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H W Murray, ... , A M Granger, R D Schreiber

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Research Article

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Requirement for T Cells and Effect of Lymphokines in Successful Chemotherapy for an Intracellular Infection

Experimental Visceral Leishmaniasis

Henry W. Murray, Melissa J. Oca, Angela M. Granger, and Robert D. Schreiber*

Division of Infectious Diseases, Cornell University Medical College, New York 10021; and *Department of Pathology, Washington University School of Medicine, St. Louis, Missouri 63110

Abstract

Although directly microbicidal, pentavalent antimony has failed as treatment for visceral leishmaniasis in patients who also have AIDS or are receiving immunosuppressive therapy. To define the role of T cells in the successful host response to chemotherapy, we examined the efficacy of pentavalent antimony (sodium stibogluconate, Pentostam) in normal and T cell-deficient BALB/c mice infected with *Leishmania donovani*. In euthymic (nu/+) mice, single injections of 250 and 500 mg/kg of Pentostam induced the killing of 67% and 89% of intracellular liver amastigotes, respectively. In contrast, in athymic nude (nu/nu) mice, up to three injections of 500 mg/kg achieved no *L. donovani* killing and did not retard visceral parasite replication. Once nude mice were reconstituted with nu/+ spleen cells, however, Pentostam exerted strong leishmanicidal activity, an effect that appeared to be transferred by either L3T4⁺ or Lyt-2⁺ cells. Responsiveness to chemotherapy could also be induced by providing nude mice with either interferon- γ or interleukin 2 alone. The absence of this T cell- and probably lymphokine-dependent mechanism is a likely explanation for treatment failures in immunocompromised patients infected with *L. donovani* and perhaps other systemic intracellular pathogens as well.

Introduction

Despite the use of chemotherapeutic agents with direct microbicidal activity, treatment failures are nevertheless regularly encountered in patients with quantitative or functional T cell defects who develop opportunistic intracellular infections. Clinical experience in patients with the acquired immunodeficiency syndrome (AIDS) has graphically magnified these difficulties, and served to reemphasize the long-held assumption that control over such infections also requires an intact cell-mediated immune response.

Recent reports have indicated that spontaneously controlled (subclinical) infections caused by the intracellular protozoan, *Leishmania donovani*, may reactivate in immunocompromised patients (1-7). In addition, primary treatment

failures using ordinarily effective pentavalent antimony (e.g., sodium stibogluconate, or Pentostam [proprietary name of Burroughs Wellcome]) as well as prompt relapses have also been described in *Leishmania donovani*-infected patients who have AIDS or are receiving immunosuppressive agents (5-7). Since successful host resistance in visceral leishmaniasis is T cell dependent (8-10) and probably mediated by macrophages activated by T cell-derived lymphokines (e.g., interferon- γ [IFN- γ]) (9-19), neither the preceding clinical observations nor the designation of *L. donovani* as an opportunistic pathogen are surprising.

However, considering visceral leishmaniasis treated with Pentostam as a model, three other observations suggest that the reasons why T cell-deficient patients may fail to respond to appropriate antimicrobial therapy are more complex. First, Pentostam is known to exert direct microbicidal activity against intracellular *L. donovani*, and in vitro, does not require either T cells or activating lymphokines to achieve this effect (20, 21). Secondly, despite the typical absence of either delayed-type hypersensitivity reactions or in vitro T cell reactivity to specific leishmanial antigen (8, 18, 19, 22), most otherwise healthy patients with visceral leishmaniasis respond well to pentavalent antimony treatment (23, 24). Thirdly and similarly, at a time when their T cells show no detectable responsiveness to leishmanial antigen (e.g., no proliferative activity or lymphokine secretion [9, 12]), *L. donovani*-infected euthymic mice readily respond to Pentostam with a rapid decrease in visceral parasite burdens (21).

To test the hypothesis that host T cell activity, albeit "undetectable," is nevertheless required for a successful response to therapy for an intracellular infection, we examined Pentostam treatment for visceral leishmaniasis in nude (athymic) mice before and after reconstitution with either T cells or recombinant (r)¹ T cell lymphokines. In this model of the T cell-deficient host, our results demonstrate that the efficacy of antimicrobial chemotherapy is strictly T cell dependent and can be enhanced by IFN- γ and interleukin 2 (IL-2).

Methods

Mice, in vivo infection, and treatment. Congenitally athymic nude (nu/nu) 20-30-g female mice bred on a BALB/c background and their euthymic (nu/+) littermates were purchased from Life Sciences (St. Petersburg, FL), and maintained under similar conventional conditions. To establish infection, mice were injected by tail vein with 0.2 ml of infected hamster spleen homogenate containing 1×10^7 *L. donovani* amastigotes (one Sudan strain) (9, 12). The level of visceral infection was determined by examining Giemsa-stained liver imprints and mi-

Address reprint requests to Dr. Murray, Division of Infectious Diseases, Cornell University Medical College, 1300 York Avenue, New York, NY 10021.

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1. Abbreviations used in this paper: IP, intraperitoneal; LDU, Leishman-Donovan unit; r, recombinant.

crossopically quantitating hepatic parasite burdens as Leishman-Donovan units (LDUs) (9, 12). 2 wk after infection (day 0), when mice were heavily parasitized (9, 12), groups of three mice were treated with intraperitoneal (IP) injections of sodium stibogluconate (Pentostam, Burroughs Wellcome Co., Research Triangle Park, NC) (21). Liver parasite burdens were determined on day +7, 1 wk after the initiation of therapy (21). Amastigote killing was calculated by comparing day +7 LDUs in treated mice to day 0 LDUs in untreated control mice; inhibition of parasite replication was determined by comparing day +7 LDUs in treated mice to day +7 LDUs in untreated control mice (21). Differences were analyzed by a two-tailed *t* test for independent samples.

In vivo reconstitution with spleen cells. 1 d before *L. donovani* infection, nude mice received by tail vein 1×10^7 spleen cells obtained from either uninfected nu/+ mice or immune nu/+ mice which had resolved a prior *L. donovani* infection (9, 10). The spleen cells transferred were unfractionated or were first depleted by > 85% of either L3T4⁺ or Lyt-2⁺ cells using a standard negative-selection technique (10). The latter employed anti-L3T4 and anti-Lyt-2 monoclonal antibodies derived from culture supernatants of the GK 1.5 and TIB 105 hybridoma cell lines (American Type Culture Collection, Rockville, MD), respectively, and complement treatment (10).

In vivo reconstitution with recombinant lymphokines. Infected nude mice were treated before and after Pentostam with four IP injections of 10^5 U of human rIL-2 (*E. coli*-derived, 2×10^7 U/mg of protein, Hoffmann-La Roche Inc., Nutley, NJ) or 10^5 U of murine rIFN- γ (*E. coli*-derived, 1.9×10^7 U/mg of protein, Genentech, Inc., South San Francisco, CA) (9). A single dose of IP Pentostam was given after 2 wk of infection (day 0), and injections of rIL-2 or rIFN- γ were administered twice before (days -2 and -1) and twice after Pentostam (days +3 and +4) (9, 21). rIL-2 and rIFN- γ were diluted in saline containing 1 mg/ml of bovine serum albumin (BSA) and injected in a volume of 0.2 ml. Control mice received BSA alone.

In vivo depletion of L3T4⁺ and Lyt-2⁺ cells. Euthymic (nu/+) mice were treated once a day for 5 d prior to *L. donovani* challenge with IP injections of 1 ml of the GK 1.5 and TIB 105 hybridoma culture supernatants. Parallel experiments demonstrated that this treatment depleted the appropriate spleen T cell subset by $\geq 90\%$ (10). After *L. donovani* infection, mice received thrice-weekly 1-ml injections of antibody (10) during the 2 wk before and the 1 wk after single-dose Pentostam treatment.

In vitro activity of Pentostam. Resident peritoneal macrophages were obtained from nu/+ and nude mice, cultivated overnight on 12-mm round glass coverslips, and then challenged with 5×10^6 *L. donovani* amastigotes (12). After 1 h (time zero), uningested parasites were removed by washing, and cells were reincubated at 37°C in 5% CO₂-95% air in tissue culture medium containing 20 μ g/ml of Pentostam (21). 48 h later, duplicate coverslips were fixed and stained, and intracellular killing was assessed by counting the number of amastigotes per 100 cells present at time zero and at 48 h (21).

Results

In vivo efficacy of Pentostam. 2 wk after *L. donovani* challenge, liver parasite burdens in both euthymic (nu/+) and nude mice increase logarithmically (9, 12, 21), spleen cells from both types of mice do not secrete detectable levels of IFN- γ or IL-2 upon in vitro stimulation with leishmanial antigen (9), and IFN- γ is not measurable in the serum of normal animals (K. Squires, R. Schreiber, and H. Murray, unpublished observations). At this time in euthymic mice, single injections of Pentostam achieved two dose-dependent effects (Fig. 1): inhibition of visceral parasite replication at 100 mg/kg, and 70–90% killing at 250 and 500 mg/kg (21). In contrast, in nude mice, treatment with up to 500 mg/kg of Pentostam failed to achieve any discernible antileishmanial effect. Treatment failure was

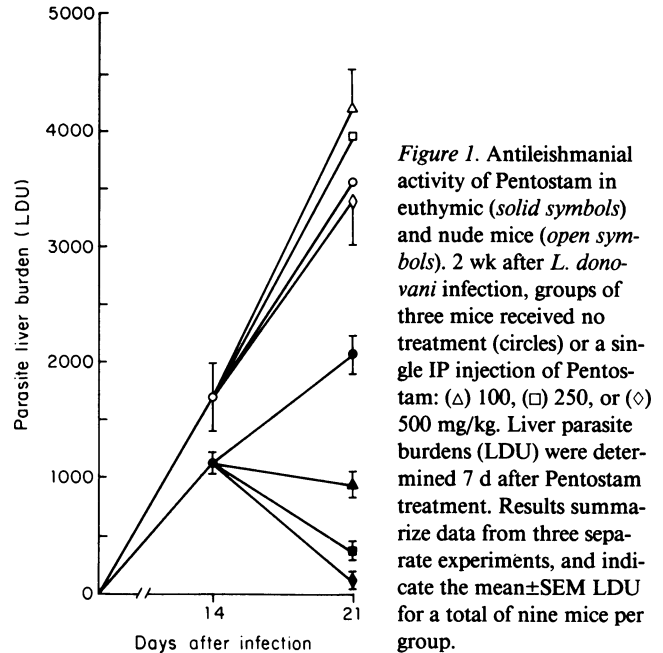


Figure 1. Antileishmanial activity of Pentostam in euthymic (solid symbols) and nude mice (open symbols). 2 wk after *L. donovani* infection, groups of three mice received no treatment (circles) or a single IP injection of Pentostam: (Δ) 100, (◻) 250, or (◊) 500 mg/kg. Liver parasite burdens (LDU) were determined 7 d after Pentostam treatment. Results summarize data from three separate experiments, and indicate the mean \pm SEM LDU for a total of nine mice per group.

not due to suboptimal dosing; nude mice treated for 1 wk with three injections of 500 mg/kg every other day still showed no antileishmanial response (three experiments, data not shown).

In vitro efficacy of Pentostam. To examine a possible defect in nude mice in the interaction of Pentostam with its target cell, peritoneal macrophages were infected in vitro and treated with a microbicidal concentration of Pentostam. After 48 h of exposure to 20 μ g/ml (21), $\geq 69\%$ of intracellular amastigotes were killed by all macrophages tested including cells from uninfected and 2-wk infected euthymic and nude mice (two experiments, not shown). Thus, nude mice did not appear to display a defect at the level of macrophage–Pentostam interaction.

Restoration of responsiveness to Pentostam by T cell reconstitution. To determine the role of T cells in permitting Pentostam to exert its antileishmanial activity, nude mice were reconstituted with spleen cells obtained from either normal uninfected nu/+ or immune nu/+ mice (10). The latter resist rechallenge with *L. donovani* (9, 25), and their Thy-1⁺ spleen cells, which respond to leishmanial antigen with the secretion of IFN- γ and IL 2 (9), can transfer resistance to naive animals (10, 25). As shown in Table I, once reconstituted with unfractionated nu/+ cells, nude mice readily acquired the capacity to respond to single-dose Pentostam treatment with both leishmanistatic (100 mg/kg) and leishmanicidal activity (500 mg/kg). Unfractionated spleen cells from uninfected nu/+ mice were as effective as cells from immune mice indicating that prior antigen sensitization was not required to achieve these results.

Role of L3T4⁺ and Lyt-2⁺ cells. To identify the T cell subset apparently responsible for establishing the ability to respond to chemotherapy, two approaches were used. First, immune spleen cells were depleted of either L3T4⁺ and Lyt-2⁺ cells before transfer into nude mice, and secondly, euthymic (nu/+) mice were injected with monoclonal antibodies which deplete these cells in vivo (10). The data in Table I indicate that for nude mice, responsiveness to Pentostam could be comparably induced by the transfer of cells depleted of either

Table I. Response to Pentostam in Reconstituted Nude Mice

Pentostam	Parasite liver burdens on day 7: cells transferred			
	None	Unfractionated	Anti-Lyt-2 ⁺ treated	Anti-L3T4 treated
Normal cells				
0 (saline)	3,988±189	3,324±126	ND*	ND
100 mg/kg	3,625±142	1,579±87	ND	ND
500 mg/kg	3,503±129	642±66	ND	ND
Immune cells				
0 (saline)	2,498±162	2,779±108	2,380±118	2,849±183
100 mg/kg	2,113±94	1,419±189	1,031±148	1,301±43
500 mg/kg	1,983±121	306±102	677±112	446±68

1 d before *L. donovani* challenge, 1×10^7 unfractionated normal or immune nu/+ spleen cells or immune spleen cells depleted of either L3T4⁺ or Lyt-2⁺ cells were transferred into nude mice. 2 wk later (day 0), groups of three mice received a single injection of saline or Pentostam, and liver parasite burdens were determined 7 d later. On day 0, mean LDUs of control and reconstituted mice were similar and ranged from 1,199±52 to 1,323±81. Results shown are from one experiment representative of two performed with normal spleen cells and three performed with immune cells, and indicate mean±SEM LDU for three mice.

* ND, not done.

T cell subset. Similarly, for euthymic mice, in vivo depletion of one T cell subset or the other did not affect responsiveness to Pentostam (three experiments, data not shown). Since the transfer of resistance to *L. donovani* in this model is dependent on Thy-1⁺ T cells (25), both of the preceding results suggest that L3T4⁺ and Lyt-2⁺ cells can probably mediate the in vivo expression of antileishmanial chemotherapy. These results do not, however, formally exclude the unlikely possibility that other cells such as B cells or natural killer cells also play some role.

Effect of IFN- γ and IL 2. Although otherwise healthy patients with visceral leishmaniasis and euthymic mice acutely infected with *L. donovani* typically respond to Pentostam, their peripheral blood mononuclear cells or spleen cells, respectively, fail to respond to leishmanial antigen with the secretion of IL-2 and IFN- γ (9, 18, 19). Since IFN- γ and IL 2, key macrophage- and T cell-activating lymphokines (11), may nevertheless still be produced in vivo (presumably within the microenvironment of the infected tissue granuloma [9]), we completed this analysis by providing nude mice with these lymphokines before and after Pentostam injection. Using doses and schedules employed in prior studies in euthymic mice (9, 17, 21), treatment of nude mice with either rIFN- γ or rIL-2 partially restored responsiveness to Pentostam (Table II). The beneficial effects of these lymphokines were observed using high-dose Pentostam (500 mg/kg), and while associated with only moderate microbicidal activity, permitted the expression of pronounced Pentostam-induced leishmanistatic activity.

Discussion

This study was initiated to explore the question of why T cell-deficient patients with intracellular infections may fail to respond to appropriate microbicidal antibiotic therapy. The

Table II. Effect of IFN- γ and IL-2 Treatment on Response to Pentostam in Nude Mice

Treatment	Liver burden on day 7	
	IFN- γ (day 0 = 1,193±105)	IL-2 (day 0 = 1,457±75)
None (control)	2,898±106	3,964±231
Pentostam 100	3,302±158	3,856±190
500	2,652±189	3,521±135
BSA alone	2,980±202	4,012±154
Lymphokines alone	3,244±130	3,583±214
Lymphokines + Pentostam 100		
+ lymphokines	2,903±89	3,236±192
BSA + Pentostam 100 + BSA	3,126±142	3,912±148
Lymphokines + Pentostam 500		
+ lymphokines	621±123*	1,026±188*
BSA + Pentostam 500 + BSA	3,010±78	3,419±102

2 wk after infection (day 0), nude mice (three or four mice per group) were treated with either 100 or 500 mg/kg of Pentostam, and liver burdens (LDU) were determined 1 wk later (day +7). BSA and lymphokines, 10^5 U of rIFN- γ or rIL-2, were injected on days -2, -1, +3, and +4 (21), and saline or Pentostam was injected on day 0. Mean day 0 LDU for all groups were within 12% of the day 0 LDU value shown for the controls. Results summarize data from three separate experiments using IFN- γ and three experiments using IL-2, and indicate mean±SEM LDU for a total of 9–12 mice per group (IFN- γ) or 9 mice per group (IL-2).

* Compared to day 7 LDU for control mice, day 7 IFN- γ and day 7 IL-2 results indicate 79% and 74% inhibition of replication of *L. donovani*, respectively ($P < 0.02$). Day 7 LDU results for treated mice were not significantly different ($P > 0.05$) from day 0 LDU of control mice.

specific question addressed was why, if a chemotherapeutic agent such as Pentostam is both directly microbicidal towards intracellular *L. donovani* in vitro (20, 21) and effective in healthy *L. donovani*-infected patients and animals whose T cells show no specific immunologic reactivity (9, 18, 19, 22), should it fail in immunosuppressed patients or T cell-deficient individuals with AIDS (5–7)? As judged by this analysis, the best answer appears to be that while antigen-specific T cell reactivity may not be detectable in the Pentostam-responsive host (using current in vitro assays), functional T cells are nonetheless strictly required for a successful in vivo response to chemotherapy. This latter finding probably explains treatment failures in *L. donovani*-infected patients who have AIDS or are receiving therapy directed at suppressing T cell function (5–7). Whether our observations are also relevant to treatment failures and the propensity for relapse in patients with visceral leishmaniasis in certain parts of the world (e.g., Kenya [22–24]) remains to be determined.

We were not surprised to find that both L3T4⁺ (helper) and Lyt-2⁺ (cytotoxic) cells appeared capable of mediating in vivo responsiveness to Pentostam or that T cell-derived lymphokines may play important roles. In a previous study, for example, we demonstrated that acquired resistance to *L. donovani* and effective control over visceral parasite replication requires both L3T4⁺ and Lyt-2⁺ cells (10). In addition, both subsets rapidly populate and are necessary for the functional development of the tissue granuloma provoked by *L. donovani*-para-

sitized macrophages (26). The primary role of the sensitized L3T4⁺ cell in this mechanism is likely to be antigen-triggered secretion of the T cell-activating lymphokine, IL-2, and in particular, the production of the macrophage-activating lymphokine, IFN- γ (10). The sensitized Lyt-2⁺ cell, perhaps stimulated by IL-2 (27), may act in a cytotoxic fashion towards parasitized macrophages (28), but in addition, can also secrete activating lymphokines including IFN- γ (10).

Although our experiments were not intended to define the optimal doses or regimens for treating nude mice with IL-2 and IFN- γ , the results indicated that both lymphokines by themselves could act in vivo to allow expression of Pentostam's antileishmanial activity. While it is possible that more intensive treatment with IFN- γ (29) or IL-2 might have better mimicked the effects of cellular reconstitution and permitted Pentostam's leishmanicidal activity to become more evident, preliminary studies in infected nude mice suggest that functional T cells may also be required for optimal in vivo responsiveness to IFN- γ (H. Murray, unpublished observations).

In addition to directly activating macrophages to kill intracellular *L. donovani* (14, 15, 17), IFN- γ also enhances the capacity of these cells to accumulate Pentostam (21) and acts synergistically with Pentostam in the intact euthymic animal (21). IL-2 does not directly activate the macrophage (30), but may augment the effects of cytotoxic T cells and natural killer cells (27, 31), and in addition, induce the secretion of IFN- γ (32–35). In the presence of responsive natural killer cells (35–37), for example, the induction of IFN- γ may explain the observation that rIL-2 enhanced the efficacy of Pentostam in nude mice.

Together, these findings serve to reemphasize that, in addition to a microbicidal drug, the successful host response to chemotherapy in systemic intracellular infections such as visceral leishmaniasis requires the active participation of T cells and may involve one or more of their secretory products. Other chemotherapeutic agents in addition to Pentostam may similarly act in concert with the host immune response (38). The type of analysis presented here may also prove useful in defining the immunologic determinants of the effective response to antibiotic drug treatment for other intracellular infections, and lead to new immunochemotherapeutic antimicrobial regimens for the T cell-deficient patient.

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