The Cured Immune Phenotype Achieved by Treatment of Visceral Leishmaniasis in the BALB/c Mouse with a Nonionic Surfactant Vesicular Formulation of Sodium Stibogluconate Does Not Protect against Reinfection

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Single-dose treatment with sodium stibogluconate solution (SSG) and treatment with a nonionic surfactant vesicular formulation of sodium stibogluconate (SSG-NIV) were compared for the ability to protect BALB/c mice against infection with Leishmania donovani. Prophylactic treatment with SSG-NIV protected against infection, although its effects were time and organ dependent; protection was not obtained with SSG. Protection against reinfection with L. donovani was observed only in mice cured by treatment with SSG-NIV. However, this protective effect was probably due to the presence of residual drug rather than an immune effect, since prophylactic SSG-NIV treatment gave similar results. Transfer of enriched spleen T-cell populations from L. donovani-infected mice or from infected SSG-NIV-treated mice gave no protection against L. donovani infection in the recipients. T cells from infected mice, but not from infected SSG-NIV-treated mice, were infectious to recipients. SSG-NIV treatment was equally effective against visceral leishmaniasis in immunocompetent and SCID mice, whereas SSG treatment was less effective in the latter. The results of this study suggest that the high antileishmanial activity of SSG-NIV is due to favorable modification of SSG delivery and does not require a fully functional immune response. Cure of visceral leishmaniasis by SSG-NIV treatment in the BALB/c mouse did not protect against reinfection.

A major advance in the therapy of any infectious disease would be the ability to not only cure the infection but also to confer resistance to reinfection. In the case of visceral leishmaniasis (VL), relapse after treatment may occur because of reinfection, if patients live in regions where VL is endemic, or because of multiplication of residual parasites which survive drug therapy.

Human immunodeficiency virus (HIV)-positive patients show high relapse rates after antileishmanial therapy (1, 3). In a retrospective nonrandomized open-trial study of secondary Leishmania prophylaxis in HIV-positive patients (22), annual relapse rates were 65% where there was no prophylaxis, 56% following allopurinol treatment (300 mg every 8 h), and 18% following monthly single antimonial injections (each equivalent to 850 mg of pentavalent antimony [SbV]). It is well known that a successful outcome of antimonial chemotherapy is dependent on an intact patient immune response (21), and since the immune response in HIV-positive patients can be discounted, the low relapse rate after antimonial prophylaxis is most likely due to the presence of drug depots at the sites of infection. However, given the short in vivo half-life of antimonials (12, 26), the prophylactic effect of single monthly antimonial injections is unexpected, although there is evidence that sodium stibogluconate (SSG) persists in tissues for prolonged periods. For example, in mice, prophylactic treatment with SSG (equivalent to 80 to 100 mg of SbV/kg of body weight) 6 days before infection with Leishmania donovani suppressed liver parasite burdens (11).

SSG entrapped in nonionic surfactant vesicles (NIV) is more effective than the free drug, and in BALB/c mice infected with L. donovani, treatment with a single dose of SSG-NIV gave >96% parasite suppression in the liver, spleen, and bone marrow (2). These treated mice displayed the immunological responses typical of a cured phenotype (15, 24), which indicated that SSG-NIV treatment had reversed the parasite-induced immunosuppression of VL. Other workers have used drug-abrogated infections to determine what effect limited exposure to the parasite has on immunity to a subsequent infection (4, 16).

The aim of the present study was to compare the abilities of prophylactic treatment with the free and NIV forms of SSG to protect against infection. In addition, since in VL host immunity influences treatment outcome, the effect of exposure to previous infection on any protective effect was investigated.

**MATERIALS AND METHODS**

Materials. SSG (Pentostam) equivalent to 29.94% (wt/wt) SbV was obtained from the Wellcome Foundation, London, United Kingdom (UK). Mono-n-hexadecyl ether tetraethylene glycol was purchased from Chesham Chemicals Ltd., Harrow, UK. Antimony standards, dichyl phosphate, and ash-free cholesterol were purchased from Sigma, Poole, UK. Fetal calf serum, RPMI medium, penicillin-streptomycin, and l-glutamine were purchased from Gibco BRL, Paisley, UK. All other reagents were of analytical grade.

**Vesicle formation and characterization.** A 150 μM concentration of surfactant-lipid, consisting of a 3:3:1 molar ratio of mono-n-hexadecyl ether tetraethyleneglycol, cholesterol, and dichyl phosphate, was melted by heating at 135°C for 2 min. The molten mixture was cooled to 70°C and hydrated with a preheated 5-ml volume of either phosphate-buffered saline (PBS) (pH 7.4) or 100 mg/ml SSG solution and homogenized with a Silverson mixer (model L4R SU; Silverson Machines, Chesham, UK) fitted with a 5/8” tubular work head (Silverson) and operated at 8,000 rpm for 15 min. Vesicle suspensions were sized with a Zetasizer 4 (Malvern Instruments Ltd., Malvern, UK).

**Animals.** In-house-bred Golden Syrian hamsters (Mesocricetus auratus) were used for parasite maintenance. Experimental studies used age-matched 8- to 10-week-old in-house-inbred female BALB/c mice or SCID mice with a BALB/c
Parasite numbers were not affected by SSG-NIV treatment on parasite burdens, but the mean percent suppression in per 1,000 host nuclei (LDU) was calculated for the liver and spleen by using the following formula: LDU = number of Leishman-Donovan units (LDU) per organ. The suspension was passed through a sieve to remove large debris and then pelleted by centrifugation. The supernatant was pelleted by centrifugation and then resuspended in 10 to 15 ml of medium, and then gently centrifuged at approximately 250 × g. The supernatant was pelleted by centrifugation and then resuspended in 10 to 15 ml of medium, and the number of amastigotes/milliliter was determined with a hemocytometer. Throughout, mice were infected by intravenous injection (tail vein, no anesthetic) of 0.5 × 10^7 to 2 × 10^7 L. donovani amastigotes.

### RESULTS

Pretreatment with SSG gave no significant protection against infection with L. donovani (Fig. 1). However, pretreatment with SSG-NIV 1 week before infection, significant parasite suppression was found in all three sites (spleen, liver, and bone marrow) as described by Carter et al. (9). The effect of drug treatment on parasite burdens is expressed as the mean percent suppression in per 1,000 host nuclei. Treatment 4 weeks preinfection was not suppressive. Controls were given 0.2 ml of PBS at each time point. On day 0 mice were infected with 1 × 10^7 amastigotes and sacrificed 14 days later and the parasite burdens in the spleen, liver, and bone marrow were determined.

![Graph](https://via.placeholder.com/150)

**Fig. 1.** Effect of prophylactic treatment with free-SSG or SSG-NIV formulations on spleen (a), liver (b), and bone marrow (c) burdens of L. donovani parasites. Uninfected mice were given a single 0.2-ml dose of SSG (100 mg of SSG/ml) or SSG-NIV (100 mg of SSG/ml) 4, 3, 2, or 1 week before infection. Controls were given 0.2 ml of PBS at each time point. On day 0 mice were infected with 1 × 10^7 to 2 × 10^7 L. donovani amastigotes; they were sacrificed 14 days later and the parasite burdens in the spleen, liver, and bone marrow were determined.

**Statistical analysis of data.** The effect of drug treatment on parasite burdens was analyzed by using a one-way analysis of variance or a Student t test on the log transformed data (using LDU values for the spleen and liver data and the number of parasites/1,000 host cell nuclei for the bone marrow data). All other data were analyzed by the nonparametric Mann-Whitney U test.

In vivo efficacies of formulations. Uninfected mice were treated once intravenously with either 0.2 ml of free SSG solution (100 mg of SSG/ml) or 0.2 ml of SSG-NIV (100 mg of SSG/ml) at 4, 3, 2, or 1 week before infection. Controls were given 0.2 ml of PBS at each time point. All mice were sacrificed 14 days after infection.

To determine if after drug treatment infected mice displayed immunity against reinfection, mice were infected on day 0 and then treated on day 7 with 0.2 ml of either SSG solution (100 mg of SSG/ml) or PBS (controls). Age-matched uninfected animals were similarly treated on day 7 with SSG, SSG-NIV, or PBS. On day 30, 31, or 38, half of the animals in each group were infected (challenge infection) and then sacrificed at various time points.

SCID mice were infected on day 0: treated on day 7 with 0.2 ml of either SSG solution (100 mg of SSG/ml), SSG-NIV (100 mg of SSG/ml), or PBS (controls); and sacrificed on day 14.

**Cell transfer experiments.** Infected controls, infected SSG-NIV-treated mice (given a single dose of SSG-NIV [100 mg of SSG/ml] on day 7), and age-matched uninfected mice were sacrificed on day 30 or 50, and their spleens were removed and teased apart with forceps. Spleen cell suspensions from mice within the same group (n = four or five) were pooled, exposed to a nylon wool column to enrich the number of T cells present (8), and adjusted to 5 × 10^7 cells/ml of RPMI 1640 medium. On day 0, uninfected female BALB/c mice (n = five) were given 0.2 ml of RPMI 1640 medium (controls) or 0.2 ml of T-cell suspension (10^7 cells) prepared from one of the donor groups. On day 1 mice were infected; they were sacrificed at various times postinfection. For each mouse, plasma samples were prepared from blood collected at sacrifice and stored at −20°C until specific antibody titers were determined.

**Parasite preparation.** L. donovani (strain MHOM/ET/67/LV82) was maintained by serial passage through hamsters as described by Carter et al. (9). To obtain a purified L. donovani amastigote preparation, the spleen of an infected hamster was removed aseptically and broken up in supplemented RPMI 1640 medium (100 µg each of penicillin and streptomycin/ml and 200 µM L-glutamine) by using a glass homogenizer. The resultant suspension was passed through a sieve to remove large debris and then pelleted by centrifugation. The pellet was resuspended in Boyle’s solution (0.007 M ammonium chloride, 0.0085 M Tris [pH 7.2]) and incubated at 37°C for 10 to 20 min to lyse erythrocytes. The suspension was pelleted by centrifugation, washed twice, resuspended in 10 to 15 ml of medium, and then gently centrifuged at approximately 250 × g. The supernatant was pelleted by centrifugation and then resuspended in 10 to 15 ml of medium, and the number of amastigotes/milliliter was determined with a hemocytometer. Throughout, mice were infected by intravenous injection (tail vein, no anesthetic) of 0.5 × 10^7 to 2 × 10^7 L. donovani amastigotes.
TABLE 1. Comparison of the abilities of SSG and SSG-NIV treatments to protect against subsequent challenge infection, as determined on day 87a

| Group | Primary infection | Treatment on day 7 | Challenge infection | Mean parasite burden ± SE |
|-------|------------------|-------------------|---------------------|---------------------------|
|       |                  |                   |                     | Spleen                  |
| A     | Yes PBS          | No                |                     | 539 ± 237               |
| B     | No None          | Yes               |                     | 197 ± 31                |
| D     | Yes SSG          | No                |                     | 209 ± 57                |
| E     | Yes SSG-NIV      | No                |                     | 26 ± 11 B              |
| F     | Yes SSG          | Yes               |                     | 206 ± 64                |
| G     | Yes SSG-NIV      | Yes               |                     | 153 ± 24 D             |
| H     | No SSG           | Yes               |                     | 190 ± 41               |
| I     | No SSG-NIV       | Yes               |                     | 229 ± 80               |

* Mice were infected on day 0 with 2 × 10⁶ L. donovani amastigotes and treated on day 7 with 0.2 ml of either SSG solution (100 mg of SSG/ml), SSG-NIV (100 mg of SSG/ml), or PBS (primary controls). Age-matched uninfected animals were similarly treated on day 7 with free SSG, SSG-NIV, or PBS (secondary controls). On day 38, half of the infected mice (i.e., primary controls, infected free-SSG-treated mice, and infected SSG-NIV-treated mice) and all the uninfected mice (pretreated with SSG, SSG-NIV, or PBS [secondary controls]) were infected with 0.5 × 10⁶ L. donovani amastigotes. Animals were sacrificed on day 87, i.e., day 45 postchallenge. Capital letters indicate statistical significance compared with values for the primary controls (group A [letters A to C]) or the secondary controls (group B [letters D and E]) as follows: A, P < 0.01; B, P < 0.005; C, P < 0.0005; D, P < 0.005; and E, P < 0.0005.

SSG-NIV treatment of infection gave no consistent protective effect against challenge infection. At day 45, liver (P < 0.05), spleen (P < 0.005), and bone marrow (P < 0.005) parasite burdens of challenged SSG-NIV-treated mice (group G) were significantly lower than those of the unchallenged group (group E [Table 2]). Spleen and liver parasite numbers of infected SSG-NIV-treated mice (group G) and secondary controls (group B) were similar (Table 2). However, bone marrow parasite burdens of challenged SSG-NIV-treated mice (group G) were significantly lower (P < 0.05) than those of secondary controls (group B), and in similar experiments using different SSG-NIV preparations and different parasite challenge inocula, challenged SSG-NIV-treated mice had lower liver and/or bone marrow parasite burdens (data not shown). However, this protective effect could be explained by the presence of residual drug, since prophylactic SSG-NIV treatment also suppressed bone marrow parasite burdens compared to those of secondary controls (compare groups E and I in Tables 1 and 2). On day 87, parasite burdens of challenged SSG-NIV-treated mice (group G) were significantly higher than those of the unchallenged group in all three sites (for group E, the P value for the spleen and liver was < 0.005 and that for bone marrow was < 0.0005 in comparison with results for group G [Table 1]) and not lower than those of secondary controls (group B) or mice given SSG-NIV prophylactically (group I).

TABLE 2. Comparison of the abilities of SSG and SSG-NIV treatments to protect against subsequent challenge infection, as determined on day 45a

| Group | Primary infection | Treatment on day 7 | Challenge infection | Mean parasite burden ± SE |
|-------|------------------|-------------------|---------------------|---------------------------|
|       |                  |                   |                     | Spleen                  |
| A     | Yes PBS          | No                |                     | 357 ± 26               |
| B     | No None          | Yes               |                     | 8 ± 2                   |
| D     | Yes SSG          | No                |                     | 163 ± 29               |
| E     | Yes SSG-NIV      | No                |                     | 13 ± 5 B               |
| F     | Yes SSG          | Yes               |                     | 166 ± 33               |
| G     | Yes SSG-NIV      | Yes               |                     | 30 ± 15                |
| H     | No SSG           | Yes               |                     | 11 ± 3                 |
| I     | No SSG-NIV       | Yes               |                     | 11 ± 2                 |

a Mice were treated as described in Table 1 footnote a, except that mice were sacrificed on day 45 (day 7 postchallenge). Capital letters indicate statistical significance compared with values for the primary controls (group A) as follows: A, P < 0.01; B, P < 0.005; and C, P < 0.0005.
Prophylactic treatment with SSG-NIV was more effective than treatment with SSG. Assuming that in all three tissue sites examined the parasiticidal concentration of SSG is the same, then the three sites could be ranked liver > spleen > bone marrow based on the ability to retain parasiticidal drug concentrations. Previous studies (13, 14) have shown that after administration of SSG-NIV more of the drug dose is directed to the liver, and higher tissue levels of antimony are obtained, than when free SSG is given at the same dose. This is not surprising, since a function of this organ is to clear particulate material from the circulation (17). A similar manipulation of drug activity at the level of particular tissues (11, 18, 27) using a variety of colloidal formulations showed that interorgan differences are formulation sensitive. The ranking of the three sites established in this study, however, probably reflects both interorgan differences in initial drug levels (delivery) and subsequent excretion rates.

It seems that drug uptake and persistence in certain tissue locations, rather than an immune effect, could explain the protection observed in response to challenge of infected SSG-NIV-treated mice. The fact that the level of protection was similar to that obtained by prophylactic SSG-NIV treatment supports this hypothesis. The greater prophylactic activity of SSG-NIV treatment than of free-SSG treatment reflects the former’s ability to direct a large proportion of the injected drug dose to tissues (14). The high efficacy of SSG-NIV depends on the quantity of drug entrapped (27); interexperimental variability could reflect NIV preparation-dependent drug entrapment efficiency, which in turn influences delivery to different tissues.

Protective immunity was not transferred with spleen cell suspensions prepared from SSG-NIV-treated mice, since on infection, cell recipients had parasite burdens which were similar to those of controls. Failure to transfer immunity may be a consequence of the innate susceptibility of BALB/c mice to L. donovani infection (5–7) or of the limited exposure of cell donors to L. donovani infection before drug treatment. It has been suggested that L. donovani-infected mice are resistant to reinfection (19, 20, 25). The results of this study confirmed that challenge of L. donovani-infected mice does not result in higher parasite burdens than those obtained in unchallenged animals. However, challenge of infected mice which had been treated with either free SSG or SSG-NIV raised parasite burdens in the liver (day 45 in free-SSG-treated mice and days 45 and 87 in SSG-NIV-treated mice), spleen

**DISCUSSION**

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**TABLE 3. Efficacies of different formulations of free SSG and SSG-NIV in SCID BALB/c mice and their immunocompetent counterparts**

| Mouse type and treatment | % Suppression (mean ± SE) |
|--------------------------|--------------------------|
|                          | Bone marrow | Liver | Spleen |
| BALB/c SCID              |             |      |       |
| SSG                     | 99 ± 1      | 96 ± 2| 0 ± 5  |
| SSG-NIV                 | 99 ± 1      | 96 ± 2| 0 ± 5  |
| Normal BALB/c            |             |      |       |
| SSG                     | 20 ± 8      | 98 ± 1| 6 ± 4  |
| SSG-NIV                 | 98 ± 2      | 100 ± 0| 100 ± 0|

*Animals were infected on day 0 with 10⁷ L. donovani amastigotes and then treated on day 7 with a single dose of SSG solution or SSG-NIV (equivalent to 296 mg of Sb/kg). The experiment was terminated on day 14, and parasite burdens in the liver, spleen, and bone marrow were determined.*
(days 45 and 87 in SSG-NIV-treated mice), and bone marrow (day 87 in SSG-NIV-treated mice) compared to levels in unchallenged mice. It may be no coincidence that the raised burdens occurred in sites where parasite numbers had been lowered by drug treatment. The presence of an upper limit on parasite load in chronically infected mice would explain why parasite burdens of primary and secondary controls were similar by day 87. Surprisingly, challenge of L. donovani-infected animals with a second infection did not result in enhanced IgG1 or IgG2a antibody titers compared to those of primary controls. Perhaps the high antibody titers of primary controls (>1:100,000) meant that on challenge the mice could not produce any more pathogen-specific antibody since the total specific B-cell population had been stimulated. This could explain why by day 87 antibody levels in primary and secondary controls were similar.

Sterne et al. (25) found that in nude mice it was possible to transfer immunity against L. donovani with unfractionated T cells from euthymic L. donovani-infected mice. The failure to transfer immunity by using cells from infected mice in this study may be due to differences in experimental protocols. In this study, cells transferred to euthymic murine recipients were collected earlier postinfection (after 30 to 50 days instead of 16 to 24 weeks), and parasite burdens in recipients were determined earlier postinfection (day 14 instead of week 4 or 8). The ability to produce specific antibody was, however, transferred with the spleen cells, since specific IgG1 (cells from both infected controls and infected SSG-NIV-treated mice) and IgG2a (cells from infected controls only) was detected in the plasma of uninfected recipients. Previous studies have shown that spleen cells from SSG-NIV-cured, L. donovani-infected mice respond to specific stimulation in vitro (2) by day 24 posttreatment (day 31 postinfection), which suggests that potentially protective memory lymphocytes should have been present.

At the same dose, the superiority of vesicular over free SSG was clearly demonstrated in this study. It elicited greater parasite suppression in the spleen, liver, and bone marrow, and free SSG did not have the prophylactic activity of SSG-NIV, which was as effective in immunocompetent and immunocompromised animals. The activity of SSG-NIV in the immunocompromised host may give this formulation a significant advantage over currently available antileishmanial formulations, since high relapse rates occur in AIDS patients with VL after treatment with antimonial (1) or amphotericin B (3, 23) drug formulations. Studies to develop an SSG-NIV formulation for possible clinical use are under way.

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