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Genetic Control of Drug-induced Recovery from Murine Visceral Leishmaniasis

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Abstract—The influence of host genetic background on the response of *Leishmania donovani*-infected mice to chemotherapy was studied using the *H-2*^{d} and *H-2*^{b} haplotypes on a BALB or a B10 genetic background. Animals were treated with free or liposomal sodium stibogluconate and parasite burdens in the liver, spleen and bone marrow were assessed. In all the mouse strains and their congenic derivatives examined, the liver responded best to therapy regardless of drug formulation, whilst the spleen and the bone marrow respectively were increasingly less responsive to chemotherapy. Treatment with free drug was more effective in congenic mice carrying the *H-2*^{b} haplotype than in those carrying the *H-2*^{d} haplotype and in mice carrying the same *H-2* haplotype, animals from a BALB background were better responders than those from a B10 genetic background. Liposomal drug was more effective than free drug treatment in all four mouse strains and produced a similar significant suppression (>99%, $P<0.001$) in liver parasite burdens to that obtained using a six times greater free drug dose. This liposomal drug dose was more effective than free drug in reducing bone marrow parasite burdens in all four mouse strains and equally (BALB/c mice) or more effective ($P<0.01$, BALB/B, B10 and B10.D2 strains) in reducing spleen parasite numbers. Liposomal drug, particularly in the B10 genetic background, effectively negated the *H-2* dependent influences apparent using free sodium stibogluconate. These results are discussed in relation to the genetic factors which are known to control the course of *L. donovani* infection in mice.

Recent evidence would indicate that the outcome of therapy with sodium stibogluconate is dependent on pharmacokinetic and host factors as well as its innate antiparasitic activity (Baillie et al 1989). Some of the host factors which may have an influence on drug efficacy are likely to be genetically determined and these in turn may effect pharmacological factors in the drug-treated individual. It is well established that the ability of a person to metabolize certain drugs is genetically controlled (Jacqz et al 1986; Wilkinson et al 1989). For example, the human cytochrome P450 enzyme P450IID6, which catalyses the oxidation of certain commonly used drugs, is under single gene control and its phenotypic expression is polymorphic within populations (Brosen & Gram 1989). In a similar manner it has been suggested that the antimony present in antimonial drugs, used in anti-leishmanial chemotherapy, has to be converted from the pentavalent to the trivalent form to exert an antiparasitic effect (Goodwin & Page 1943). Therefore, it is quite likely that patients may vary in their ability to convert the antimony to its active form, a factor which may be extremely important in such a rapidly excreted drug (Rees et al 1980), since the time the drug is available for conversion is likely to be limited.

The immunological susceptibility or resistance to a disease such as leishmaniasis is also under genetic controls which have been well-characterized in murine disease models (Bradley et al 1979; Blackwell 1985; Blackwell et al 1985). It is also well established that successful chemotherapeutic treatment of leishmaniasis is dependent upon a potential or latent immune response (Alvar 1989; Iwobi et al 1991). Therefore, it is likely that genetic factors controlling immunological susceptibility or resistance to leishmaniasis will influence the outcome of chemotherapy. In mice innately susceptible (*Lsh*^{s}) to *Leishmania donovani* three MHC (*H-2*) controlled phenotypic patterns of infection are found: early cure (s and r haplotypes); cure (b haplotype); and non-cure (d, f and q haplotypes) (Ulczak & Blackwell 1983). How the *H-2* genes influence the disease process is not fully understood but their action seems to be T cell-dependent, as cure has been shown to be T cell-mediated (Ulczak et al 1988) and correlates with a positive delayed-type hypersensitivity response (Rezai et al 1980). Non-cure is also associated with the generation of CD4$^+$ T cells (Blackwell & Ulczak 1984) and cure mice can be rendered non-cure by infecting with large parasite numbers (Ulczak & Blackwell 1983).

The following study in *Lsh*^{s} mouse strains was to determine whether *H-2* genes influenced the outcome of drug therapy. Both non-cure, and cure haplotypes rendered non-cure by administering a high infection dose, were studied and in order to determine whether other genetic controls could be involved, mice carrying identical haplotypes on both B10 and a BALB genetic background were used.

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Materials and Methods

Materials

Sodium stibogluconate (Pentostam) equivalent to 0·32 mg Sb mg^{-1} was obtained from the Wellcome Foundation, UK, synthetic (>99% pure). L-α-Phosphatidylcholine (DPPC) and ash-free cholesterol were obtained from Sigma. Liposomes comprised 70% DPPC and 30% cholesterol on a molar basis. Briefly, multilamellar liposomes were produced by dissolving 150 μmol of DPPC/cholesterol 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 were produced by probe-sonicating the multilamellar preparation at 60°C for 3 min using an M.S.E. 150 W sonicator, fitted with a titanium probe, set at approximately 10–15% of maximum power output.

The sonicated vesicular 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 diameter of the sonicated liposomes used in these studies was found to be 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.

Animals

The following strains of 8–10 week-old female mice, 19–23 g, obtained from Harlan Olac Ltd, Shaw's Farm, Blackthorn, Bicester, UK, were used in experiments: BALB/c which carry the *H-2*^{d} haplotype, their congenic derivative BALB/B mice which carry the *H-2*^{b} haplotype; C57BL/10ScSn (B10 mice), which carry the *H-2*^{b} haplotype and congenic B10.D2 mice which carry the *H-2*^{d} haplotype. In-house-bred Golden Syrian hamsters (*Mesocricetus auratus*) which originated from the Bantim and Kingman colony (The Field Station, Aldborough, Hull, UK) were used to maintain the parasite.

Parasite

Leishmania donovani (MHOM/ET/67: LV9) was harvested and maintained as described by Carter et al (1988). Mice were infected via the tail vein (without anaesthetic) with 1–2×10^7 *L. donovani* amastigote parasites in 0·2 mL RPMI-1640.

Parasite distribution

The method of determining parasite burdens (numbers/1000 host cell nuclei) in the 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 per 1000 host cell nuclei × the organ weight (g) (Bradley & Kirkley 1977).

Parasite suppression

Infected mice were treated by injection via the tail vein (without anaesthetic) on days 7 and 8 post-infection with 0·2 mL of one of the following: phosphate-buffered saline (controls); sodium stibogluconate solution (equivalent to 1·25, 2·5, 3·75, 5, 25, 50 or 75 mg Sb mL^{-1}); or liposomal drug suspension (equivalent to 0·8 Sb mg mL^{-1}). On day 14 post-infection, animals were killed and parasite numbers in the spleen, liver and bone marrow determined.

Presentation and statistical analysis of data

The effect of therapy is expressed as the % parasite suppression, calculated as the % decrease in the parasite burden in a tissue site for an individual mouse relative to the mean parasite burden for that site in the appropriate control group. Mean % suppression in parasite burdens ± standard errors (s.e.) are shown. However, the parasite data were analysed using an independent *t*-test or a one way analysis of variance on the log-transformed data using the LDU/organ for the spleen and liver data and the number of parasites/1000 host cell nuclei for bone marrow data.

Results

A comparison of the therapeutic outcome of free (total dose equivalent to 88·8 mg Sb kg^{-1}) and liposomal (total dose equivalent to 14·2 mg Sb kg^{-1}) sodium stibogluconate treatment in BALB/c, BALB/B, B10 and B10.D2 mouse strains revealed significant differences in drug efficacy at the dose levels used which were not only strain-dependent, but also site- and formulation-dependent.

Free and liposomal drug treatment were equally effective in suppressing liver parasite numbers ($\geq 99\%$ compared with the corresponding controls) in all four mouse strains ($P < 0{\cdot}001$, Table 1), even though the sodium stibogluconate dose administered to animals in the liposomal form was only

Table 1. The mean percentage suppression (±s.e., $n \geq 5$) in spleen, liver and bone marrow parasite burdens in *L. donovani*- infected BALB/c, BALB/B, B10 and B10. D2 mice, treated on days 7 and 8 post-infection with free (equivalent to a total dose of 88·8 mg Sb kg^{-1}) or liposomal (equivalent to a total dose of 14·2 mg Sb kg^{-1}) sodium stibogluconate, compared with control values.

	Mean % parasite suppression (±s.e.)			
	BALB/c	BALB/B	B10	B10.D2
Spleen				
Free	41±16*	50±13*	26±8	4±42
Liposomal	56±17***	87±4***	87±2***	84±5***
Liver				
Free	100±0·2***	100±0·2***	98±1·0***	100±0·0***
Liposomal	100±0·0***	100±0·0***	100±0·0***	100±0·2***
Bone marrow				
Free	0±24	59±6**	4±15	17±34
Liposomal	48±13*	79±8**	77±8**	85±3**

*$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$, compared with its own control.

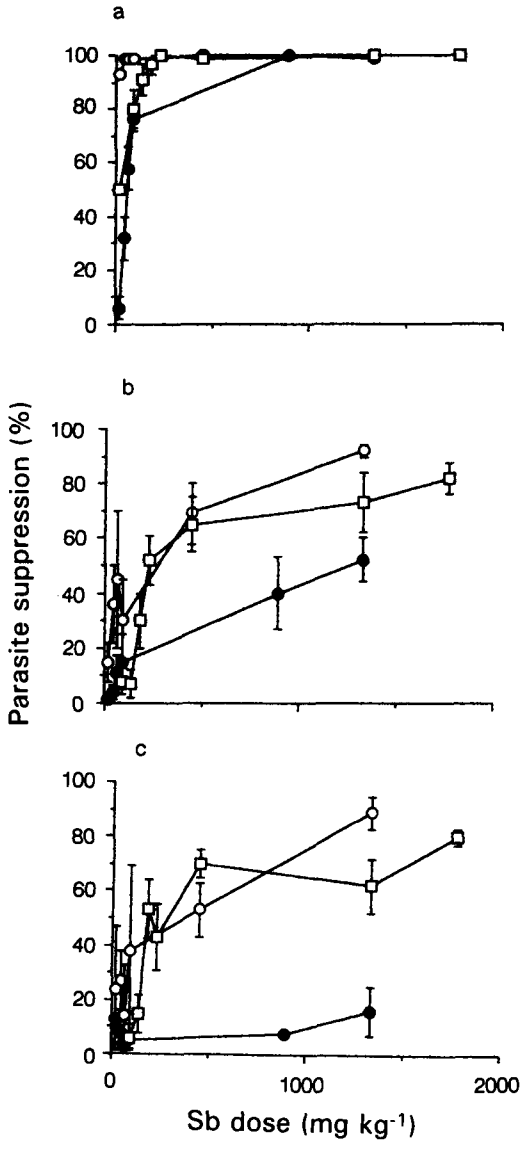

FIG. 1. The dose response curves for free stibogluconate based on the parasite burdens in liver (a), spleen (b) and bone marrow (c) of *Leishmania donovani*-infected B10 (○), B10.D2 (●) and BALB/c (□) mice.

a sixth of the free formulation. Free drug treatment significantly reduced spleen parasite burdens in BALB/B ($P < 0{\cdot}01$, Table 1) and BALB/c mice ($P < 0.05$, Table 1) but in B10 and B10.D2 drug-treated animals parasite burdens were similar to control values (Table 1). Free drug treatment only significantly suppressed bone marrow parasite burdens of BALB/B mice ($P < 0{\cdot}01$) compared with controls. Liposomal drug had greater efficacy against spleen and bone marrow parasites since it significantly lowered splenic ($P < 0{\cdot}001$) and bone marrow ($P < 0{\cdot}05$) parasite numbers in all four strains of mice compared with their controls (Table 1). Whereas *H-2* dependent differences following chemotherapy were noted in the BALB genetic background, they were not obvious in B10 congenic mice, which on the whole responded poorly in the spleen and bone marrow to free drug but well to the liposomal formulation.

It was possible that the *H-2* dependent efficacy of free drug treatment on the BALB genetic background was not demonstrated on the BI0 background because an inappropriate drug dose was administered to animals; therefore B10, B10.D2 and BALB/c animals were treated with a range of free stibogluconate doses. An *H-2* dependent effect became apparent (at low drug doses in the liver and at high drug doses in the spleen and bone marrow) in mice with a B10 genetic background: B10 mice were more amenable to drug treatment than B10.D2 mice (Fig. 1). This experiment also confirmed that *H-2* matched mice with a BALB genetic background were more responsive to free sodium stibogluconate therapy than mice with a B10 genetic background, since greater suppressions in liver, spleen and bone marrow parasites burdens were obtained in BALB/c ($H\text{-}2^d$) compared with B10.D2 ($H\text{-}2^d$) mice.

Discussion

As demonstrated in previous studies (Carter et al 1988, 1989), the therapeutic effects of free and liposomal sodium stibogluconate on *L. donovani* infection of the liver, spleen and bone marrow were greatest in the liver and least in the bone marrow in four mouse strains.

The present study also showed that susceptibility of *L. donovani* in all three infection sites to free stibogluconate depended not only on the *H-2* haplotype of the host but also on other background genes. Thus, although mice carrying the $H\text{-}2^b$ haplotype responded better to chemotherapy than mice carrying the $H\text{-}2^d$ haplotype on both the BALB and B10 genetic backgrounds, non-*H-2* genes were also important as *H-2* matched mice with a BALB genetic background responded better to drug treatment than mice with a B10 genetic background.

The genetic dependency of the efficacy of free sodium stibogluconate therapy may be explained by either immunological or pharmacokinetic mechanisms. In this study we did not measure any pharmacokinetic parameters therefore we cannot comment on their importance. This study did, however, demonstrate that *H-2* genes do influence the outcome of sodium stibogluconate chemotherapy. It has already been shown that the *H-2* genes influence the response of mice to a primary *L. donovani* infection where mice carrying the b *H-2* haplotype have a cure profile and those carrying the d *H-2* haplotype, a non-cure profile (Blackwell et al 1980). These haplotypes could be described as good and poor responders to drug treatment, respectively. The *H-2*-controlled immunological mechanisms involved in development of resistance to the parasite may well work synergistically with stibogluconate therapy since it has already been shown that combined treatment with antimonial drugs and immunological mediators can improve therapeutic outcome compared with giving drug alone (Adinolfi et al 1985; Murray et al 1988, 1989, 1991; Badero et al 1990). The cure/non-cure phenotypes normally take at least 30 days to develop in a primary infection (Blackwell et al 1980), whereas the good/poor response phenotypes obtained in this study could be detected on day 14 post-infection. The ability of the drug to lower the parasite load may allow *H-2* genes to exert their effects more quickly.

Treatment with liposomal drug at a sixth of the free drug dose was more effective at reducing parasite burdens than the

free drug in all four strains of mice and the same genetic dependency in responsiveness to drug treatment was not obtained. Thus, carrier-mediated therapy may reduce or circumvent the contribution usually required by host genetic factors on the outcome of drug therapy. Evidence that carrier systems such as liposomes can alter the host's immune responses comes from other studies, where carriers have been used as adjuvants to negate the genetically restricted non-responsiveness to an antigen (Allison & Gregoriadis 1990).

In summary, this study demonstrated that both *H-2* and non-*H-2* genes can influence the outcome of drug treatment, and that carrier systems may circumvent or negate these genetic influences. Non- or poor responsiveness to anti-leishmanial chemotherapy does occur in patients (Chung et al 1990; Thakur et al 1991) and this may in part be due to genetically-controlled immunological non-responsiveness. Therefore using a carrier drug delivery system could improve present treatment regimens.

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