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Visceral leishmaniasis in the BALB/c mouse: sodium stibogluconate treatment during acute and chronic stages of infection

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Summary

The effect of initiating drug treatment (free, liposomal or surfactant vesicle forms of stibogluconate) at different times post-infection in *Leishmania donovani* infected BALB/c mice was studied. It was found that free stibogluconate therapy was less effective at the later stages of infection, i.e. there was a right shifting of the dose response curve. Treatment with either carrier form of the drug was less sensitive to the length of infection so that the benefit obtained by using this form of the drug dramatically increased over the course of infection. The liposomal and surfactant vesicle forms of the drug were equally effective and they were always more suppressive than the free drug. It is proposed that the successful therapeutic outcome of drug treatment is dependent on the interaction of the innate antileishmanial activity of the drug and a host factor(s), which can either augment or reduce the inherent activity of the drug.

Introduction

Visceral leishmaniasis was among those disease states which, when liposomes were first proposed as a means of targeting drugs, were described as suitable candidates for treatment by this novel therapeutic approach (reviewed by Alving, 1986). However, progress has been slow and the clinical use of liposomal formulations for any class of drug is not widespread. In an attempt to define in greater detail the advantages and the limitations of carrier mediated therapy of infectious diseases we have made extensive use of visceral leishmaniasis as a model (Carter et al., 1988, 1989a–c). The leishmania parasite lives within the parasitophorous vacuole (Alexander and Vickerman, 1975) of macrophages and as a consequence of this intracellular habitat there are few effective drugs, and even those in clinical use, such as the pentavalent antimonials, involve a multiple dosage schedule (WHO, 1984). The attractions of a carrier based therapy where the drug is delivered to the infected organ, and perhaps to the interior of the infected cells within this organ, should be obvious.

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Various animal models have been developed to study the effects of chemotherapy against *Leishmania donovani* (reviewed by Neal, 1987). In the majority of such studies, the success of a particular drug treatment has been based on its effect against an acute infection (Black et al., 1977; New et al., 1981; Baillie et al., 1987; Hunter et al., 1988; Carter et al., 1988), where treatment outcome was assessed by day 20 post-infection at the latest. There has been limited use of models which are based on a chronic infection, although the incubation period of *L. donovani* in man, is between 4–10 months (Manson-Bahr, 1987) and the chronic infection is the disease status which presents in the clinic for treatment. A feature of chronic *L. donovani* infection is specific immunosuppression. In man there is no response to parasite antigen in delayed hypersensitivity skin tests (Manson-Bahr, 1959) and in vitro, the lymphocytes from patients are unresponsive to specific blastogenesis (Sacks et al., 1987). In mice, this immunosuppression has been shown to be caused by a population of T suppressor cells, which express the Lyt-1^+-2^- phenotype, and which is not induced until after day 15 post-infection (Blackwell and Ulczak, 1984). Therefore a mouse model for the assessment of the effects of chemotherapy in which drug treatment was carried out before day 15 post-infection, i.e. before the onset of specifically induced immunosuppression, might be regarded as inadequate since it ignores the influence of an important component of the disease.

We have already demonstrated that in BALB/c mice, *L. donovani* liver parasites are more susceptible to chemotherapy with the free or carrier forms of stibogluconate than those residing in the spleen and bone marrow (Carter et al., 1988). For delivery of liposomal drug to these latter infection sites a small vesicle size is required (Carter et al., 1989b). Expression of the Lsh^s and $H\text{-}2^d$ genes in the BALB/c mouse provides a susceptible, non-cure strain in which visceral leishmaniasis is not self limiting (Blackwell et al., 1985) and as a consequence, an appropriate chronic infection model. This study examines the outcome (parasite suppression in liver, spleen and bone marrow) of stibogluconate treatment of a chronic *L. donovani* infection in BALB/c mice and hence indirectly, the influence of the parasite-induced immunosuppression, known to obtain late in infection, on the outcome.

Materials and Methods

Materials

Sodium stibogluconate (Pentostam) equivalent to 0.32 mg pentavalent antimony (Sb^v) mg^{-1} was obtained from the Wellcome Foundation, U.K. Throughout, drug dose is expressed in terms of Sb^v. Synthetic (> 99% pure) L-α-phosphatidylcholine (DPPC) and ash free cholesterol (CHOL) were obtained from Sigma. The single chain non-ionic surfactant (Surfactant I, Baillie et al., 1985) was obtained from L'Oréal, France. Liposomes and non-ionic surfactant vesicles comprised 70% amphiphile (DPPC or non-ionic surfactant) and 30% CHOL, on a molar basis, and were prepared using procedures already described (Baillie et al., 1986). Briefly multilamellar liposomes were produced by dissolving 150 μmol of DPPC/CHOL mixture in 10 ml chloroform in a 50 ml round-bottomed flask. The solvent was removed at room temperature (20 °C), under reduced pressure and the resulting film hydrated with 5 ml drug solution at 50–60 °C with gentle agitation. Sonicated liposomes and surfactant vesicles were produced by probe sonicating the multilamellar preparations at 60 °C for 3 min using an M.S.E. 150W sonicator, fitted with a titanium probe, set at approximately 10–15% of maximum power output.

The sonicated suspensions were sized by photon correlation spectroscopy at a 90° scattering angle using a Malvern Instruments type 7027 60 channel correlator in conjunction with a He/Ne laser (Siemens; wavelength 632.8 nm, nominal power output 40 mW). The mean hydrodynamic diameters of the sonicated vesicles used in these studies were found to be: non-ionic surfactant vesicles, 106 nm, polydispersity factor, 0.26; liposomes, 116 nm, polydispersity factor, 0.30. Derived from the z average diffusion coefficient (Koppel, 1972), the diameter is weighted towards the larger vesicles in the sample so that a large proportion of the vesicles will have diameters of < 100 nm. Materials were used as received and all other reagents were of analytical grade.

Animals

Eight to ten week-old female inhouse bred BALB/c mice were used throughout experiments and inhouse bred Golden Syrian hamsters (*Mesocricetus auratus*) were used to maintain the parasite.

Parasite

L. donovani (LV9) obtained from Dr. G. Coombs, Glasgow University, was harvested and maintained as described by Carter et al. (1988). Mice were infected by injection into the tail vein (without anaesthetic) of $1\text{–}2 \times 10^7$ amastigotes in 0.2 ml.

Parasite distribution

The method of determining parasite burdens (numbers/1000 host cell nuclei) in liver, spleen and bone marrow has been described by Carter et al. (1988). The number of Leishman-Donovan units (LDU) was calculated per organ for the liver and spleen using the formula: LDU = number of amastigotes/1000 host cell nuclei × organ weight (g) (Bradley and Kirkley, 1977).

Parasite suppression

Infected BALB/c mice were injected intravenously via the tail vein (without anaesthetic) on two consecutive days with 0.2 ml of one of the following: distilled water (controls); sodium stibogluconate solution (equivalent to 5, 12.5, 25, 50, 75 or 100 mg $Sb^v \cdot ml^{-1}$); vesicular, liposomal or surfactant vesicle, drug suspension (equivalent to 0.10, 0.20, 0.40, 0.60 or 0.80 mg $Sb \cdot ml^{-1}$). Six days after the second injection parasite numbers in the spleen, liver and bone marrow of controls and drug treated mice were determined. The time between infection of the animals (designated day 0) and the initiation of therapy was varied. Throughout, the duration of infection refers to the day on which the animals were killed.

Presentation and statistical analysis of data

The effect of sodium stibogluconate therapy on parasite burdens in each tissue site is expressed as the mean % parasite suppression in experimentals compared with controls. This was calculated by obtaining the group mean value of the decrease in parasite burden for each individual experimental mouse (based on LDU values in the spleen and liver, and as the mean number of amastigotes/1000 host cell nuclei in the bone marrow) relative to the mean parasite burden for that site in the appropriate control group. Parasite suppression data were analysed using the non-parametric Mann-Whitney test.

Results

In untreated mice there was a marked hepatosplenomegaly (Table 1) which progressed with increasing duration of infection so that by day 84, a 4-fold increase in mean spleen weight and a doubling of mean liver weight were observed. In drug treated animals however, liver and spleen weights also increased with infection time and in spite of the significant drug induced decreases in liver parasite burdens obtained, there was no significant difference between the organ weights of control and drug treated animals at any time post-infection.

In untreated mice, amastigote numbers in liver and spleen increased markedly between days 14 and 38 when parasite burdens were apparently close to maximum (Table 1). Bone marrow burdens were similar to those described for the spleen. The liver parasite numbers were similar to those already described for this organ in BALB/c mice by Blackwell et al. (1985).

On the basis of the fixed dose used to treat animals there was a decrease in the efficacy of the free drug with duration of the infection, which was only apparent in the liver (Table 1). Dose response curves for free drug against liver parasites show this time dependency, i.e. treatment of infections of longer duration was less successful, more clearly (Fig. 1). There was effectively a right shifting of the dose response curve which was dependent on the duration of infection. Thus the Sb^v dose for 50% suppression was at day 14, 20 $mg \cdot kg^{-1}$; day 55, 45 $mg \cdot kg^{-1}$; day 85, 160 $mg \cdot kg^{-1}$. Although the parasites in bone marrow were more resistant to free stibogluconate than those in the liver, a similar right shifting was seen. In the spleen, no significant effect of duration of infec-

TABLE 1

The effect of free (88.8 mg Sb^v / kg), liposome or surfactant vesicle (14.2 mg Sb^v / kg) drug treatment on the hepatosplenomegaly (organ weights ± S.E., n ≥ 5) and on the parasite burdens (LDU ± S.E., n ≥ 5) caused by Leishmania donovani infection in BALB / c mice

The total doses indicated were given as two divided doses at 7 and 6 days before assessment of the treatment, parasite burdens and organ weights, which was carried out on the day post-infection shown in the table. *P* values are for the comparison of the appropriate experimental group data with that for the controls which were dosed with water in place of drug solution. n.s., no significant difference (95% confidence limit) found, n.d., not determined

Treatment	Day post-infection			
	14	38	58	84
Control				
Spleen wt. (g)	0.23 ± 0.03	0.61 ± 0.10	0.88 ± 0.93	0.93 ± 0.03
LDU	34 ± 9	541 ± 300	375 ± 54	256 ± 36
Liver wt. (g)	0.99 ± 0.07	1.57 ± 0.11	1.65 ± 0.14	1.83 ± 0.19
LDU	2337 ±364	5014 ±1070	902 ±187	1322 ±611
Free drug				
Spleen wt. (g)	0.26 ± 0.02	0.76 ± 0.07	0.87 ± 0.05	1.03 ± 0.10
LDU	43 ± 15	174 ± 53	455 ± 67	401 ± 9
	n.s.	n.s.	n.s.	n.s.
Liver wt. (g)	1.01 ± 0.05	1.83 ± 0.11	1.74 ± 0.09	2.10 ± 0.10
LDU	282 ± 55	543 ± 279	981 ±239	1415 ± 73
	$P < 0.001$	$P < 0.001$	n.s.	n.s.
Liposomes				
Spleen wt. (g)	0.30 ± 0.02	0.70 ± 0.07	0.78 ± 0.06	0.93 ± 0.06
LDU	25 ± 1	188 ± 47	186 ± 33	253 ±104
	n.s.	n.s.	$P < 0.01$	n.s.
Liver wt. (g)	1.09 ± 0.04	1.69 ± 0.14	1.49 ± 0.09	1.89 ± 0.12
LDU	30 ± 17	356 ± 179	52 ± 18	277 ± 63
	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.05$
Surfactant vesicles				
Spleen wt. (g)	0.27 ± 0.02	0.82 ± 0.04	0.69 ± 0.08	n.d.
LDU	22 ± 4	240 ± 38	126 ± 33	n.d.
	n.s.	n.s.	$P < 0.01$	
Liver wt. (g)	0.95 ± 0.06	1.96 ± 0.06	1.46 ± 0.13	n.d.
LDU	0	180 ± 62	24 ± 6	n.d.
	$P < 0.001$	$P < 0.001$	$P < 0.001$	

tion on free drug efficacy could be discerned.

At all infection times studied, the efficacy of either carrier form of the drug (liposomes or surfactant vesicles), was higher than that of free drug. In addition, compared to the free drug, the antileishmanial activity of stibogluconate in the carrier form was less sensitive to the duration of infection. The data for the carrier forms were also more variable and it was not possible to detect a significant difference among the dose response curves obtained for day 14, 55 and 85, infections. As a consequence of the differential susceptibility of free and vesicular drug efficacies to the duration of infection, the increase in efficacy obtained by using the carrier forms was greater against infections of longer duration. Thus against the day 84 infection, increases in efficacy of 200 × were attained. Either type of carrier system gave these significant efficacy increases when used as a means of delivering the drug.

Discussion

It seems reasonable to assume that the appearance of a suppressor T cell population

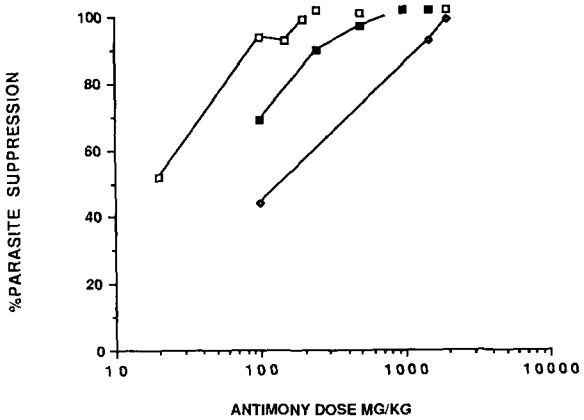

Fig. 1. The parasite suppression (%) achieved in the liver by free sodium stibogluconate treatment of *Leishmania donovani* infected BALB/c mice. Infected animals were treated with the total dose of Sb^v shown, given as two divided doses at 7 and 6 days before assessment of parasite burdens on day 14 (□), 55 (■) or 84 (◆) post-infection. Suppressions for individual treated mice were calculated relative to the appropriate mean control values (dosed with water in place of drug solution, $n \geq 5$) and mean suppression is shown ($n \geq 5$).

(Blackwell and Ulczak, 1984), accompanying the chronic progress of *L. donovani* infection in the non-cure BALB/c mouse, is associated with the observed decrease of free stibogluconate efficacy against infections of long duration. Indeed it was perhaps to be expected that the onset of such immunosuppression would be antagonistic to the antileishmanial activity of the drug. The marked resistance of visceral leishmaniasis to chemotherapy in AIDS patients (Alvar, 1988) also supports the theory that the immune status of the host influences the outcome of leishmaniasis chemotherapy.

However, the different susceptibilities of treatment with the free and carrier forms of stibogluconate to duration of infection suggest that factors other than the host's immune status may also be involved. Our previous findings, which show that, in the mouse, the antileishmanial activity of stibogluconate is site-dependent (Carter et al., 1988, 1989a, b), provide the basis for describing the outcome of chemotherapy (parasite suppression) at any particular site of infection (e.g. liver, spleen or bone marrow) in the form of a simple expression: outcome = innate drug activity + pharmacokinetic factors. The results of the present study, however, indicate that this expression is incomplete since it inadequately explains why the difference in efficacy between the carrier and free forms of stibogluconate is dependent on the duration of infection.

The expression must be modified by an additional term to take into account the influence of a host factor(s) such that:

outcome = innate drug activity + pharmacokinetic factors + host factor(s).

The host factor may have a positive or negative value, respectively augmenting or suppressing the inhibitory effects of the drug treatment. In the chronic infection, the host factor has a negative value, shown by the lower activity of the free drug against this form of the infection. We suggest that this may well be due to the T cell induced immunosuppression which is antagonistic to the antiprotozoan activity of the drug. Support for this suggestion comes from studies which show that manipulation of the host's immune response does influence the outcome of drug therapy and exogenous immunostimulants such as muramyl dipeptide (Adinolfi et al., 1985) and interferon γ (Murray et al., 1988) act synergistically with stibogluconate against *L. donovani*.

The poor therapeutic outcome achieved with free drug in the chronic model may, at any infection site, be addressed by attaining and/or sustaining higher levels of drug, by the use of a carrier which enhances drug delivery to the site. The lower sensitivity of the vesicular forms of the drug to the duration of the infection is thus a consequence of the ability of the carriers to modify drug distribution. In spite of mechanistic considerations, the dramatic increases in efficacy observed in the later stages of infection with the carrier forms of the drug provide further support for the use of a carrier mediated therapy for the treatment in the field of the human disease, which will inevitably be chronic.

A logical improvement to the therapy of visceral leishmaniasis would combine the advantages of the drug delivery approach with manipulation of the host's immune response. Whether such a treatment would be successful against *Leishmania* parasites at all infection sites and would function

in a chronic model awaits investigation, as does the investigation of its safety and utility in man. If such a regimen was successful, those patients with (genetically determined) immunodeficiencies, who would as a result be expected to respond poorly to conventional drug therapy, would stand to benefit most from this novel immuno-pharmacological approach.

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