mg).
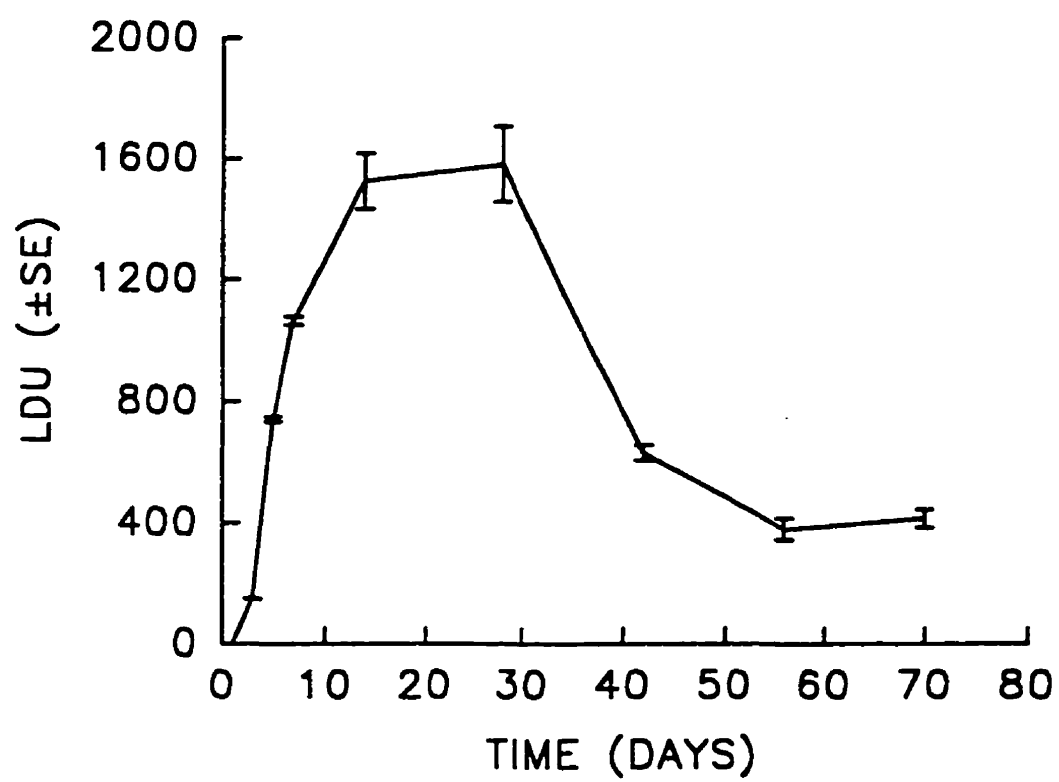
burden induced by the lymphokine-stimulated cells prepared from the splenocytes of infected mice; supplementary lymphokine treatment did not, however, enhance the reduction induced by IL-2-stimulated cells prepared from normal splenocytes. An effector activity to reduce the infection in vivo by IL-2-stimulated splenocytes from syngeneic mice inoculated 2 wk earlier with 5×10^6 CFU of *Mycobacterium bovis* BCG (BCG-CANCER; Institut Armand-Frappier, Laval, Quebec, Canada) was identical to that of stimulated cells from normal mice, even when treatment of the recipient infected animals was supplemented by daily i.p. injections of the MLA 144 supernatant; there may be, thus, some specificity in the ability of lymphokine-stimulated splenocytes to reduce experimental visceral leishmaniasis in vivo. The therapeutic effectiveness of the stimulated splenocytes required a minimum of 10^5 to 10^6 cells to obtain a significant reduction in the hepatic parasite burden (Fig. 3); Rosenberg et al. (1986) have reported similar minimal cell requirements for tumor immunotherapy with lymphokine-activated killer (LAK) cells, or with tumor-infiltrating leukocytes (TIL).

A significant feature of mice infected by *Leishmania donovani* is that their T cells have a markedly-depressed proliferative response to stimulation by *Leishmania* antigen, or by Con A (Murray et al., 1987). Support for the existence of an unreactive state in visceral leishmaniasis is also found in the present report as the fact that unstimulated splenocytes from infected animals were no more able to activate a reduction of the

Figure 2: The ability of syngeneic IL-2-stimulated splenocytes to reduce the

hepatic parasite burden in C57BL/6 mice infected by Leishmania

donovani: 10^6 stimulated splenocytes from infected or normal mice

were transfered intravenously once into animals that had been

inoculated with 10^7 amastigotes 17 days earlier. Some animals were

also treated with the MLA 144 lymphokine intraperitoneally (IP) on

each of the 7 days following adoptive transfer. The intensity of

infection is expressed as an infection index: LDU in infected mice

treated with stimulated splenocytes ÷ LDU in mice treated with

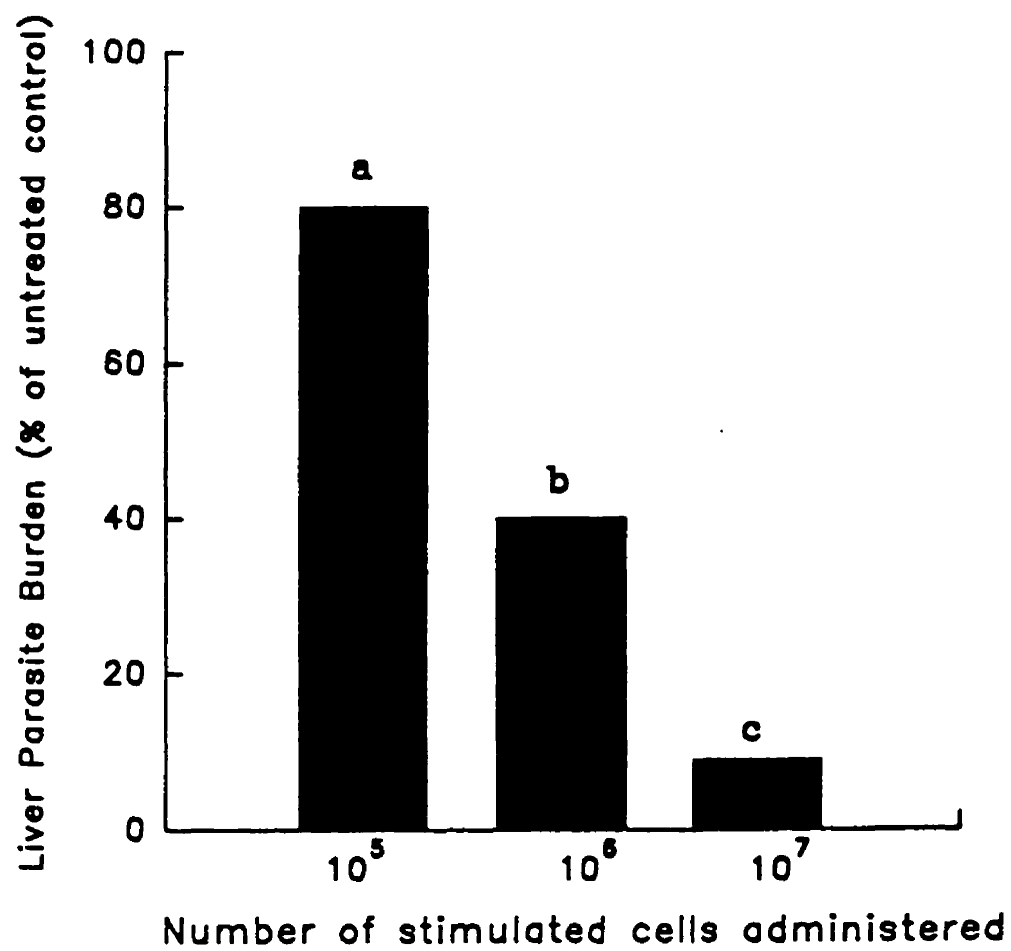unstimulated splenocytes x 100.

The infected animals were treated with 1: normal splenocytes; 2:

splenocytes of infected animals; 3: MLA 144 supernatant; 4:

stimulated splenocytes of normal mice; 5: stimulated splenocytes

of infected mice; 6: splenocytes of infected mice stimulated in the

presence of antigen; 7: stimulated splenocytes of normal mice,

plus the MLA 144 lymphokine IP; 8: stimulated splenocytes of

infected mice, plus the MLA 144 lymphokine IP; 9: splenocytes

of infected mice stimulated in the presence of antigen, plus the

MLA 144 lymphokine IP; 10: stimulated splenocytes from BCG-

infected mice, plus the MLA 144 lymphokine IP. Columns

identified by the same letter are not significantly ($P<0.05$)

different from each other.
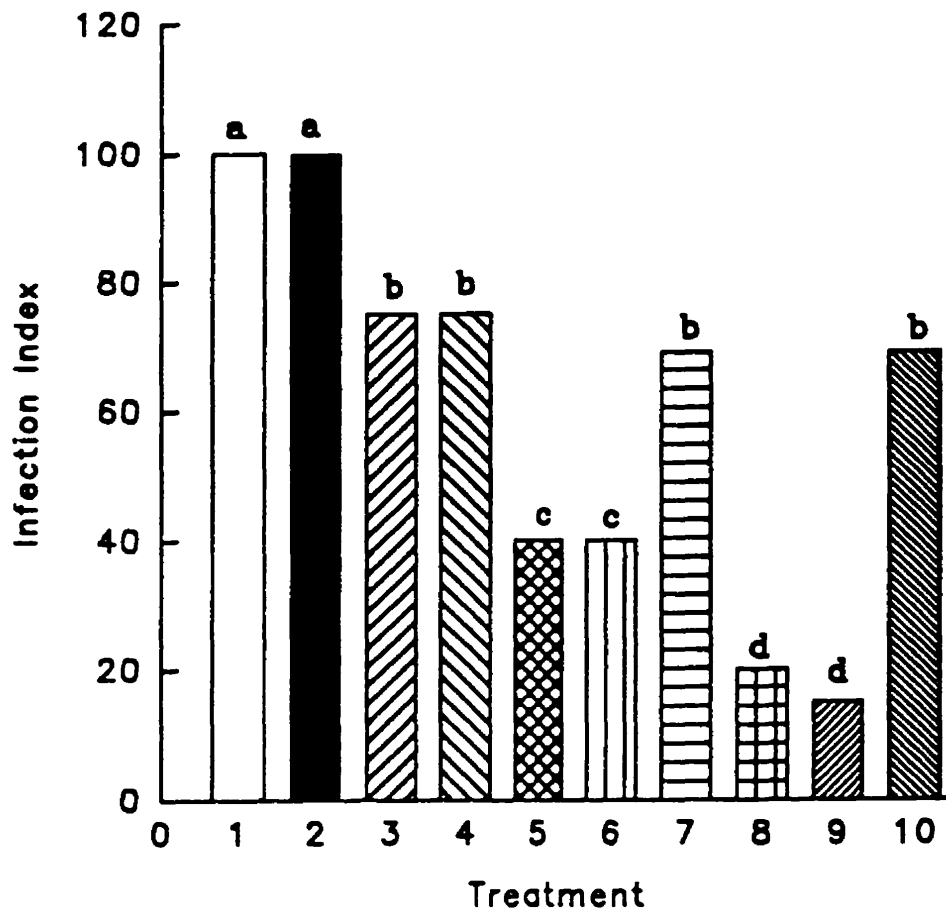
infection than were unstimulated cells obtained from normal mice. Despite this unresponsiveness, however, we demonstrate that a significant anti-leishmanial curative activity can be induced in infected mice by the adoptive transfer of IL-2-stimulated, macrophage-depleted syngeneic splenic cells. It is interesting that the presence of promastigote antigen during lymphokine stimulation *in vitro* did not significantly further enhance the curative ability of these cells, even when the treated animals received supplementary i.p. injections of the IL-2-containing MLA 144 supernatant. The lack of enhancement by antigen of the curative ability of the lymphokine-stimulated cells in vivo could have been due to the possibility that the splenocytes of infected mice are already fully-primed by antigen (or as fully-primed as they become) during infection and, thus, the addition of exogenous antigen during stimulation in vitro was redundant. Support for this latter statement can be found in the fact that lymphokine-stimulated normal splenocytes were not as effective as the stimulated cells of infected animals in reducing the intensity of the infection. Our evidence that supplemental i.p. treatment with the IL-2-containing supernatant of MLA 144 cells significantly-enhanced the curative effect of stimulated splenocytes in vivo is in agreement with the reports (Cheever et al., 1982; Mule et al., 1985; Rosenberg et al., 1986) that effector activity in tumor immunotherapy with LAK or TIL cells is enhanced by the administration of exogenous lymphokine. The injection of the supernatant alone significantly reduced the parasite burden in infected mice, in support of the report by Murray et al. (1993).

A deficiency in the production of cytokines (Reiner and Finke, 1983; Olivier and

Figure 3: The hepatic parasite burden in C57BL/6 mice inoculated with 10^7

amastigotes and treated intravenously (IV) once 17 days later with 1

of 3 different doses of IL-2-stimulated splenocytes from infected

mice; these animals did not receive supplementary treatment with the

supernatant of the MLA 144 cell line. Intensity of the infection is

expressed as a percent of the LDU in control untreated mice infected

for 3 wk. Columns identified by the same letter are not significantly

($P<0.05$) different from each other.

Tanner, 1989) is certainly a root cause of the reduced ability of the immune system to mediate effective control in murine visceral leishmaniasis. Although effector cells prepared from the IL-2-stimulated splenocytes of infected mice had a highly-significant anti-leishmanial activity in C57BL/6 mice infected by *L. donovani*, these splenocytes did not, paradoxically, proliferate in vitro in response to promastigote antigen, or to Con A (results not shown), in confirmation of the report of Murray et al. (1987). It is quite probable that the regulation of experimental leishmaniasis could involve several activation pathways; a case in point is the evidence that IFN-γ alone (Murray et al., 1987, 1993) or soluble products released by IL-2-stimulated splenocytes (Eslami et al., 1995) can activate parasiticidal action in infected macrophages in vitro and in vivo (Fig. 2). IL-2-stimulated splenocytes induce leishmanicidal activity in infected cells in vitro even in the presence of monoclonal anti-IFN-γ (Eslami et al., 1995) suggesting that the production of IFN-γ by the stimulated cells is not necessarily required for the activation of the infected macrophages. It has been suggested (Eslami and Tanner, 1994) furthermore that infected macrophages elaborate soluble substances that could play an important role in regulating infection by *L. donovani*, facilitating the establishment and proliferation of visceral leishmaniasis. The results of this study show that, although infection by *L. donovani* significantly down-regulates immunity, infected animals contain T cells (Eslami et al., 1995) that retain an intrinsic potential to be activated by IL-2, and which can induce a curative leishmanicidal activity *in vivo*. This study suggests that the rescue of

infected animals from parasite-induced immunosuppression might be an important part of a strategy to control experimental visceral leishmaniasis.

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CONNECTING STATEMENT III

It was reported above that the course of intracellular infection by *L. donovani* describe a cycle *in vitro* which is characterized by an initial non-proliferative phase which lasts about 4 days, followed by a phase of rapid division that peaks on approximately the 7th day post-inoculation. It was, therefore of interest to determine when during the intracellular life cycle of the parasite would chemotherapy with Pentostam, immunotherapy with LAK cell or the combination of immuno-chemotherapy be most effective in inducing a reduction of the infection.

Chapter V

Synergism of IL-2-Stimulated Splenocytes and Pentostam

Enhances the Killing of *Leishmania donovani in vitro*

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Running head: LAK CELLS AND PENTOSTAM
ENHANCE THE KILLING OF *L.DONOVANI*.

Key Words: visceral leishmaniasis, LAK cells, Pentostam,
immunochemotherapy

ABSTRACT

Control of infections by the obligate intramacrophage protozoan parasite
Leishmania donovani is traditionally done with pentavalent antimonial drugs. It was of
interest to determine if immunotherapy with IL-2-stimulated splenocytes would enhance
drug action in an *in vitro* model system. It is confirmed that non-toxic doses of
Pentostam decrease infection in a dose-dependent manner *in vitro* in terms of both the
number of amastigotes/100 macrophages and the % of infected macrophages; the
curative effect was most apparent when the drug was used on the 7th day of the
infection, when the parasite was in its proliferative phase. It is also confirmed that
recombinant IL-2-stimulated splenocytes induced infected macrophages to reduce
significantly their parasite burden, especially when the infection was treated in the non-
proliferative phase of the parasite, also in a dose-dependent manner. Leishmanicidal
action in the infected macrophages was induced by cytokine(s) released from the
lymphokine-stimulated cells. Immunotherapy in the non-proliferative phase, combined
with drug treatment in the proliferative phase, reduced the infection to levels significantly
well below those produced by either treatment alone. Immunotherapy with IL-2-
stimulated splenocytes in combination with Pentostam is, therefore, an excellent candidate
treatment for the effective reduction of experimental infections by *Leishmania donovani*.

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INTRODUCTION

The obligate intramacrophage protozoan parasites of the genus *Leishmania* cause a spectrum of lesions in their vertebrate hosts which depend on the parasite species involved and on the intensity and kind of the host's immune response. The ability of *Leishmania donovani* to proliferate in vertebrates depends also upon the interaction of a number of factors which have been associated, among other things, with a capacity of the organism to down-regulate immunity (1), and with the innate genetic susceptibility/resistance phenotype of the host animal (2). Control of infections by *L. donovani* in humans is done traditionally with amastigote-specific (3) pentavalent antimonial drugs, and the prognosis of the infection is frequently death unless such treatment is successful (4). Treatment of visceral leishmaniasis with antimonial drugs presents a problem in that these drugs can produce toxic side-effects, especially when high doses or prolonged treatment is required (5); therefore, this drug treatment must be done under careful management and with appropriate supervision. It has, in addition, been long recognized that antimonial therapy can sometimes be ineffective and that relapse sometimes occurs despite apparent cure (6;7;8), due in part to the innate drug resistance of the strain of *Leishmania* which is responsible for producing the infection (9). Because of these difficulties, there is an interest in understanding the mechanisms by which the killing of *L. donovani* is effected in infected cells and in alternative approaches to the control of visceral leishmaniasis.

We wished to confirm previous reports (10) concerning the effect of different doses of Pentostam on experimental visceral leishmaniasis *in vitro* and to add data to

those reports on changes in the percentage of infected macrophages following drug treatment. The course of intracellular infection by *L. donovani* describes a cycle *in vitro* which is characterized by an initial non-proliferative phase which lasts about 4 days, followed by a phase of rapid division which peaks on approximately the 7th day post-inoculation (11). It was, therefore, also an interest of this study to determine when, during the intracellular life cycle of the parasite, is drug therapy most effective. We have also reported that IL-2-stimulated splenocytes prepared from normal mice, or from mice infected by *L. donovani*, can activate significant leishmanicidal activity in macrophages, reducing the percentage of parasitised cells and the number of amastigotes per macrophage *in vitro* and the number of amastigotes *in vivo* in the liver of infected mice (13). We were curious, therefore, to determine when, during the course of the parasite's life cycle *in vitro*, would such lymphokine-stimulated cells be most effective in inducing a reduction of the infection. It was also natural to investigate the effectiveness of a treatment regimen that combined immuno- with chemotherapy.

The results of this study suggest that Pentostam's action was greatest when the drug was administered during the phase of the rapid intracellular division of *L. donovani* *in vitro*, and that lymphokine-stimulated splenocytes were most effective when administered on the 3rd day, during the non-proliferating phase of the parasite. This study also demonstrates that immunotherapy begining on day 3 of the infection, followed by drug therapy during the proliferative phase, was considerably more effective than either treatment alone in reducing the intensity of the infection *in vitro*.

MATERIALS AND METHODS

Animals and the parasite.

Female C57BL/6 (H-2^b; *Lsh^s*) mice aged from 4 to 6 weeks and female Syrian hamsters (Lak:Lvc) were purchased from the Charles River Laboratories, Inc. (St-Constant, Québec). The LV9 strain of *Leishmania donovani* was maintained in the laboratory by the periodic passage of amastigotes in hamsters; amastigotes obtained from the spleens of CO_2-euthanised infected hamsters were transformed to promastigotes for the inoculation of macrophage monolayers, as previously described (11). All animals were euthanised with CO_2 before parasites or tissues were recovered from them.

Collection and infection of mouse peritoneal cells.

Resting cells were harvested from normal, unstimulated C57BL/6 mice by lavage of the peritoneal cavity with 5 ml of ice-cold RPMI 1640 medium (Gibco, Grand Island, NY), supplemented by 10% fetal bovine serum (Gibco), 100 μg/ml streptomycin (Sigma, St.Louis, MO) and by 1000 U/ml penicillin (Sigma), as described by Mishell and Shiigi (14); this supplemented medium was used throughout and will be referred to hereafter as RPMI. After collection, the peritoneal cells were counted in a hemacytometer with trypan blue and made up to 10^7 viable cells/ml RPMI. One hundred μl aliquots of this suspension were placed on individual 18 x 18 mm cover slips and the macrophages in the cell suspension were allowed to adhere overnight, each in a well of 3.5 cm dia x 1 cm deep 6-well Linbro culture plates (ICN Biomedicals, St-Laurent, Québec) containing 2 ml RPMI; all cell cultures in this study were incubated at 37°C in humidified 5% CO_2 in air. The non-adherent cells were then removed by gentle washing with warm (37°C)

RPMI and each cover slip was placed in a well of another 6-well Linbro culture plate (ICN Biomedicals, St-Laurent, Québec) containing 2 ml RPMI; the macrophages were cultured for 2 hrs prior to exposure to 10^7 metacyclic promastigotes. The parasites were allowed to internalize for 2 hrs and free promastigotes were then removed by careful washing with warm RPMI. The intensity of the infection *in vitro* was determined, after methanol fixation and staining with Giemsa, in 200 macrophages chosen at random in non-contiguous fields (x 1000); infection was expressed (11) both as the number of amastigotes per 100 infected cells and as the percent of infected macrophages.

Treatment with Pentostam.

It is known that Pentostam can sometimes have cytotoxic effects *in vitro*, inducing the detachment of macrophages from monolayers (10). In order to determine if any of the doses of the drug used in this study had a cytotoxic effect on the host cells, three days after infection of the macrophage monolayers (see above), the supernatant medium was replaced once with 100 μl of RPMI containing either 5, 10, 15, or 20 μg Pentostam/ml. A total of 100 infected cultures were treated with the drug; 50 infected cultures were incubated without the drug as controls. Five monolayers from each treatment group and 10 control cultures were terminated 1, 2, 3, 4, or 8 days after treatment; they were air-dried, fixed in methanol and stained with Giemsa. Two hundred macrophages were examined at random on each cover slip culture as above and the percentage of infected macrophages were determined, as well as the number of amastigotes per infected cell. This control for cytotoxicity showed a normal decline in the number of uninfected, untreated cells over time; the number of viable macrophages

was not significantly changed by the presence of any of the four different doses of Pentostam studied, suggesting that none of the doses of the drug used in this study had a significant cytotoxic effect (the results are not shown, but they are available on demand). Although not specifically confirmed in this study, Pentostam also appears not to have a cytotoxic effect on infected macrophages in the range of doses used in this study, at least not in short term incubations with the drug (15).

Splenocytes.

Splenocytes from infected or from mice which had been inoculated intracardially 14 days earlier with 10^7 amastigotes were obtained by passing the spleen aseptically through a sterile stainless-steel screen (60 mesh, 80 gauge) into ice-cold RPMI with a glass pestle; erythrocytes in the cell suspension were lysed with Gey's salts (14). Before stimulation with rIL-2, the number of macrophages in all splenocyte suspensions was reduced by adherence overnight at 37°C in petri dishes to diminish the number of potential suppressor cells (16). Non-adherent spleen cells were recovered from the adhered macrophages by washing the cell carpet with warm RPMI; the non-adherent spleen cells were then washed twice with cold RPMI, counted in a hemacytometer with trypan blue and adjusted to 5 x 10^6 viable cells/ml. The viability of all cell preparations was at least 95%, as determined by trypan-blue exclusion.

Stimulation of splenocytes with rIL-2 and immunotherapy.

Several aliquots of 1 ml RPMI containing 5 x 10^6 viable non-adherent splenocytes from uninfected or from infected C57BL/6 mice inoculated 7 days earlier with 10^7 amastigotes (see above) were seeded into each well of 6-well Linbro culture plates (ICN)

and stimulated with sufficient rIL-2 (Cedarlane, Hornby, Ontario) to give, in appropriate

wells, doses of 125, 250 or 500 U of the recombinant lymphokine/ml. The splenocytes

were harvested 72 hrs after stimulation; cells stimulated with the same amount of

lymphokine were pooled, washed 3x with RPMI and each pool adjusted to 5×10^{6} viable

cells/ml. To determine when during the intracellular proliferative cycle of *L. donovani*

is the parasite most susceptible to the microbicidal action induced by the lymphokine-

stimulated cells, washed infected macrophage monolayers 3 or 7 days after inoculation

of 10^{7} promastigotes were cultured in 2 ml RPMI alone, or in 2 ml RPMI containing a

total of 10^{7} viable rIL-2-stimulated splenocytes obtained from infected or normal mice.

To confirm that activation was due to IL-2, RPMI containing the anti-IL-2 monoclonal

immunoglobulin from S4B6 cells (ATCC HB 8794) was added, in the proportion of 1

μg of antibody protein per unit of rIL-2, to some washed splenocyte suspensions

containing 5×10^{6} viable cells/ml, to which 250 U of the stimulating recombinant

lymphokine was added subsequently. The antibody was prepared from pooled RPMI

supernatants of S4B6 cells precipitated 3x at 50% saturation with ammonium sulphate

(pH 7.2) and extensively dialysed against several changes of a large exess of PBS; the

protein concentration of this preparation was determined by the Lowry method (14). In

some cultures, splenocytes stimulated with 250 U of rIL-2 were placed in 0.45 μm

Millicel-HA chambers (Millipore, Mississauga, Ontario) to confirm that activation of

leishmanicidal activity in infected macrophages was mediated by a lymphokine released

from the stimulated splenocytes (13). The curative effect of the IL-2-stimulated

splenocytes was determined 48 hrs after treatment; in some cultures, the infected

macrophages were treated with the lymphokine-stimulated cells throughout the 8-day treatment period.

Statistical analysis.

The results of this study were analysed by the non-parametric Kruskal-Wallis ANOVA test and the statistical significance of the difference between paired data (eg. control vs. treatment; treatment 1 vs. treatment 2) was obtained from the Student-Newman-Keul test using the SigmaStat statistical software (17). The difference between two results was taken as statistically significant when the P value was 0.05 or lower.

RESULTS

Time course of the infection in vitro.

The percentage of infected macrophages and the number of amastigotes/100 macrophages in control untreated infected macrophage monolayers is shown in Fig. 1. The course of the infection with *L. donovani in vitro* illustrated in this figure does not differ from that previously reported (11) and illustrates again the intriguing fact that, although the number of amastigotes per macrophage described an intracellular proliferative cycle, the percentage of infected macrophages changed relatively little during the course of the infection.

Pentostam reduced infection in vitro in a dose-dependent fashion.

Three days after the inoculation of resting peritoneal macrophage monolayers with *L. donovani* promastigotes, these cells were treated with 100 μl of 5, 10, 15 or 20 μg/ml doses of Pentostam; the number of amastigotes/100 macrophages and the percent of infected cells were determined 1, 2, 3, 4, or 8 days after treatment. The results of this study indicate that, except for the smallest dose of 5 μg/ml, all the doses of Pentostam examined significantly reduced in a dose-dependent fashion the number of amastigotes/100 macrophages and the percentage of infected cells as early as the first day after treatment was started (Fig.2). The lowest dose of 5 μg Pentostam/ml did, nevertheless, show antiparasite activity on the second day following the start of the drug treatment.

Pentostam was most effective when used in the proliferative phase of the infection.

To determine when during the intracellular proliferative cycle are

Figure 1. Time course of the *L. donovani* infection *in vitro*.

The intensity of infection in resting peritoneal C57Bl/6 macrophage

monolayers inoculated with 10^7 metacyclic promastigotes of *Leishmania*

donovani. Cover slips were fixed with methanol on days 4, 5, 6, 8, and

11 after inoculation and stained with Giemsa. The numbers of

amastigotes/100 macrophages and the percent infected macrophages

($\pm$SE) were determined in 200 macrophages chosen at random in non-

contiguous fields (x 1000).
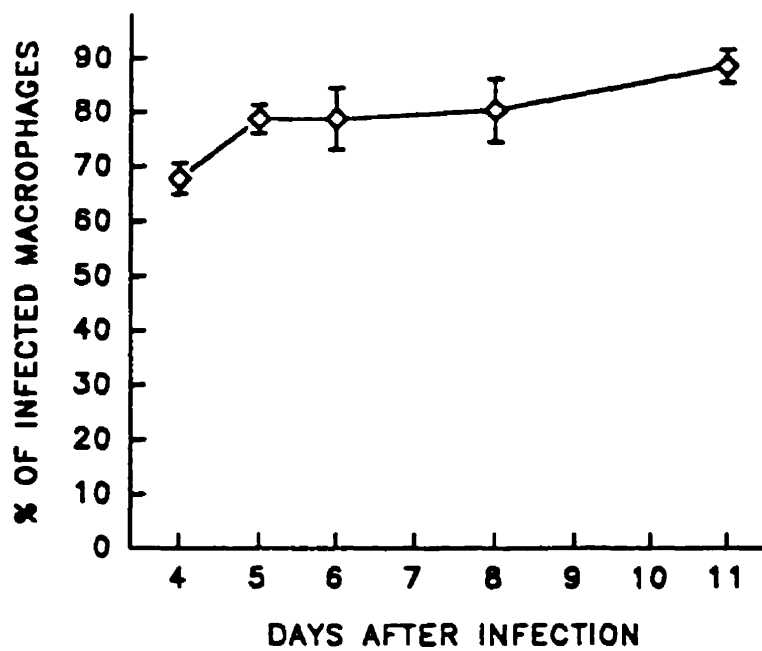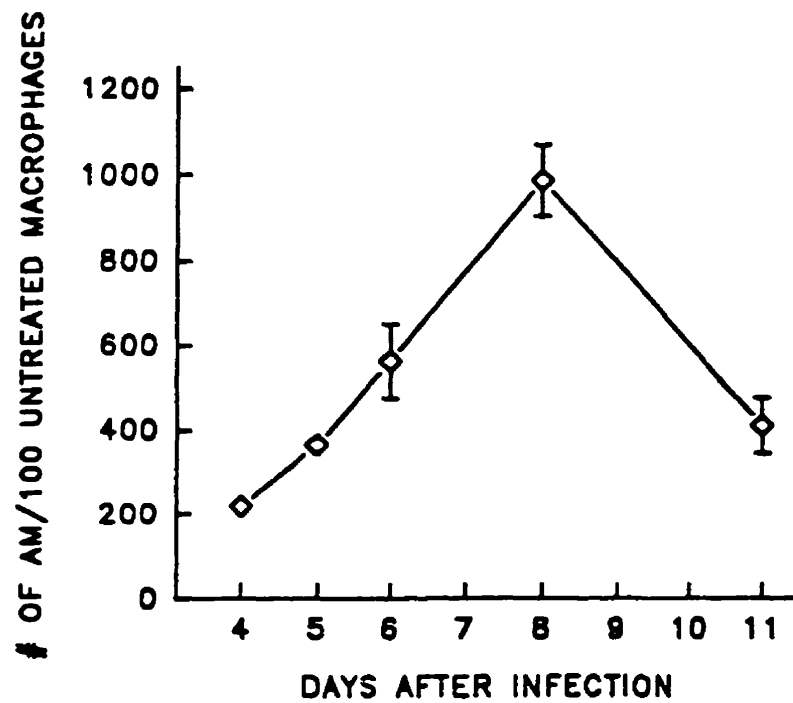
Figure 2. Pentostam reduces the infection *in vitro* in a dose-dependent fashion.

The number of amastigotes/100 macrophages and the percentage of infected macrophages ($\pm$ SE) in C57BL/6 resting peritoneal macrophage cultures inoculated with 10^7 metacyclic promastigotes and treated, from day 3 of the infection, for different periods of time with 100 μl of RPMI containing 5 (☐), 10 (☐), 15 (▨) or 20 (▤) μg Pentostam/ml; untreated control cultures (◼).

TIME (DAYS AFTER TREATMENT)

TIME (DAYS AFTER TREATMENT)

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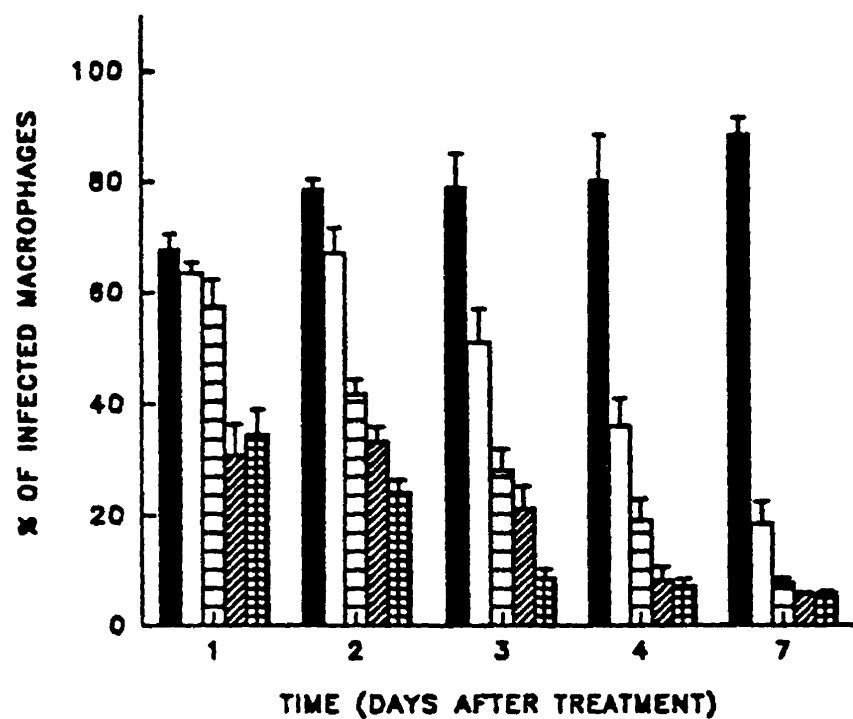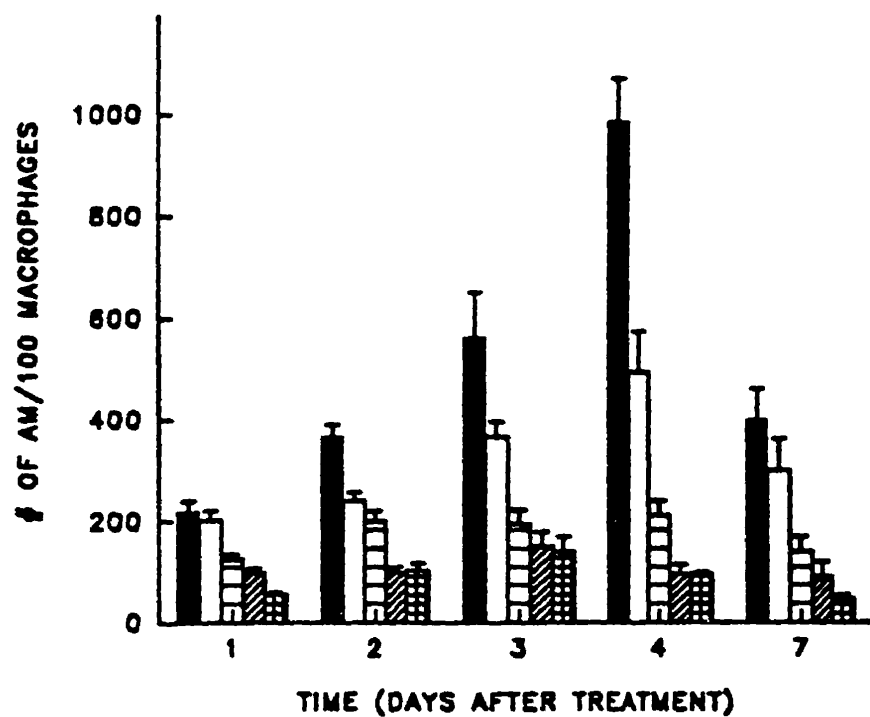
amastigotes most susceptible to the parasiticidal action of Pentostam. Infected macrophage monolayers were treated with 15 μg/ml of the drug during the non-proliferative phase or in the proliferative phase of the parasite (11); this dose of Pentostam was very effective in reducing the number of amastigotes in infected macrophages (Fig. 2). Control cultures were not treated. The results of the treatment were assessed 24 hrs later and are expressed, as above, as the number of amastigotes/100 macrophages and the percent of infected macrophages. The results of this study (Fig. 3) indicate that, when treatment with Pentostam was done in the proliferative phase, the number of parasites in the infected macrophages and the percent of the infected cells were decreased to a significantly greater degree than when drug treatment was done in the non-proliferative phase on day 3.

Splenocytes stimulated with rIL-2 reduced the infection in vitro.

We were interested (1) to confirm that IL-2-stimulated splenocytes have the ability to reduce infections by *L. donovani in vitro*, (2) to determine an optimum dose for the recombinant lymphokine with which to best stimulate an effector function in splenocytes, (3) when would immunotherapy be most effective and (4) confirm that the activation of parasiticidal activity in the infected macrophages could be due to a soluble factor(s) released by the lymphokine-stimulated cells. The results presented in Fig.4 indicate that stimulated cells prepared from the splenocytes of infected mice and stimulated by as little as 125 U/ml of rIL-2 were able to significantly reduce both the number of amastigotes/100 macrophages and the percent of infected macrophages in *L. donovani* infections *in vitro* in the 48 hrs that followed the immunotherapy. There was no

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significant difference in the ability of splenocytes stimulated with 125 U/ml of rIL-2 to reduce the percent of infected macrophages *in vitro* when the immunotherapy was done either on day 3 or day 7; treatment with splenocytes stimulated with this dose of the lymphokine were, however, more effective in reducing the number of parasites in the infected cell when the treatment was done on day 3 than on day 7. Reduction of the infection by splenocytes stimulated by 250 or 500 U/ml of rIL-2 was significantly more effective when the treatment was done on day 3 in the non-proliferating phase of the infection than when the parasites were at the peak of their proliferative cycle on day 7. Spleen cells from infected animals, whether unstimulated by the lymphokine or incubated with rIL-2 in the presence of anti-IL-2, had no effect to reduce the infection; rIL-2 itself, at any of the doses used for stimulation had no apparent cytotoxic effect on the spleen cells (data not shown). The amount of monoclonal anti-IL-2 used in this study was able to neutralize the action of even 500 U of the lymphokine used to stimulate the splenocytes. It should be pointed out that the therapeutic effect of the lymphokine-stimulated cells was greater in reducing the number of parasites in the infected cells than in reducing the percentage of infected cells, especially when the splenocytes had been stimulated with the lowest dose of rIL-2 (125 U) used in this study. The results of this study also indicate that containing the lymphokine-stimulated cells in Millipore chambers did not interfere with the ability of these splenocytes to reduce the infection, confirming that the macrophage-activating capacity of these cells was due to the action of a soluble lymphokine(s) released by the stimulated cells (12).

Figure 3. Pentostam is most effective when used in the proliferative phase of the infection.

Anti-leishmanial effect ($\pm$SE) on infected C57BL/6 resting peritoneal macrophage monolayers treated with 15 μg Pentostam/ml on days 3 or 7 after the inoculation of 10^7 promastigotes; when treated, the infections were, respectively, in their non-proliferating or proliferating phases. The effect of the drug was assessed 24 hrs after treatment on methanol-fixed, Giemsa-stained smears.
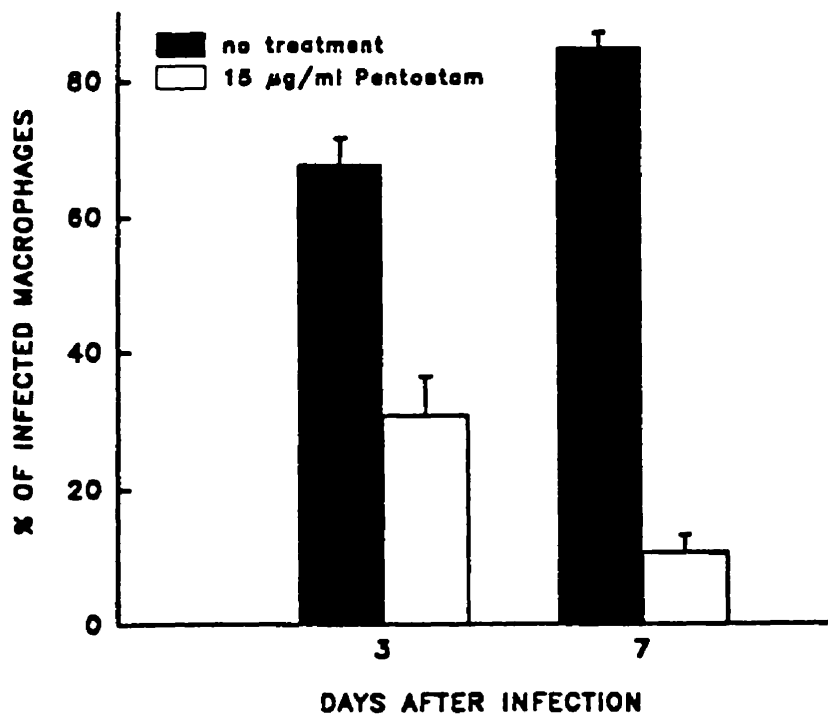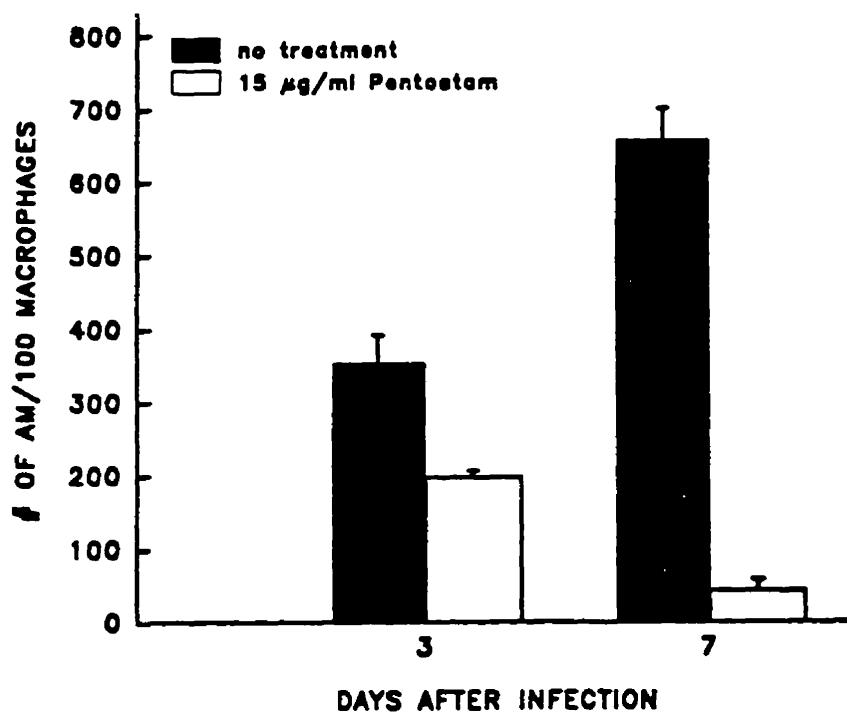
Figure 4. Splenocytes stimulated with rIL-2 reduce the infection in vitro best when used in the non-proliferating phase of the parasite.

Curative effect of rIL-2-stimulated splenocyte treatment on infected C57BL/6 resting peritoneal macrophage monolayers during the non-proliferating (3 days) or proliferating (7 days) phases of *L. donovani* infections *in vitro*; the monolayers were inoculated with 10^7 promastigotes. The splenocytes were obtained from mice inoculated 14 days earlier with 10^7 amastigotes intracardially and were stimulated with different amounts of rIL-2 in the presence or absence of an anti-IL-2 monoclonal antibody. Some of the stimulated splenocytes were placed in Millipore chambers. The infected manolayers were treated during the non-proliferating (3 days) or the proliferating (7 days) phases of the infection *in vitro*. The curative action of the rIL-2-stimulated splenocytes was determined 24 hrs after treatment.

A: untreated infected macrophages; **B, C, D:** treatment of infected macrophages with splenocytes stimulated by 125 U/ml r IL-2; **C:** stimulated splenocytes held in Millipore chambers; **D:** splenocytes incubated with IL-2 and anti-IL-2 monoclonal antibody; **E, F, G:** treatment with splenocytes stimulated by 250 U/ml rIL-2; **F:** as in **C**; **G:** as in **D**; **H, I, J:** splenocytes stimulated by 500 U/ml rIL-2; **I:** as in **C**; **J:** as in **D**.

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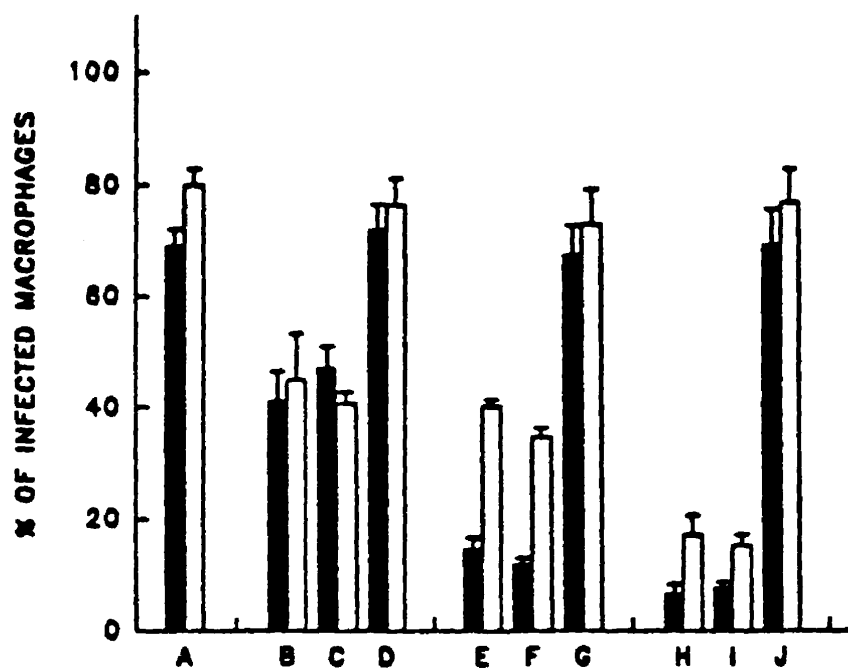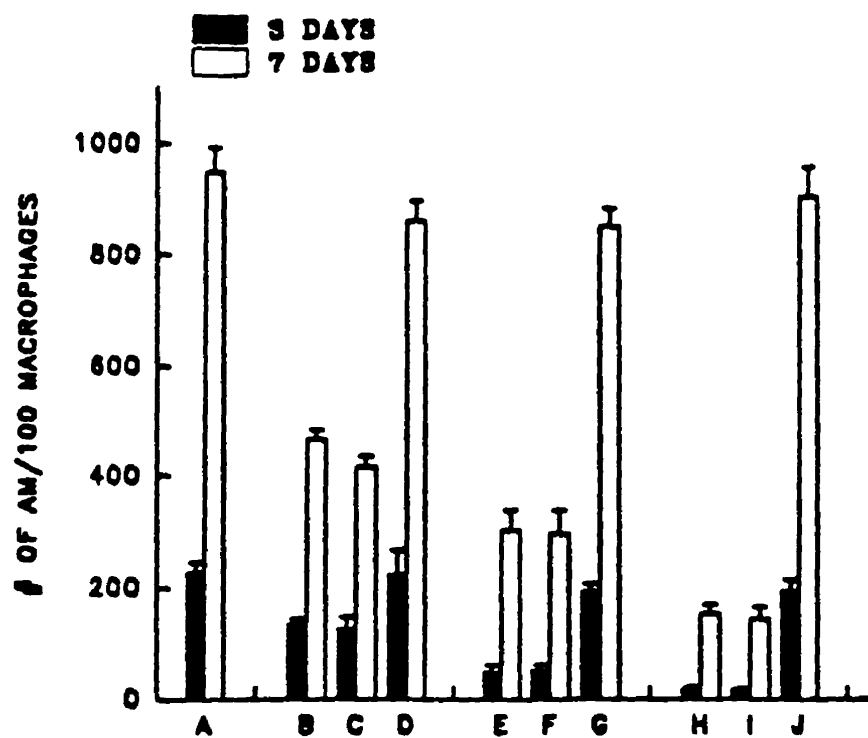
Treatment with rIL-2-stimulated splenocytes plus Pentostam is significantly more curative than either treatment alone.

Control assays in our previous report clearly indicate that lymphokine-stimulated cells prepared from splenocytes obtained from infected animals were superior effector cells in inducing leishmanicidal activity in the infected macrophages than those obtained from uninfected mice, especially when immunotherapy of the infected macrophages was in the non-proliferating phase and was followed continuously until day 11 (12). The combination strategy of immunotherapy with rIL-2-stimulated spleen cells on day 3, with the additional treatment of 15 μg/ml Pentostam starting on day 5 during the proliferative phase of the infection, reduced the number of parasites/100 macrophages and the percentage of infected cells *in vitro* on day 11 treatment was to levels (Fig. 5) significantly below those produced by either treatment alone (Figs. 2 & 3). The results illustrated in Fig.5 also indicate that immunotherapy done with stimulated cells prepared from the splenocytes of infected animals (+Pentostam) was more effective than when the effector cells were prepared from splenocytes of uninfected mice (+Pentostam). Removing the stimulated spleen cells from the cultures 24 hrs before Pentostam treatment was started on day 5 did not alter the effectiveness of the combined therapy to reduce the infection (data not shown). A greater curative effect was also not obtained when the infected macrophages were treated continuously with stimulated splenocytes from day 3 of the infection until the end of the experiment, in addition to Pentostam therapy starting on day 5.

Figure 5. Treatment with of rIL-2-stimulated splenocytes on day 3 and Pentostam on day 5 is significantly more curative than either treatment alone.

The curative effect on infected macrophages of splenocytes obtained from uninfected or infected C57BL/6 mice and stimulated by 250 U of rIL-2. The infected macrophage cultures were treated during the non-proliferative phase of the infection, starting on day 3, with the lymphokine-stimulated splenocytes and, starting on day 5, with 15 μg/ml Pentostam during the proliferative phase of the infection *in vitro*. The effect of the combined immuno- and chemotherapy on the infections *in vitro* was determined on days 4, 5, 8 and 11 after the monolayers following inoculation of the parasite.

(■) untreated infected macrophages; (⊟) infected macrophages treated for 48 hrs with splenocytes of uninfected mice stimulated by rIL-2 + Pentostam; (▨)infected macrophages treated for 8 days (until day 11) with splenocytes of infected mice stimulated with rIL-2 + Pentostam; (▦) infected macrophages treated for 48 hrs with splenocytes of infected mice stimulated with rIL-2 + Pentostam.
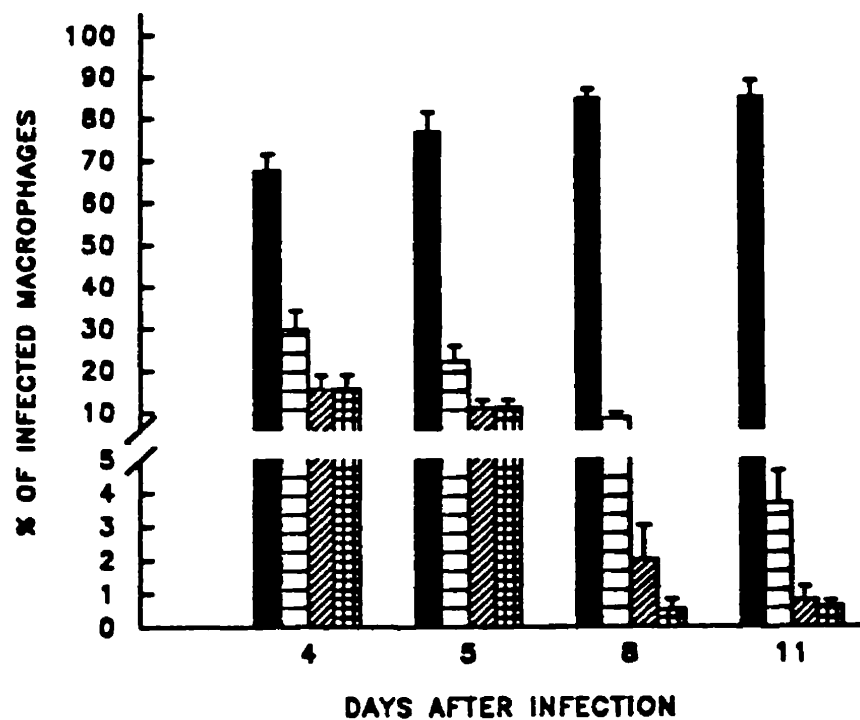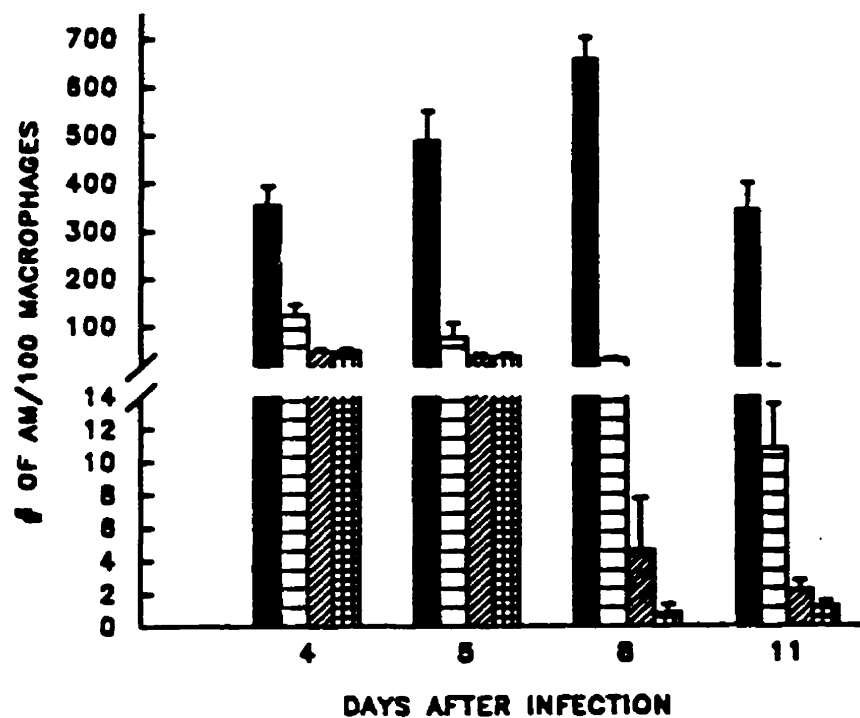
DISCUSSION

Although used for decades as the treatment of choice for visceral leishmaniasis, pentavalent antimonials have a number of well-recognized limitations which include potentially toxic adverse reactions and the possibility that the infecting strain is resistant to the drug. We confirm that the treatment of infected macrophages *in vitro* with non-toxic doses of Pentostam was effective in significantly reducing the number of parasites in infected macrophages (10) and we add the evidence that the drug in these doses also reduced the percentage of infected macrophages. The number of parasites per macrophage declined significantly in a dose-dependent fashion as the length of the treatment increased, especially in those macrophages treated with the two highest doses (15 or 20 μg) of the drug. This study also indicates that Pentostam was particularly effective when treatment *in vitro* was done in the proliferative phase of the parasite's intracellular life cycle; the rapid proliferation of the organism may have increased the uptake of the antimonial drug, and its consequent intraparasite concentration. The effectiveness of Pentostam depends, therefore, not only on the dose of the drug used, but also on the stage of the parasite's intracellular cycle when treatment is applied.

A significant feature of experimental and clinical visceral leishmaniasis is the down-regulation of immune functions (1), as exemplified by the suppressed response of T cells to stimulation by *Leishmania* antigen, or by Con A (13;18). Notwithstanding this fact, the results of this present study confirm the report that the suppressed splenocytes of infected animals can be rescued from immunosuppression by rIL-2, releasing a lymphokine(s) which activates infected macrophages to reduce their intracellular parasite

burden (12). The results reported here add, furthermore, the evidence that lymphokine-stimulated cells are especially effective when used for immunotherapy on the 3rd day of the infection *in vitro*, in the non-proliferative phase of the organism's intracellular life cycle (11). It has been proposed that IFN-γ is the principal lymphokine signal from the stimulated cells that activates leishmanicidal action in infected macrophages (18); the nature of the activating signal requires clarification, however, since we have presented evidence that monoclonal anti-IFNγ did not interfere with the ability of IL-2-stimulated splenocytes to activate infected macrophages to reduce their parasite burden (12). The supernatant of Con A-activated splenocytes is also able to cure infected macrophages *in vitro* (19), and it is curious that IL-2 alone does not have a curative effect *in vitro* (19), although it does reduce the infection *in vivo* (13), indicating that the mechanism by which cure is obtained *in vitro* is probably quite different than it is *in vivo*. Studies of the effect of the anti-IL-2 antibody to eliminate the stimulation of splenocytes for effector activity on infected macrophages were not controlled by a normal or an irrelevant immunoglobulin of the same isotype of the monoclonal anti-lymphokine. However, the possibility that the inactivation of the rIL-2 was achieved by factors released non-specifically from leukocytes following the binding of the immunoglobulin by its Fc receptor on the cells can be largely discounted. The number of macrophages in the suspensions of the spleen cells were reduced by adherence overnight and, thus, the number of cells (including some stimulated B cells) bearing FcγRI were diminished before the rIL-2 was added, reducing the possibility that non-specific anti-lymphokine would have been produced by attachment of the antibody by its Fc receptor.

This study confirmed that IL-2-stimulated splenocytes isolated from infected mice were more effective in inducing leishmanicidal activity in macrophages than lymphokine-stimulated cells from normal mice (12); despite the down-regulation of lymphocyte function that the parasite induces in infected animals (1), these cells continue to retain an ability to be stimulated by IL-2 (12). The fact that the cells isolated from infected mice are more efficacious in inducing leishmanicidal action in infected cells following lymphokine stimulation than the splenocytes of normal mice suggests that *L. donovani* "primes" the IL-2 signal pathways of lymphocytes to produce an enhanced susceptibility to activation by the lymphokine. It should be pointed out that this study was done using as infected target macrophages the cells of the innately-susceptible C57BL/6 mice; these macrophages are more difficult to cure from their infection with this parasite by immunotherapy than infected cells of innately-resistant mice (12). The results of this study indicate that immunotherapy on the non-proliferative phase of *L. donovani in vitro*, followed by chemotherapy with Pentostam in the parasite's proliferative phase, produced a greater reduction of the parasite burden than either treatment alone. The effectiveness of this strategy suggests that a rescue by immunotherapy from the immunosuppression induced by the parasite, followed by a direct drug attack on the organism, may very well provide a most effective means of eliminating infections by *Leishmania donovani*.

ACKNOWLEDGEMENTS

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CONNECTING STATEMENT IV

The results from the studies described above indicate that infection by *L. donovani*
can be reduced very effectively by treating infected mice *in vivo* or infected macrophages
in vitro with IL-2-stimulated splenocytes. The ability of immunotherapy with IL-2-
stimulated splenocytes to cure *L. donovani in vivo* and *in vitro* is clear evidence that
these stimulated splenocytes activate leishmanicidal pathways in infected cells and it was
of interest to determine if the cure which occurs after immunotherapy or chemotherapy
with Pentostam *in vitro* is associated with an activation of the oxygen and/or NO_2
responses.

Chapter VI

rIL-2-STIMULATED SPLENOCYTES ACTIVATE THE NO$_2^-$-PRODUCING ABILITY OF MACROPHAGES INFECTED BY LEISHMANIA DONOVANI[1]

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Running head: NO$_2^-$ production in leishmaniasis is activated by rIL-2-stimulated cells.

ABSTRACT

The obligate intramacrophage parasite *Leishmania donovani* has a multiplication

cycle in it's host cell: a non-proliferative phase until day 3-4, and a proliferative phase

from 5 to 8 days after infection. Although infection with *L. donovani* down regulates

immunity and parasite clearance by macrophages, rIL-2-stimulated splenocytes activate

leishmanicidal action *in vitro* in peritoneal macrophages and *in vivo* in C57BL/6 (Lsh^r)

mice. Treatment with rIL-2-stimulated splenocytes for 48-72 hrs *in vitro* was effective

in stimulating infected macrophages to produce NO$_2^-$, whereas Pentostam had no effect

on this pathway. The "oxygen burst" response is eliminated in *L. donovani*-infected

macrophages; neither Pentostam nor rIL-2-stimulated splenocytes were able to activate

this microbicidal mechanism. Pentostam is a specific anti-amastigote drug which probably

has a direct effect on the parasite; rIL-2-stimulated splenocytes may produce their

curative effect in *L. donovani* leishmaniasis by stimulating the parasiticidal nitrogen oxide

mechanism in macrophages.

INTRODUCTION

The *Leishmania* are parasitic protozoans that produce diseases worldwide that, depending on the parasite species involved, range from relatively "benign" cutaneous infections to the potentially lethal visceral disease produced by *Leishmania donovani* (1). The members of this genus present a remarkable example of intracellular parasitism because their amastigote stage only parasitizes macrophages, a principal phagocytic cell and the leukocyte which is responsible for initiating cellular and humoral immune defenses against invading microrganisms (2-4). *L. donovani* survives in its intramacrophage habitat, at least in part, because of its ability to profoundly down regulate the infected cell and the immunological functions of the host's lymphocytes (5).

The fact that macrophages are the specific host of *L. donovani* clearly indicates that intracellular microbicidal pathways, the more important ones of which are the oxygen and nitrogen intermediates (6), are not activated during the course of the infection. It has been shown that oxygen radicals are not produced in macrophages infected by *L. donovani* (7,8), the parasite inhibiting the stimulus-response coupling through calcium- and PKC-dependent pathways (9-11). The L-arginine-dependent production of inorganic nitrogen oxides (11,14,18) is also an effector pathway that can act against *L. major* (15-17) and, when stimulated by IFN-γ and TNF-α, it also reduces infections with *L. donovani* (18). Infection by *L. donovani* can also be reduced very effectively by treating infected mice *in vivo* (19) or in infected macrophages *in vitro* (20)

with spleen cells previously stimulated by IL-2 *in vitro*, especially when the infected macrophage is also treated with a non-toxic dose of the leishmanicidal drug Pentostam (21). The ability of immunotherapy with IL-2-stimulated splenocytes to significantly reduce *L. donovani in vivo* and *in vitro* is clear evidence that these stimulated splenocytes activate leishmanicidal pathways in infected cells and it was of interest to determine if the cure which occurs after immunotherapy is associated with an activation of the oxygen and/or the NO_2^- responses. We (22) have shown that *L. donovani* goes through a proliferation cycle in macrophages and it was also of interest, therefore, to determine when, during this cycle, are reactive intermediates most readily activated by IL-2-stimulated splenocytes. A study was also made to determine if the leishmanicidal action of Pentostam is due to direct toxicity of the drug on the parasite, or whether it acts by activating down regulated microbicidal responses. The results of this study show that immunotherapy failed to activate H_2O_2 and the O_2^- responses, but did activate the production of NO_2^-. Anti-IFN-γ failed to completely block the activation of the NO_2^- response induced by the IL-2-stimulated splenocytes. Pentostam did not reactivate the production of either the reactive oxygen, nor the NO_2 intermediates.

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MATERIALS AND METHODS

Animals and the parasite

Female C57BL/6 (H-2^b; Lsh^s) mice, aged from 4 to 6 weeks, and female Syrian

hamsters (Lak:Lvc) were purchased from the Charles River Laboratories (St-Constant,

Québec). The LV9 strain of *Leishmania donovani* was maintained in the laboratory by

the periodic passage of amastigotes in hamsters; amastigotes obtained from the spleens

of infected hamsters were transformed into promastigotes for the inoculation of

macrophage monolayers, as previously described (22). All animals were euthanized with

CO_2 before the parasite or tissues were recovered from them.

Collection and infection of mouse peritoneal cells

Resting cells were harvested from normal, unstimulated C57BL/6 mice by lavage

of the peritoneal cavity with 5 ml of ice-cold RPMI 1640 medium (Gibco, Grand Island,

NY), supplemented by 10% fetal bovine serum (FBS;GIBCO), 100 μg streptomycin

(Sigma, St. Louis, MO) and by 100 U/ml penicillin (Sigma), as described by Mishell and

Shiigi (23); unless otherwise indicated, this supplemented medium was used throughout

this study and will be referred to hereafter as RPMI. After collection, the normal

peritoneal cells were counted in a hemacytometer with trypan blue and made up to 10^6

viable cells/ml RPMI. One hundred μl aliquots of this suspension were placed in each

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well of 96-well culture plates (ICN Pharmaceuticals Ltd., Montreal, Québec) and the macrophages were allowed to adhere overnight; all cell cultures in this study were incubated at 37°C in humidified 5% CO_2 in air. The non-adherent cells were then removed by gentle washing of the cell carpet with warm (37°C) RPMI. After washing, the adhered macrophages were cultured for a further 2 hrs prior to exposure to 10^7 metacyclic promastigotes. The parasites were allowed to internalize for 2 hrs and free promastigotes were then removed from the cultures by gentle washing with warm RPMI (22).

Splenocytes

Splenocytes from uninfected mice, or from mice that had been inoculated intracardially 2 wks earlier with 10^7 amastigotes, were obtained from these animals by passing the spleen aseptically through a stainless-steel screen (60 mesh, 80 gauge) with a glass pestle into ice-cold RPMI; erythrocytes in the cell suspension were lysed with Gey's salts (23). Before stimulation with rIL-2, the number of macrophages in the splenocyte cell suspensions was reduced by adherence overnight at 37°C in petri dishes, to diminish the number of potential suppressor cells (24). Non-adherent spleen cells were recovered from the adhered macrophages by washing the cell carpet with warm RPMI; the non-adherent spleen cells were then washed twice with cold RPMI, counted in a hemacytometer with trypan blue and adjusted to 5×10^6 viable cells/ml. The viability of all cell preparations was at least 95% by trypan-blue exclusion.

Stimulation of splenocytes with rIL-2

Several aliquots of 1 ml RPMI, containing 5×10^6 viable splenocytes (from which

the number of macrophages had been reduced by adherence), obtained from uninfected or from infected C57BL/6 mice inoculated 7 days earlier with 10^7 metacyclic promastigotes, were seeded into each well of 6-well Linbro culture plates (ICN) and stimulated with rIL-2 (Cedarlane, Hornby, Ontario) at a concentration of 250 U rIL-2/ml RPMI (19). The splenocytes were harvested 72 hrs after stimulation, pooled, washed 3x with RPMI and re-adjusted to 5×10^6 viable cells/ml.

Chemo- and immunotherapy

It was of interest to determine if the production of NO_2^- and $H_2O_2-O_2^-$ by macrophages was a function of when, during the intracellular proliferative cycle of *L. donovani*, infected cells were treated with lymphokine-stimulated splenocytes, or with Pentostam. Normal peritoneal macrophage monolayers in 96-well Linbro culture plates (ICN) were, therefore, inoculated with 10^7 promastigotes (22) and, 3 or 7 days after inoculating the parasite, they were washed and treated with RPMI alone, or with 10^6 viable rIL-2-stimulated splenocytes obtained from uninfected or from infected mice. Some infected macrophage monolayers were treated on days 3 or 7 of the infection with a dose of 15 μg/ml of Pentostam alone, or in combination with the immunotherapy. Other infected and uninfected macrophage monolayers were treated with washed rIL-2-stimulated splenocytes in the presence of the monoclonal anti-IL-2 antibody produced by S4B6 cells (ATCC HB 8794) to neutralize any IL-2 produced by the lymphokine-stimulated cells which, reacting with possible IL-2 receptors on macrophages, might have enhanced the action of the immunotherapy.

Measurement of the production of nitrites, superoxide and hydrogen peroxide

The supernatant of infected macrophage monolayers, prepared as described above, was removed and discarded 72 hrs after treatment with lymphokine-activated splenocytes and/or Pentostam on days 3 or 7 after infection by the parasite (22). Treated or untreated infected or uninfected monolayers were stimulated to produce NO_2^- by the addition of 100 μl of a solution of 10 ng of LPS (Sigma)/ml RPMI, plus 100 μl of a solution containing 100 U rIFN-γ (Genzyme)/ml RPMI. In some cultures 25 ng/ml of anti-IFN-γ had been added to inhibit any stimulation of the infected macrophages by IFN-γ produced by the lymphokine-stimulated splenocytes.The cultures were incubated in the presence of the stimulators for 24 or 48 or 72 or 96 hrs and the supernatant was tested for the presence of NO_2^-, or O_2^- or H_2O_2. Nitrites were determined by using freshly-prepared Griess reagent (18); this reagent, which contains 1% sulphanilamide (Sigma) and 0.1% naphthalenediamine HCl (Sigma), was added 1:1 to the supernatants and the reaction was read 5 minutes later at 570 nm using a Dynatech MR 5000 microplate reader (Fisher Scientific, Montréal, Québec); sodium nitrate standards in the range of 1 to 100 μM were included on each plate. Aminoguanidine inhibits the metabolism of L-arginine and, thus, the production of NO_2^-; some assays were, therefore, done in the presence of 0.5 mM of this inhibitor to determine the specificity of the reaction observed in this study.

The production of H_2O_2 into the supernatants of infected macrophages was determined by the relative ability of the supernatant to catalize the oxidation of 0.01% phenol red by horseradish peroxidase (25); the production of the superoxide intermediate O_2^- was determined by the relative ability of the supernatants to reduce ferric cytochome C (26). Quantitation of these reactions was done spectrophotometrically at 610 nm for

H_2O_2 and at 550 nm for O_2^- using a Dynatech 5000 (Fisher) microplate reader.

MTT colorimetric assay

The relative number of viable parasites in infected and control macrophage monolayers, prepared as described above, were determined with the MTT assay for *L. donovani* promastigotes (27) before or after drug treatment or immunotherapy. Treated infected peritoneal macrophage monolayers cultures were washed once with RPMI, lysed with SDS (28) and then incubated for 72 hrs in RPMI containing 17% FBS (Gibco). Ten μl of a stock solution of 5 mg MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-phenyltetrazolium bromide; Sigma) per ml PBS was then added to each culture (29) which now contained the promastigotes into which the amastigotes released from the infected macrophages during lysis had transformed. The stock solution had been sterilized by passage through a 0.22 μm Millipore filter and was kept at 4°C for no more than 14 days after preparation. The reactions were incubated at 23°C for another 16 hrs when the blue formazan crystals produced by mitochondrial dehydrogenase activity were solubilised (30) in the 100 μl of 10% SDS added to each well of the culture plate. The cultures were incubated for further 16 hrs at 37°C and then the optical density of each well was read at 570 nm with a Dynatech 5000 (Fisher) microplate reader; the reference beam was set at 630 nm and the blank contained RPMI+MTT+10% SDS. An excellent direct relationship exists between the adsorption values obtained at 570 nm and the numbers of promastigotes present in culture (31).

Statistical analysis

The results of this study were analyzed by the non-parametric Kruskal-Wallis

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ANOVA test and the statistical significance between paired data (eg: control vs treatment; treatment 1 vs treatment 2) was obtained from the Student-Newman-Keul test using the SigmaStat (18) statistical software. The difference between two results was taken as significant when the P value was 0.05, or lower.

RESULTS

rIL-2-stimulated splenocytes and/or Pentostam do not stimulate the production of H_2O_2 or O_2 by macrophages infected by L. donovani

The data obtained in this study (Tables 1A and 1B) indicate that internalization of *L. donovani* abolishes the ability of normal, unstimulated mouse peritoneal macrophages to produce reactive oxygen intermediates *in vitro*. The data also indicates that neither curative treatment with rIL-2-stimulated splenocytes and/or Pentostam was unable to induce the infected cells to produce either H_2O_2 or O_2^-. Control untreated, uninfected macrophages did, however, produce a significant amount of both H_2O_2 and O_2^- after treatment, especially after the third day in culture. The production of the two oxygen intermediates by uninfected cells was enhanced by treatment with rIL-2-stimulated splenocytes alone, or in combination with Pentostam; drug treatment of uninfected cells also produced a significant reactive oxygen intermediate response, although not as intense as that stimulated by the immunotherapy.

NO_2^- production by peritoneal macrophages infected by L. donovani is activated by treatment with rIL-2-stimulated splenocytes at different phases of the parasite's proliferative cycle in vitro

Uninfected peritoneal macrophages, or peritoneal macrophages infected *in vitro* with *L. donovani* did not produce NO_2^- (Figure 1A), but the production of this microbicidal intermediate was activated by treatment, either with splenocytes obtained from infected, or uninfected mice that had been stimulated *in vitro* with rIL-2, or by

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Table 1A: Production of H_2O_2 into the supernatants of infected macrophages treated with different immuno- and/or chemotherapy regimes ($?M/hr/mg$ protein)

DAY	I	U	I+L	U+L	I+NL	U+NL	I+L+P	U+L+P	I+NL+P	U+NL+P	I+P	U+P
1	24±5	51±10	-	-	-	-	-	-		-	-	-
2	46±11	96±12	-	-	-	-	-	-	-	-	-	-
3	48±8	106±16	52±5	265±41	47±24	237±41	-	-	-	-	-	-
4	52±11	260±33	76±16	336±49	51±20	304±16	48±5	180±11	-	135±15	46±10	145±15
5	47±12	155±20	64±7	340±43	48±6	287±14	55±11	260±35	44±10	190±37	37±5	217±12
6	44±6	109±10	62±12	296±10	36±10	265±15	50±20	220±18	40±8	150±30	36±7	185±22
7	40±15	101±15	50±15	215±32	35±4	195±30	42±9	145±20	38±6	145±45	32±8	135±17

I: infected macrophages; U: uninfected macrophages; I+L: infected macrophages treated with rIL-2-stimulated splenocytes recovered from infected mice (LAK); U+L: uninfected macrophages treated with LAK cells; I+NL: infected macrophages treated with rIL-2-stimulated splenocytes recovered from uninfected mice (NLAK); U+NL: uninfected macrophages treated with NLAK cells; I+L+P: infected macrophages treated with LAK cells and Pentostam; U+L+P: uninfected macrophages treated with LAK cells and Pentostam; I+NL+P: infected macrophages treated with NLAK and Pentostam; U+NL+P: uninfected macrophages treated with NLAK and Pentostam; I+P: infected macrophages treated with Pentostam; U+P: uninfected macrophages treated with Pentostam

Table 1B: Production of O_2^- into the supernatant of infected macrophages treated with different immuno- and/or chemotherapy regimes ($^mM/hr/mg$ protein)

DAY	I	U	I+L	U+L	I+NL	U+NL	I+L+P	U+L+P	I+NL+P	U+NL+P	I+P	U+P
1	17±5	55±23	-	-	-	-	-	-	-	-	-	-
2	26±15	73±15	-	-	-	-	-	-	-	-	-	-
3	37±8	94±30	40±6	112±11	39±4	95±20	-	-	-	-	-	-
4	49±20	120±11	50±10	190±20	48±15	150±15	80±10	345±34	53±31	324±26	46±11	256±14
5	47±12	94±5	52±12	280±41	48±10	220±20	83±13	309±15	49±4	300±16	48±15	173±21
6	36±12	66±20	48±20	200±30	43±5	195±35	68±15	272±33	40±14	294±24	40±12	135±23
7	35±17	55±23	43±8	156±25	38±8	135±30	55±20	241±20	39±9	199±15	38±3	99±15

I: infected macrophages; U: uinfected macrophages; I+L: infected macrophages treated with rIL-2-stimulated splenocytes recoverd from infected mice (LAK); U+L: uninfected macrophages treated with LAK cells; I+NL: infected macrophages trated with rIL-2-stimulated splenocytes recovered from uninfected mice (NLAK); U+NL: uninfected macrophages treated with NLAK cells; I+L+P: infected macrophages treated with LAK cells and Pentostam; U+L+P: uninfected macrophages treated with LAK cells and Pentostam; I+NL+P: infected macrophages treated with NLAK cells and Pentostam; U+NL+P: uninfected macrophages treated with NLAK and Pentostam; I+P: infected macrophages treated with Pentostam; U+P: uninfected macrophages treated with Pentostam

treatment with LPS+IFN-γ. However, the presence of 0.5 mM aminoguanidine, a competitive inhibitor of L-arginine metabolism, reduced the ability of macrophages to produce NO_2^- (Figure 1B), following immunotherapy and/or Pentostam treatment. Although immunotherapy with lymphokine-stimulated splenocytes was somewhat less effective in activating the NO_2^- pathway in infected macrophages than LPS+IFN-γ (Figure 1A), the results obtained in this study indicate that significantly more NO_2^- was produced when treatment with the rIL-2-stimulated splenocytes was done in the non-proliferative (day 3) phase of the parasite's life cycle *in vitro*. As a matter of fact, when the immunotherapy was done in the proliferation phase of the parasite's replication *in vitro* on day 7, the amount of NO_2^- produced by the infected cells was reduced to the level (Figure 1A: D&F) of aminoguanidine-inhibited cultures.
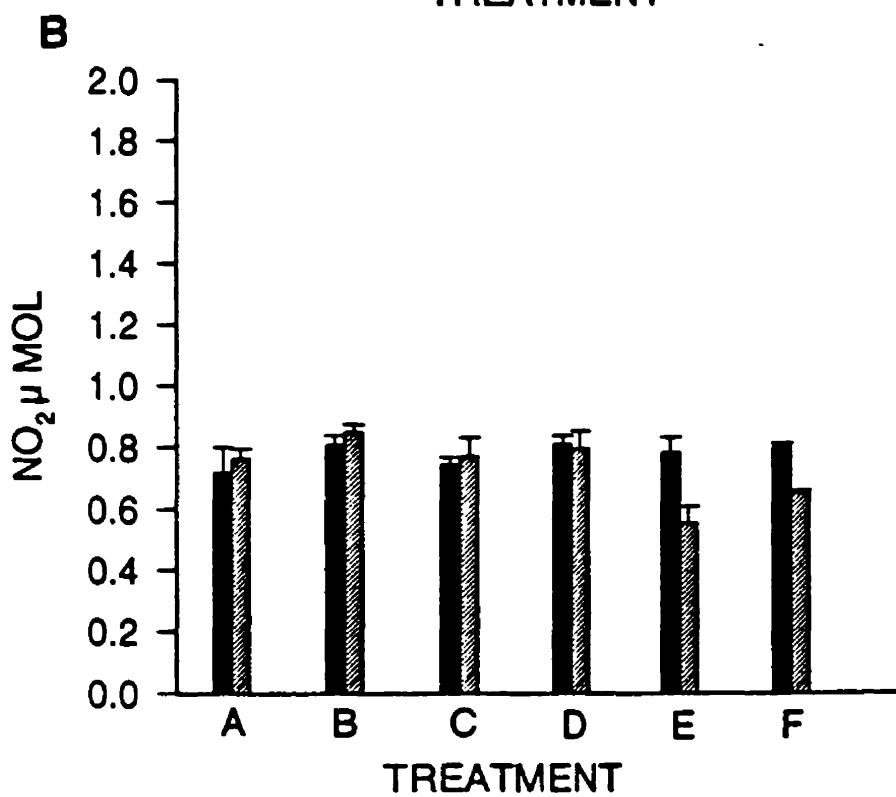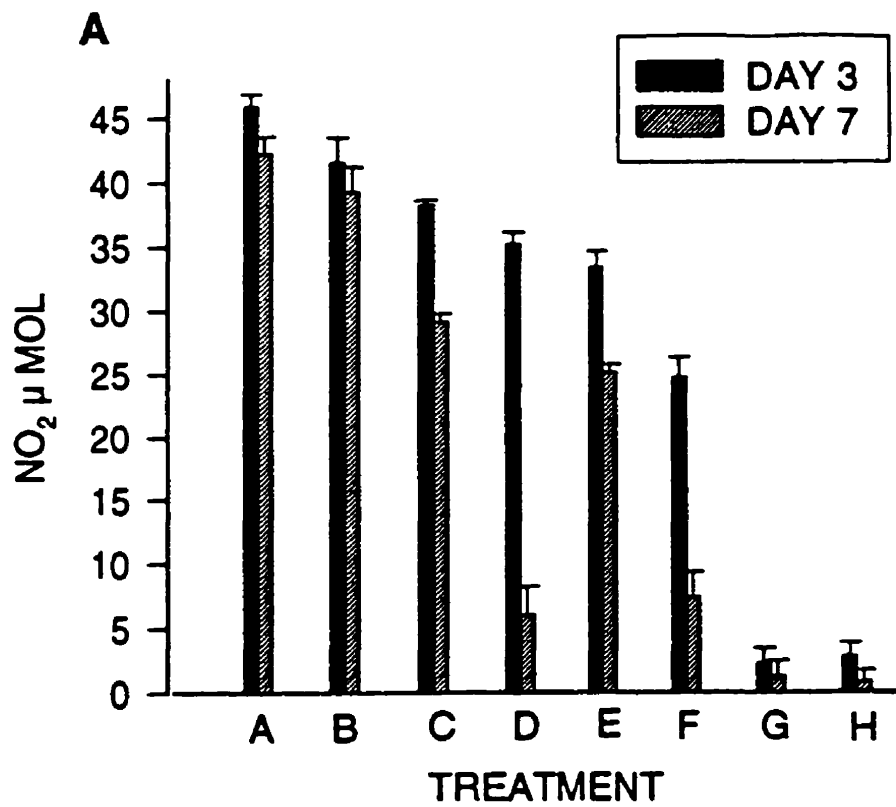
Time course of the production of NO_2^- by L. donovani-infected peritoneal macrophages activated by rIL-2-stimulated splenocytes, or by LPS and IFN-γ

The results presented in Figure 2 A and B clearly show that the production of NO_2^- by uninfected or infected macrophages treated with rIL-2-stimulated splenocytes or by LPS+rIFN-γ rises gradually in the days following treatment, to produce a maximum response 72 hrs later; untreated infected or uninfected macrophages did not produce NO_2^-. LPS+rIFN-γ was a better stimulator of the production of NO_2^- from infected or uninfected peritoneal macrophages than were the rIL-2-stimulated splenocytes. Lymphokine-stimulated splenocytes from infected mice (LAK) were more effective stimulators of the production of NO_2^- from uninfected or infected macrophages than were the stimulated splenocytes that had been obtained from uninfected animals (NLAK). A

Figure 1. The ability of peritoneal macrophages, infected or not by *L. donovani*, to produce NO_2^- when activated during the non-proliferating or proliferating phases of the infection *in vitro* in the absence (**A**) or presence of 0.5 mM aminoguanidine (**B**). *

* Resting peritoneal macrophage monolayers in 96-well Linbro plates were inoculated with 10^7 promastigotes and then, 3 or 7 days after inoculation they were each treated with 10^6 rIL-2-stimulated splenocytes obtained from uninfected (NLAK) or infected (LAK) C57BL/6 mice, or with 10 ng LPS + 100U of rIFN-γ.

A: uninfected macrophages + LPS & rIFN-γ; B:infected macrophages + LPS & rIFN-γ ; C: uninfected macrophages + LA K; D: infected macrophages + LAK; E: uninfected macrophages + NLAK; F: infected macrophages + NLAK; G: uninfected macrophages; H: infected macrophages.

Figure 2. Time course of the production of NO_2 by *L. donovani*-infected

peritoneal macrophages activated by rIL-2-stimulated splenocytes, or

by LPS + IFN-γ.

(○) infected macrophages; (●) uninfected macrophages; (▽)

infected macrophages + LAK; (▼) uninfected macrophages+LAK;

(□) Infected macrophages+NLAK; (■) uninfected

macrophages+NLAK; (△) Infected macrophages+LPS+IFNγ;(▲)

uninfected macrophages+LPS+IFNγ.
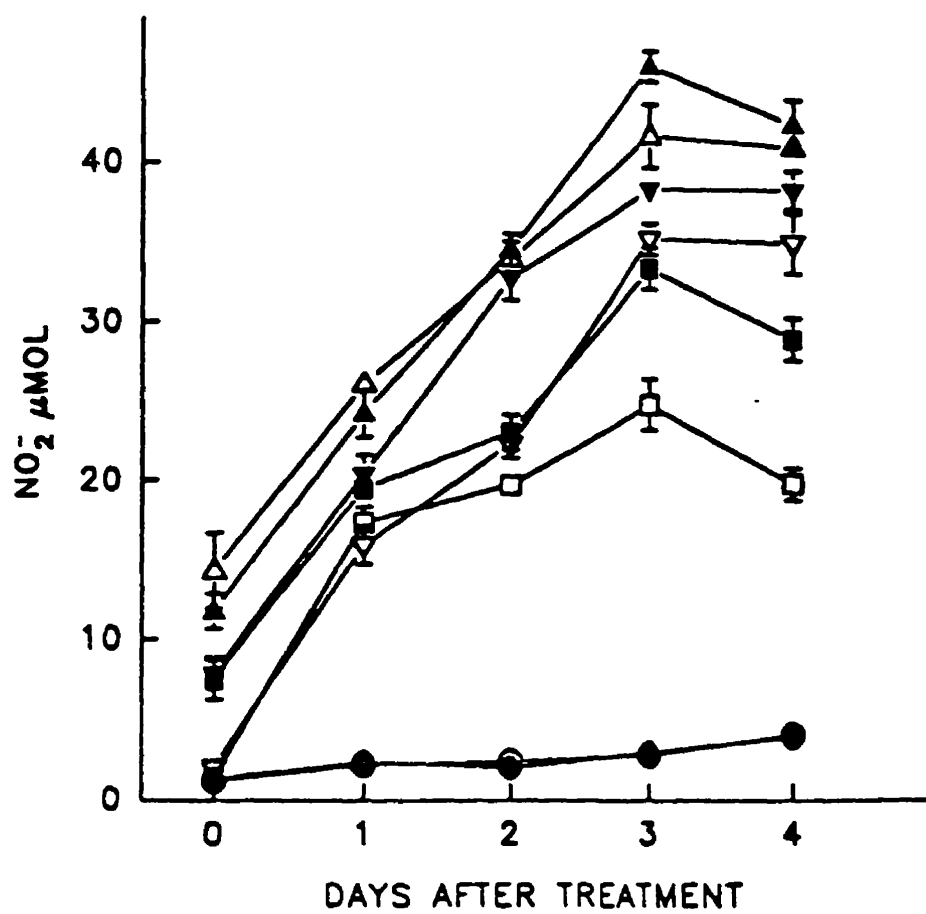
requirement for the production of NO_2^- of a first IFN-γ signal preceding LPS stimulation is indicated by the fact that the anti-IFN-γ monoclonal antibody was able to block the production of the NO_2^- microbicidal intermediate (Figure 3). Anti-IL-2 antiserum used as an antibody control at the time of treatment with LAK cells had no effect to enhance or to reduce the activation of the NO_2^- response by LPS+rIFN-γ, nor by washed rIL-2-stimulated splenocytes (Figure 4 A and B). However, the ability of immunotherapy with IL-2-stimulated splenocytes to activate the production of NO_2^- was, however, reduced to about 50% by the use of the monoclonal anti-IFN-γ.

Assessment of the leishmanicidal activity of rIL-2-stimulated splenocytes and Pentostam by the MTT reaction at different times of the infection

The MTT leishmanicidal assay, as a measure of the dehydrogenase activity in promastigotes derived from infected peritoneal macrophages, can be a convenient means of determining the fate of an infection by *L. donovani in vitro*. The results presented in Figure 5 confirm previous results (19,21) that both rIL-2-stimulated splenocytes from infected or from uninfected animals trigger a significant leishmanicidal activity and that this activity is greatest when the immunotherapy is done at day 3 in the non-proliferative phase of the parasite's life cycle *in vitro*. The results of this study also confirm (21) that Pentostam has an effect to produce an enhanced curing action *in vitro* when combined with immunotherapy. The combined use of Pentostam with the rIL-2-stimulated splenocytes appears, by the MTT assay, to have the added advantage that the combined treatment is equally effective when used in both the non-proliferative or the proliferative phases of the parasite's life cycle *in vitro*.

Figure 3. Time course of the production of NO_2 by *L. donovani*-infected

pritoneal macrophages treated with LPS + IFN-γ in the presence, or

absence of anti-IFN-γ monoclonal antibody, or with an anti-IL-2

monoclonal antibody.

(○) Infected macrophages+LPS+IFNγ; (●) Uninfected

macrophages + LPS + IFNγ; (▽) Infected

macrophages + LPS + IFNγ + Anti IFNγ; (▼) Uninfected + LPS

+ IFNγ + Anti IFNγ; (□) Infected macrophages + LPS + IFNγ

+ AntiIL-2; (■) uninfected + LPS + IFNγ + Anti IL-2.
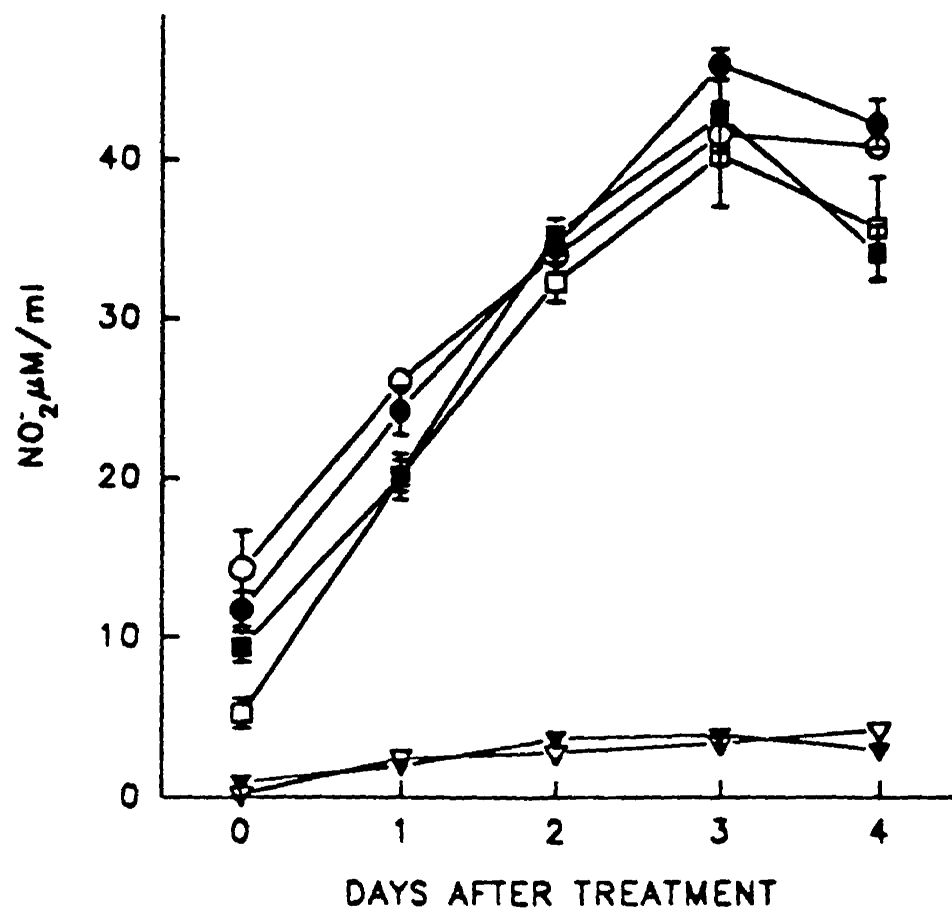
Figure 4.   Time course of the effect of an anti-IL-2 monoclonal antibody (A) and an anti-IFN-γ monoclonal antibody (B) on the ability of rIL-2-stimulated splenocytes to induce the production of NO^2 from resident peritoneal macrophages infected by *L. donovani*.

(●) Infected macrophages+LAK; (■) Uninfected macrophages + LAK; (▲) infected macrophages + LAK + Anti IL-2; (▼) Uninfected macrophages+LAK+Anti IL-2; (○) Infected macrophages+LAK+Anti IFNγ; (□) Uninfected macrophages + LAK + Anti IFNγ; (△) Infected macrophages + LAK + Anti IL-2+ Anti IFNγ; (▽) Uninfected macrophages + LAK+ Anti IL-2 + Anti IFNγ.
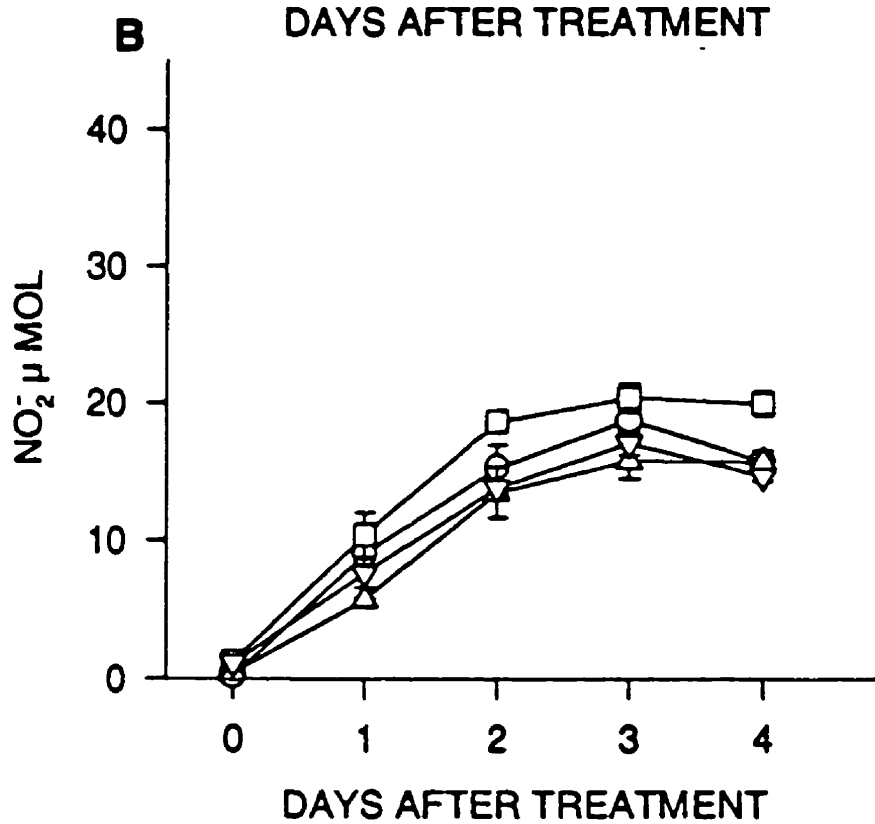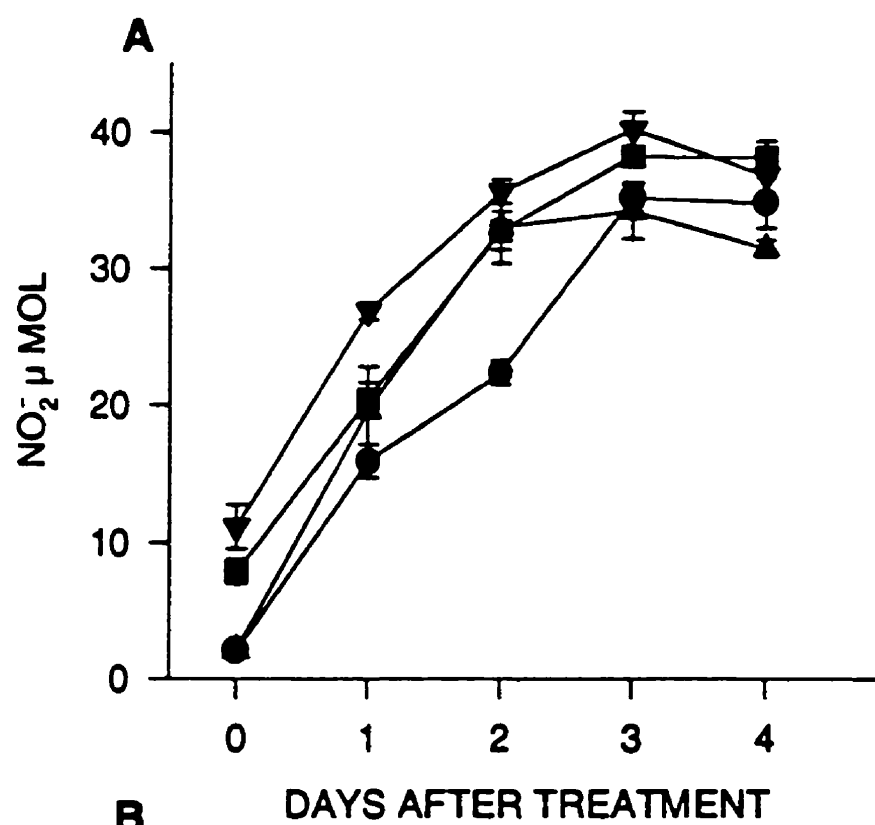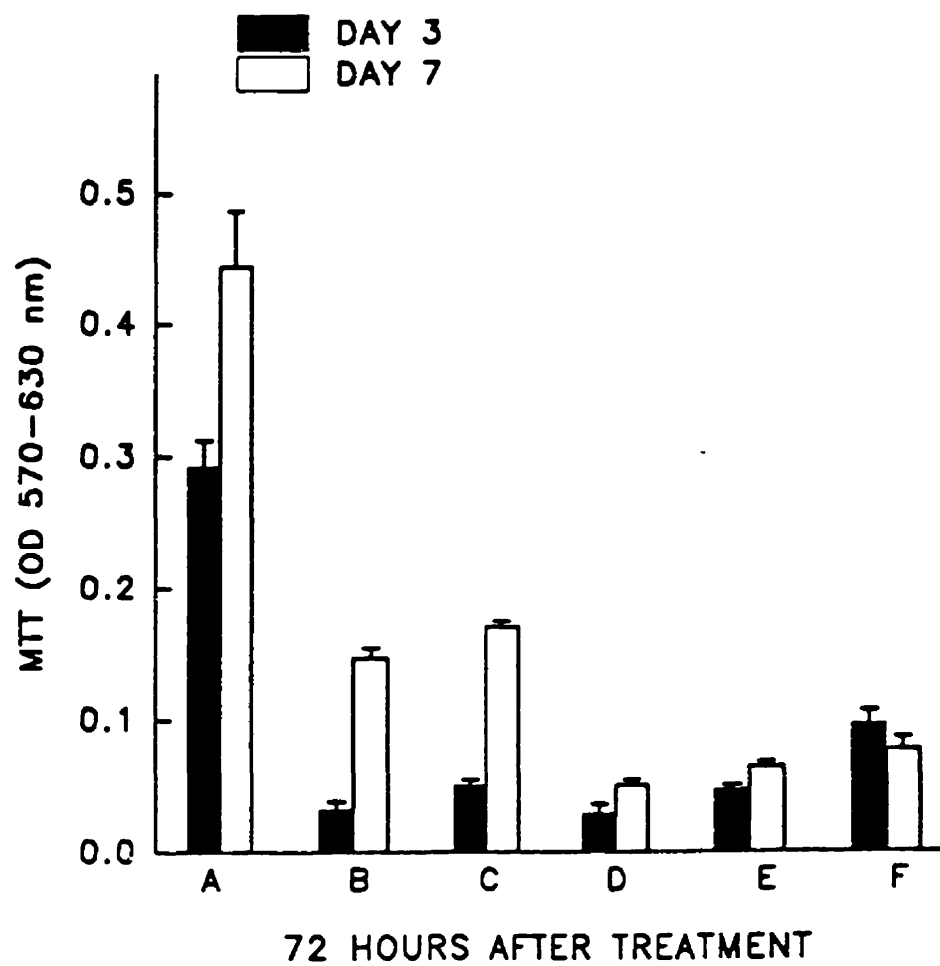
Figure 5. Assessment of the leishmanicidal action by the MTT reaction of treatment by rIL-2-stimulated splenocytes and/or Pentostam during the reproductive cycle of *L. donovani in vitro*.

A= untreated; B= LAK; C= NLAK; D= LAK + Pentostam;

E= NLAK + Pentostam; F= Pentostam.

72 HOURS AFTER TREATMENT

DISCUSSION

The production of nitric oxide by lymphokine-activated macrophages has been correlated with a resistance to infection with *Leishmania*, or with other obligate intracellular pathogens, such as *Toxoplasma gondii* and *Mycobacterium bovis* (32,33). Macrophages stimulated by IFN-γ produce a moderate level of NO_2^-, but exposure to a second signal from LPS, or TNF-α or β, results in an enhanced significant up regulation of the production of the nitrogen intermediates to significant microbicidal levels (14, 34-36). Our results confirm that LPS + IFN-γ are excellent stimulators of the production of NO_2^- in experimental visceral leishmaniasis produced by *L. donovani in vitro*; we also show, however, that treatment with rIL-2-stimulated splenocytes can also stimulate the secretion of high levels of NO_2^-, without the need of a second activating signal. Previous studies (22) have indicated that there is a proliferative cycle exhibited by *L. donovani* inside the macrophage: a non-proliferative phase *in vitro* that lasts until about day 4 and a proliferation phase, that begins approximately on day 5 and reaches a maximum about day 7 of the infection. Immunotherapy or a curative measure is most effective in the non-proliferative phase (21) and chemotherapy with Pentostam reduces infection with *L. donovani* most effectively when used in the proliferative phase. The results of the present study suggests that the activation of the secretion of NO_2^- is greatest when immunotherapy was done in the non-proliferative phase.

For a long time, the reactive oxygen intermediates have been thought to be the macrophage's major killing mechanism for leishmanicidal activity. Attachment and entry of promastigotes and amastigotes into the macrophage should elicit a respiratory burst

(7,8) and, although reactive oxygen intermediates have been shown to be toxic for log-phase promastigotes, intracellular amastigotes appear to be able to withstand this microbicidal mechanism (37,38). The resistance of amastigotes to reactive oxygen intermediates has been postulated to be due to the effect of a greatly up-regulated level of parasite-produced endogenous catalase, peroxidase, or superoxide dismutase (39,40). Our results confirm that, after internalization of the parasite in the macrophage, reactive oxygen products are not produced and add that immuno- or chemotherapy has no effect to activate this microbicidal agent.

Our previous results (19) indicate that monoclonal anti-IFN-γ was unable to completely eliminate the ability of rIL-2-stimulated splenic T cells to reduce *L. donovani* infection in macrophages; such a result suggests the intriguing possibility that other lymphokines might also be involved in reducing the intensity of infection by immunotherapy. Results of the studies reported here indicate that the same anti-IFN-γ treatment decreased by half the secretion of NO_2^- by macrophages treated by IL-2-stimulated splenocytes, suggesting that, either the full potential of the macrophage to produce nitrogen intermediates is not required for eliminating the intracellular infection, or that the NO_2^- pathway is not the only leishmanicidal effector induced by curative immunotherapy; an additional pathway is probably activated by its own lymphokine signal from the rIL-2-stimulated splenocytes. Such an additional leishmanicidal activity pathway is certainly not for the production of oxygen intermediates, since we clearly show that infection with *L. donovani* abolishes the ability of infected macrophages to produce H_2O_2 and O_2^- and that curative immunotherapy and/or Pentostam treatment do

not activate this microbicidal mechanism; stimulation of oxygen intermediates is, therefore, not required to cure experimental visceral leishmaniasis. *Leishmania donovani* produces an immunosuppressed state (24, 41-45) and the combination of immunochemotherapy has proved to be a significantly more effective treatment than either immunotherapy or chemotherapy alone (21). In this respect we were interested to discover that the MTT method of assessing the effectiveness of anti-leishmanial therapy generally supports our previous reports, especially that of the superior effectiveness of immunotherapy in the non-proliferating early phase of the infection. The results of this assay indicate, however, that the drug Pentostam may be equally effective in the non-proliferative as in the proliferative phase of the parasite's life cycle, eliminating the selective effect of combined immuno- or chemotherapy to a specific phase of the parasite's intracellular cycle.

This study shows again that immunotherapy using lymphokine-activated spleen cells, plus drug treatment with Pentostam is an effective agent against *Leishmania donovani* and that the action of immunotherapy is likely through the activation of the leishmanicidal NO_2^- mechanism.

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GENERAL SUMMARY AND DISCUSSION

The members of the genus *Leishmania* are obligate intracellular parasites of the macrophages of vertebrates. These organisms cause a wide range of clinical disease and infectious processes which have asymptomatic, cutaneous, mucosal, or visceral manifestations in humans, depending on the species of the parasite and on the immune response produced by the host. The *Leishmania* are inoculated into the vertebrate by phlebotomine arthropod vectors as metacyclic promastigotes, penetrating the macrophages of their hosts, where they proliferate as amastigotes in the phagolysosome of the cell.

1. It is very well-known that the progress in mice of an infection by *Leishmania donovani* can be divided into innate and chronic phases. Some of the results presented in this study show that the development of *L. donovani* in its obligate macrophage host is also characterized by distinct proliferative phases of the organism. After inoculating peritoneal macrophage cultures of C57BL/6 mice *in vitro* with as few as 5 or as many as 500 amastigotes/cell, only 3 to 6 amastigotes were present 24 hrs later in each infected host cell. This evidence suggests that the ligand(s) to which the parasite binds onto the macrophage surface is expressed only in a limited number of copies because the same number of amastigotes were internalized irrespective of the size of the inoculum. In addition to a probable restricted number of ligands for the parasite on the host cell, only

55 to 65% of the macrophages in monolayers of peritoneal cells were invaded by the parasite in the 24 hrs that followed the inoculation, despite the large excess of the inoculum, suggesting, furthermore, that not all peritoneal macrophages are innately equally susceptible to infection by the parasite.

Amastigotes did not proliferate in the macrophages in first 3 to 4 days of the intracellular infection; the parasite then proliferated rapidly for the next 2 to 3 days, reaching a peak density in numbers of parasites/cell on the 7th day of the infection *in vitro*. The non-proliferation phase of *L. donovani* is perhaps a period during which the parasite "adapts" to the physiological conditions that the organism finds in its intracellular mamalian habitat, conditions that are probably very different from those it experienced in its phlebotomid arthropod intermediary host. Initiation of a proliferation phase in the macrophage after the 4th day of the infection suggests either that the parasite requires a period of time to "adapt" to its new habitat, or that the organism requires this period to successfully "prepare" its habitat to facilitate its own proliferation. It is also possible to speculate that any parasite-induced change to the habitat to permit proliferation probably includes blocking the signal transduction pathways by which microbicidal oxygen and nitrogen intermediates are generated in macrophages. Non-proliferating and proliferating amastigotes are different from one another, as indicated by the fact that the former are more susceptible than the latter form to the leishmanicidal effect of macrophages activated by LAK cells and, presumably, to the microbicidal action of the INO that immunotherapy induces in the host cell; proliferating amastigotes, on the other hand, are more susceptible to the direct leishmanicidal action of the drug Pentostam. Following

the peak of proliferation that occurs on day 7, the number of amastigotes in C57BL/6 macrophages *in vitro* begin a slow decline which is likely the result of a recovery of innate microbicidal controls in the infected cell that can develop even in culture, in the absence of other cell types that could provide accessory activation signals.

2. A significant feature of clinical and experimental visceral leishmaniasis is the profound and extensive unresponsiveness of immune reactions that is induced by infections with *L. donovani*. This obligate intracellular protozoan parasite reduces the expression of Class I and II determinants on the membrane of macrophages and also reduces the ability of the cell to produce IL-1, to phagocytize and to present antigen epitopes; infection by this parasite also abolishes, among other things, the ability of splenic T cells from infected mice to proliferate in response to activation by Con A, or by leishmanial antigens, suggesting that, because the parasite never infects lymphocytes, a soluble down regulating factor must play an important role in controlling the development and the proliferation of the infection. This present study confirms the existence of a down regulated state in experimental visceral leishmaniasis by showing that unstimulated splenic T lymphocytes obtained from infected mice were no more able to reduce the number of infected peritoneal macrophages *in vitro* or the number of infected liver macrophages *in vivo* than were the unstimulated spleen T cells of normal mice. Although the lymphokine IL-2 did not stimulate blastogenesis in the T cells of infected mice, IL-2-stimulated (LAK) splenocytes obtained from normal or infected mice induced significant leishmanicidal activity in infected macrophages; LAK cells prepared from the

splenocytes of infected mice were more effective in activating infected macrophages to eliminate their amastigote burden than were LAK cells prepared from normal animals. The difference in the ability of IL-2-stimulated spleen cells from normal or from infected mice to induce the microbicidal activation phenomenon in infected cells suggests a priming of the signal pathway(s) for the production of cytokines by T cells during the course of infection by *L. donovani*; it is clear that the production of the lymphokines that activate leishmanicidal action in macrophages does not, however, occur in the absence of the additional stimulation of these primed cells by IL-2. It would be most interesting to study not only the nature of the priming signal(s) that the parasite provides to the T cells of infected animals, but also the nature of the signal(s) that IL-2 provides to the primed T cell that results in the production of the cytokines that produce leishmanicidal activity in infected macrophages. However, despite the down regulation of immunity that occurs in clinical and experimental kala azar, this present study confirms that the down regulated T cells of the infection retain a potentially-stimulatable, intrinsic capacity for effector activity which, provided with a stimulus of exogenous IL-2, can ellicit parasiticidal action in infected macrophages; T cell anergy in experimental visceral leishmaniasis can be, therefore, readily reversed by treatment with lymphokines.

The efficacy of LAK cells to provide a signal that can induce leishmanicidal activity in macrophages clearly depends on the *Lsh* phenotype of the infected cell: Irrespective of the *Lsh* phenotype of the activating LAK cell, a greater reduction of the intracellular infection was obtained in infected resistant (*Lsh*[r]) peritoneal macrophages

than in infected susceptible (*Lsh*^{s}) cells. This result raises the intriguing possibility that the *Lsh*=*Bcg*=*Ity* gene that regulates the susceptibility of hepatic macrophages *in vivo* to infection by *L. donovani* also probably regulates the capacity of the infected cell to be activated (and cured) by the lymphokines produced by LAK cells. The target(s) for a probable double action of the *Nramp* protein product of the *Lsh* gene would indeed be another fascinating subject for study in experimental kala azar.

3. It has been known for a long time that reactive nitrogen and/or oxygen metabolic intermediates are principal microbicidal effectors in neutrophils and macrophages. Although oxygen intermediates have been shown to be toxic for log phase promastigotes, amastigotes can withstand this microbicidal mechanism because this stage of the parasite's life cycle produces protective levels of endogenous catalase, peroxidase, or superoxide dismutase. The results of this study show that infection of macrophages by *L. donovani* promastigotes does not stimulate the production of reactive oxygen intermediates by the host cell and add that curative immuno- or chemotherapy has no effect to reactivate this microbicidal pathway. The elimination by *L. donovani* of the natural ability of macrophages to produce a microbicidal oxygen "burst" response indicates that the parasite severely affects signal transduction pathways in the infected cell and this important parasite-induced defect deserves considerable study to discover where and how it is produced.

It has also been suspected for a long time that the induction of leishmanicidal action in macrophages is driven by a lymphokine signal and the results of this study

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confirm that the lymphokine trigger for leishmanicidal activity is provided by cytokines released from LAK cells because LAK cells held in cell-impermeable Millipore chambers were as able as free LAK cells to induce macrophages to eliminate their burden of *L. donovani* amastigotes. IFN-γ has been the lymphokine suspected of being principally implicated in this activation because it has been shown that this cytokine is able to stimulate infected cells to reduce the number of *L. donovani* amastigotes they contain. Although macrophages stimulated by IFN-γ alone can produce some reactive nitrogen intermediates, a second signal from LPS, or TNF-α or TNF-ß is required for the production by the cell of microbicidal levels of this effector mechanism. The results which are reported here confirm the results of other investigators that LPS+IFN-γ is an excellent stimulator of the production of INO by macrophages; LAK cell treatment alone also activated the production of microbicidal levels of INO in infected macrophages, without, however, the need of a supplementary signal.

Monoclonal anti-IFN-γ did not greatly reduce the ability of LAK cells to activate leishmanicidal activity *in vitro* and *in vivo*, nor did it abolish the production of INO by macrophages activated by LAK cells. Anti-IFN-γ antiserum reduced the potential of LAK cells to clear the infection *in vivo* by about 20% and to clear the infection *in vitro* by approximately 30% and reduced by 50% the production of INO by infected macrophages activated by LAK cells. These results suggest that the INO reactive intermediate pathway for *leishmania* control may not be the only effector stimulated by immunotherapy, and that IFN-γ is probably not the lymphokine produced by LAK cells which has the principal function to activate leishmanicidal action in infected

macrophages.

4. The cells that activated a reduction of the infection by *L. donovani*, after stimulation by IL-2, were clearly T lymphocytes because depletion studies with anti-Thy 1.2 abolished the ability of splenocytes to be stimulated by the lymphokine. In addition, the result that the monoclonal anti-IL-2 from the S4B2 cell line blocked the stimulating action of both the rIL-2 and the IL-2-containing supernatant of the MLA 144 cells indicated that IL-2 was indeed the lymphokine that stimulated splenic T cells to induce leishmanicidal activity in infected macrophages. It is, of course, possible that cytotoxic lymphocytes could also have been stimulated as a result of the effect of IL-2 on the suspensions of splenocytes and might have also participated in the curative action of the LAK cells. An action by cytotoxic cells would have, however, required physical contact between the target infected cell and the stimulated lymphocyte, but cell-cell contact was not required to cure the intracellular infection and no significant reduction in the number of cells in culture after immunotherapy; therefore, cytotoxic lymphocytes probably played no important role in the leishmanicidal activity that is reported in this study.

5. As it has been mentioned above, a significant feature of mice and humans infected by *L. donovani* is that T cells are markedly-depressed in their ability to proliferate in response to stimulation by lymphokines, leishmanial antigen, or mitogens. Support for the existence of this unreactive state in visceral leishmaniasis is found in this

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thesis in the confirmation that unstimulated splenocytes from infected animals are no more able to activate a reduction in the infection than are the unstimulated splenocytes of normal mice. Despite this unresponsiveness, however, it is demonstrated that a significant anti-leishmanial curative activity can be induced in infected mice and in infected macrophages *in vitro* by IL-2-stimulated syngeneic spleen cells. It is interesting that the ability of LAK cells to cure the infection *in vivo* was not enhanced by the presence of promastigote antigens during stimulation of the splenic T cells by the lymphokine, even when the treated animals subsequently received supplementary intraperitoneal injections of IL-2. A lack of an enhancing effect by the parasite's antigens in the curative ability of LAK cells *in vivo* may have been due to the possibility that the splenocytes of infected mice became fully-primed by leishmanial antigens in the course of the infection and, thus, additional exogenous antigen during the lymphokine stimulation was unable to raise the level of activation. Lymphokine-stimulated splenocytes obtained from normal mice were never as effective in reducing the intensity of the infection *in vivo* and *in vitro* as were the stimulated cells of infected animals, supporting the existence of parasite-priming of T cells in leishmaniasis.

LAK cells prepared in the presence of parasite antigens were, in contrast, more effective in clearing the intracellular infection *in vitro* than were LAK cells that had been stimulated by IL-2 in the absence of antigen. This curious phenomenon suggests that the more richly-complex interacting systems in the infected animal that, for example, either provide new potential host cells by cytopoiesis, or remove dead or effete macrophage casualties of the parasite must certainly influence the effectiveness of the treatment. It

is doubtless for some of these reasons that, although the prepatent period of kala azar *in vivo* is short in susceptible mice, the proliferative period of the infection in C57BL/6 mice extends from approximately day 15 to day 35, before the parasite population begins its normal decline in the untreated animal. The evidence that supplemental intraperitoneal treatments with exogenous IL-2 significantly enhanced the curative effect of LAK cells *in vivo* is in agreement with the several reports that the effector activity of LAK or TIL cells is enhanced by exogenous lymphokine in the treatment of human neoplastic disease.

6. The treatment of choice for many years in clinical and experimental visceral leishmaniasis has been with antimonial drugs, despite reports of the existence of toxic side-effects, especially when high doses or prolonged treatment is required. It is, in addition, recognized that antimonial therapy is sometimes ineffective in human infections and that relapses sometimes occur despite apparent cure due, in part, to the inherent drug resistance of the strain of the organism responsible for producing the infection. It is confirmed in this study that the treatment of experimental visceral leishmaniasis *in vitro* with non-toxic doses of Pentostam effectively reduced not only the number of parasites in macrophages, but also the percentage of infected macrophages. The leishmanicidal action of Pentostam occurred without the activation of the microbicidal oxygen and/or nitrogen metabolic intermediates that the infection had down regulated; it would seem, therefore, that this drug acts by a direct toxic action on the amastigotes of the parasite, principally when the organism is actively dividing in its host cell. Both

indeces of the intensity of the infection (amastigotes/macrophage and number of infected cells) declined in a dose-dependant fashion in the first few days of treatment *in vitro*, especially in those macrophages treated with higher doses of the drug. However, although the percent of infected cells continued to decline until the end of the experiment, an increase in the number of amastigotes per macrophage was obtained a few days into the treatment, especially in cultures treated with low doses of Pentostam. The increase in the number of parasites in infected cells, coupled with a steady decrease in the number of infected macrophages at low doses of the drug suggests that either the surviving parasites were present in cells where the drug was not particularly effective (where, for example, drug efflux was high), or that the low dose selected resistant organisms that were able to proliferate "freely" in the presence of the drug. Pentostam was specially effective when treatment *in vitro* was done in the proliferative phase of the parasite's intracellular life cycle; the rapid proliferation of the organism may have acted to enhance an increased uptake of the drug and its consequent effective leishmanicidal action. The effectiveness of Pentostam clearly depends, therefore, not only on the dose used, but also on the stage during the parasite's intracellular life cycle when the drug is given.

7. Immunotherapy in the non-proliferation phase of *L. donovani*, followed by chemotherapy with Pentostam in the parasite's phase of active division, produced a more effective reduction of the parasite burden than either treatment alone. The effectiveness of the double immunological and drug strategy suggests that the rescue by LAK cells of

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infected macrophages from the down regulation induced by *Leishmania donovani*, followed by a direct anti-parasite attack on the organism by the antimonial drug, may very well provide a most the effective means of eliminating visceral leishmaniasis.

APPENDIX

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22 April 1996

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Dear Dr Eslami

In reply to your letter of 2 April 1996, I am happy to approve reproduction in your thesis of material from the following papers published in this Journal:

> Eslami Z. & Tanner C. E. (1994).Time course and intensity of infection *in vitro* in the resident peritoneal macrophages of resistant and susceptible mice inoculated with different doses of *Leishmania donovani*. International Journal for Parasitology 24: 743-747

> Eslami Z., Oliver M & Tanner C. E (1995).Immunotherapy with IL-2 stimulated splenocytes reduces *in vitro* the level of *Leishmania donovani* infection in peritoneal macrophages . International Journal for Parasitology 25: 975-981.

Yours sincerely

DR ALAN D DONALD
EDITOR, IJP

April 11, 1996

Zohreh Esmani
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Dear Zohreh,

You may certainly use the paper published in the Journal of Parasitology as part of your thesis.

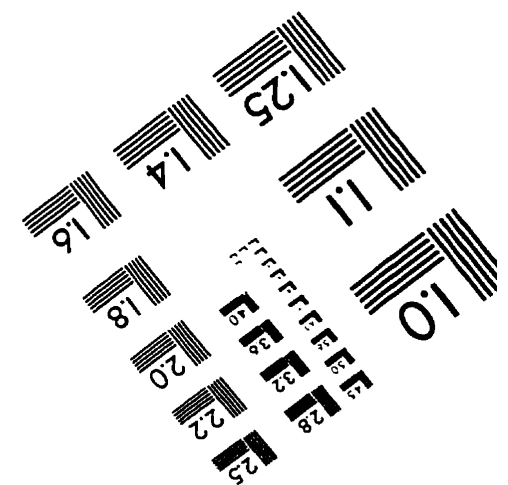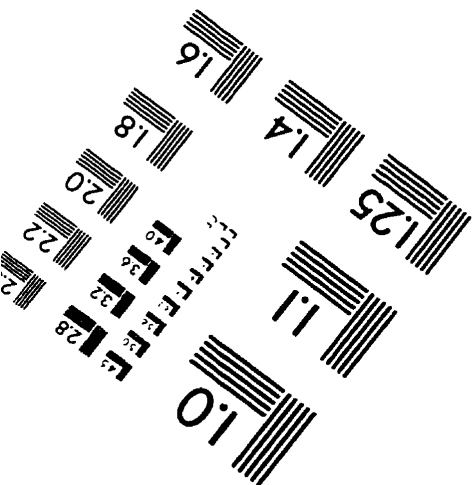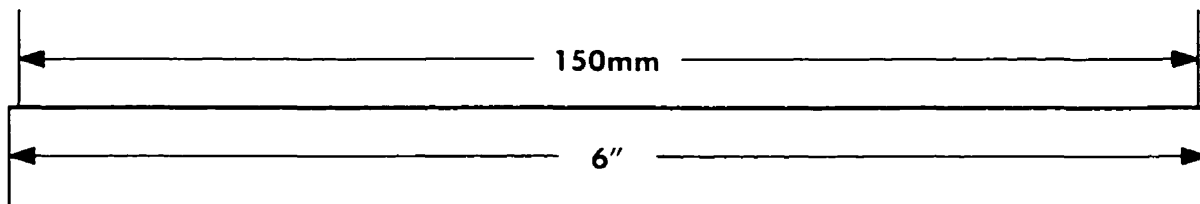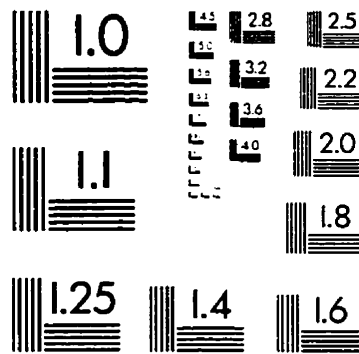Good luck in your efforts.

Sincerely yours,

Gerald W. Esch
Editor

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