SODIUM ANTIMONY GLUCONATE (SAG) MEDIATES ANTILEISHMANIAL EFFECT BY STIMULATING INNATE AND CELLULAR ARMS OF THE IMMUNE SYSTEM

JAYATI MOOKHERJEE BASU AND SYAMAL ROY

Pentavalant antimony complexes, such as Sodium antimony gluconate (SAG) is still the first choice for chemotherapy against various forms of leishmaniasis including visceral leishmaniasis or kala-azar. Although the requirement of a somewhat functional immune system for the anti-leishmanial action of antimony is known, the cellular and molecular mechanism of action of SAG is not clear. Our study very clearly showed that SAG treatment induces not only activation of important components of the intracellular signaling pathway but also enhances class I restricted antigen presentation. It was also observed that SAG treatment stimulates proliferation of both $CD4^+$ and $CD8^+$ T cells directly independent of antigen presenting cells.

Introduction

Each year, there are some 500,000 cases of visceral leishmaniasis (VL) (90% in Bangladesh, Brazil, India, Nepal and Sudan), with an estimated 50 000+ deaths, and 1,500,000 cases of cutaneous disease (90% in Afghanistan, Algeria, Brazil, Islamic Republic of Iran, Peru, Saudi Arabia and Sudan). The global mortality from visceral leishmaniasis can only be estimated, because in many countries the disease is not notifiable or is frequently undiagnosed, especially where there is no access to medication. In some cases, for cultural reasons and lack of access to treatment, the case-fatality rate is three times higher in women than in men. The disease burden is calculated at 2 090 000 disability adjusted life years (1 249 000 in men and 840 000 in women), a significantly high rank among communicable diseases. There is a shortage of information on the actual cost of leishmaniasis, although it is known that in some parts of Asia a family in which there is a case of leishmaniasis is three times more likely than an unaffected family to have sold its cow or rice field, plunging it into a vicious circle of disease-poverty-malnutrition-disease. The number of cases is increasing, mostly because of gradually increasing transmission in cities, displacement of populations, exposure of people who are not immune, deterioration of social and economic conditions in outlying urban areas, malnutrition (with consequent weakening of the immune system), and coinfection with HIV. In 34 of the 88 countries in which the disease is endemic, cases of coinfection have been reported.[1]

* Indian Institute of Chemical Biology, Kolkata- 700032, INDIA.

The First Line of Treatment of Leishmaniasis: Antimonials

Antimonials are the first choice among the chemotherapeutics available so far and being used to treat leishmaniasis for the last 60 years. Among alternative drugs, pentamidine is toxic, amphotericin B is both expensive and toxic with reported cases of resistance, the liposomal form of amphotericin B is prohibitively expensive and needs hospitalization, and oral miltefosine is very expensive with contraindications and emerging resistance. On the contrary, antimonials are very cheap and affordable for common people of Indian subcontinent.

Development of antimonials as antileishmanial chemotherapy: In a treatise published in Leipzig in 1604, antimony, which was introduced by Paracelsus as a general panacea in the 16th century, was acclaimed as one of the

Seven Wonders of the World. Sometimes banned and often argued over for another three centuries, the modern era of its usage began in 1905 when Plimmer and Thompson showed the activities of antimony against trypanosomes in rats and subsequently in the treatment of human trypanosomiasis in Africa. The first published records of use of these trivalent antimonials tarter emetic for treatment were by Macado and Vianna in 1913 for CL, and by di Cristina and Cariona in Sicily and Rogers in India in 1915 for VL[2]. Later it was found to be highly toxic as well as very unstable in tropical climate[3,4]. Thereafter, the pentavalent antimony compound urea stibamine synthesized by U. N. Brahmachari (1920) emerged as an effective chemotherapeutic agent against Indian Kala-azar (KA). The development of the less toxic pentavalent antimonials by Brahmachari, Schmidt, Kikuth and others led to the synthesis of antimony gluconate (Solustibosan) in 1937[5] and sodium stibogluconate (Pentostam) in 1945[6]. Another carbohydrate complex, meglumine antimoniate (Glucantime, Aventis) soon followed. Solutions of these two pentavalent antimonials, Pentostam (GlaxoSmithKline, UK) containing 100 mg Sb(V) ml^{-1} and Glucantime (Aventis, France) containing 85 mg Sb^V ml^{-1}, remain the first line treatments for VL and some forms of CL[7,8] . Generic antimonial products are also available; a recent clinical trial in Sudan has shown the Albert David (Calcutta) product Pentostam to be as effective, but 14 times cheaper, than the GSK product[9]. Despite their longevity and because of the absence of more suitable alternative drugs, studies on the optimal use, variations in drug sensitivity, indications of resistance and mechanisms of action are still in progress.

Mechanisms of Action

Species Sensitivity **:** Biochemical studies over the past two decades have indicated a number of potential targets for pentavalent antimonials: glycolysis[10] (in particular inhibition of ADP phosphorylation[2]), DNA I topoisomerase[11, 12] and trypanothione[13, 14]. Molecular analysis of *in vitro* established drug-resistant clones has suggested inhibition of fatty acid beta-oxidation[15]. However, the precise mechanisms of action of these drugs and the relevance of such studies will remain uncertain unless activities are related to pharmacologically achievable concentrations, remembering that the target cell is an intracellular parasite and there is a variation in strain/species sensitivity.

Recent research has started to define some ground rules. Firstly, it is important to be clear about the drug formulation being used. After 50 years the complex chemistry of meglumine antimoniate (Glucantime) has been characterized and a major moiety [MW 507] has been identified[16]. However, despite many efforts, the exact nature of the complex polymeric carbohydrate sodium stibogluconate and its molecular weight has not been determined [16, 17].

Secondly, it is important to remember the differential *in vitro* sensitivity of promastigotes and amastigotes to pentavalent antimonials in comparison to trivalent antimonials. Until recently it was unclear whether this difference, with promastigotes insensitive to concentrations of 1000 mg Sb^V/ml compared to amastigotes with an IC50 value of 10 mg Sb^V/ml, was due in part to the influence of the macrophage (MΦ) host cell. In addition, the IC50 values reported for axenic amastigotes are about 10-fold higher than would be expected for intracellular amastigotes[18]. Although these results confirm that amastigotes have greater intrinsic sensitivity to pentavalent antimonials than promastigotes, they also highlight the caution necessary with regard to formulations, species and systems used. Drug uptake could contribute to the differential activity and early studies indicated that amastigotes have a superior ability to concentrate drug than promastigotes[19, 20]. However, when comparing promastigotes with intra-macrophage amastigotes, the concentrations of antimony determined in the parasite forms following incubation at (the very different) IC50 concentrations were found to be similar. Some explanation for the difference between amastigote and promastigote sensitivity has recently been advanced[21]. It was shown that axenic *L. donovani* amastigotes were able to metabolise Sb^v to Sb^{III} more effectively than promastigotes although the mechanism was not elucidated. A third factor that influences the activity of antimonials is the interaction with the host MΦ, either through accumulation or metabolism. In a J774 MΦ-*L. panamensis* model, Pentostam was accumulated to only 33% of the concentration in the external medium after a 4-hour incubation period, in contrast to trivalent antimonials which were concentrated from the medium. However, there was evidence that MΦs could retain pentavalent antimony[22]. Importantly, considering the nature of the parasitophorous vacuole, activity was maintained[22] at low pH in keeping with previous observations [23]. Metabolism of penta- to trivalent antimonials by MΦs has recently been described to involve host cell glutathione[24].

SAG activates intracellular signaling events to generate ROS and NO : Pentavalent antimonials have never been regarded as exerting an effect solely due to direct cytotoxic action. Schmidt and Peter, (1938)[7] mention a moderate role for antimonials in the paradigm "the

reticulo-endothelial system (i.e. its stimulation by drugs etc.) is of importance for the cure". Studies on murine VL infections (BALB/c – *L. donovani*) have established that an intact T-cell population, more specifically Th1, is required for Sb^V to have a curative anti-leishmanial effect[25-27]. The drug itself is leishmanicidal *in vitro* and *in vivo*, however complete cure *in vivo* is not achieved without Th1 input. Patients co-infected with VL-HIV respond poorly to treatment[28]. After an initial response, these patients frequently relapse and require alternative treatment[29]. Dermotropic infections in man usually selfcure. This can take from 3 months to 3 years depending on the species of *Leishmania* involved. In such cases antimonial treatment augments the host's immune response to rapidly resolve the infection. Exceptional cases include DCL where in the absence of a cell mediated response antimonials prove to be ineffective[30]. Several studies have shown that endogenous IL-2[31] , IL-4[32, 33] and IL-12[34] influence the effectiveness of chemotherapy with pentavalent antimony. These findings indicate the requirement of somewhat functional T cell compartment for SAG action.

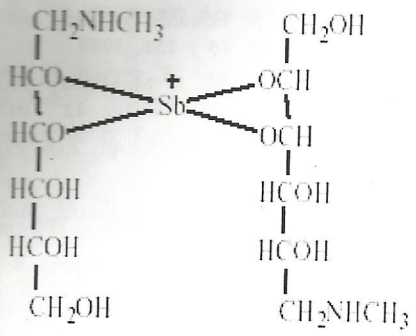
Murray[35] demonstrated that MΦ activation had a significant effect on intracellular parasite killing. Treatment with SAG has been reported to induce ROS generation in peripheral blood cells of *L. infantum* infected mice on stimulation with phorbol ester (PMA) or zymosan[36] (50), and to induce NO[37] in canine leishmaniasis. Recently it has been shown by our group that sodium antimony gluconate (SAG) alone induces both ROS and NO in murine MΦ resulting in two waves of killing of *L. donovani* amastigotes. The first phase of killing (i.e., at early time point, around 6 h post treatment) was due to induction of ROS as evident from the sensitivity to tocopherol or NAC, while the second wave of killing (i.e., at later time point, 24 h and 48 h) was mediated by NO generation as evident from the sensitivity to L-NMMA failure of SAG mediated parasite killing in *L. donovani* infected peritoneal MΦ from iNOS $^{-/-}$ C57BL/6 mice[38]. Both ROS and NO are known to be involved in parasite killing in the early stage of leishmanial infection in mice, whereas NO alone is involved in the late phase[39]. The importance of NO in the final elimination of leishmanial parasites is further underlined by the studies that treatment of *L. major* infected mice with L-NMMA drastically increased the lesion size[40], while *L. major* infection in iNOS knockout mice visceralized in a late phase of infection[41].

We also showed that SAG-induced activation of the PI3K-PKC-Ras/Raf pathway triggered ERK phosphorylation and thereby ROS generation. Pretreatment of *L. donovani* infected MΦ with inhibitors of any of these signaling molecules results in the inhibition of SAG-mediated ROS generation. On the other hand, activation of the PI3K/Akt pathway by SAG seems to trigger phosphorylation of p38 MAPK. Inhibition of PI3K or p38 MAPK strongly abrogated SAG mediated NO production in *L. donovani* infected MΦ as well as parasite killing at ~24h following SAG treatment. This suggests that NO production and subsequent parasite killing in response to SAG treatment involved mainly PI3K and p38 MAPK. It was further shown that p38 MAPK mediated NO generation by SAG treatment is an indirect mechanism. Actually p38 MAPK induces TNFα production, which in turn induces iNOS2 expression and subsequent NO generation, as SAG mediated NO generation and parasite killing could be abrogated by treatment with anti-TNFα neutralizing Ab[38].

LD infection has been reported to increase PTPase activity, mainly that of SHP1 type[42, 43, 44], which might contribute to dysregulation of PTK dependent signaling events[44] and MΦ deactivation [43]. SAG inhibits SHP1 and SHP2 classes of PTPases but not MKP1 type[45]. Sb^v and the gluconic acid backbone bound in various specific stoichiometric ratios inhibit purified SHP1 with specific efficacies[45]. SHP1 might directly dephosphorylate ERKs [46] and regulate activation of other important signaling molecules like PI3K[46]. Thus, inhibition of SHP1 by SAG might indirectly favor the tyrosine phosphorylation of PI3K, as well as thereby helping the activation of the PI3K-PKC-Ras/Raf-ERK1/2 pathway for ROS generation and the PI3K-Akt-p38 MAPK pathway for NO generation[36].

In addition, SAG up-regulates IFN-γ receptors both in uninfected and LD infected THP1 cells, as well as in monocytes derived from kala-azar patients treated with SAG[47]. Thus it is quite possible that SAG influences the host's anti-leishmanial defense by altering IFN-γ responsiveness. Indeed, SAG fails to act in IFN-γ knockout mice[48]. We have also observed that SAG and IFN-γ synergize to produce high levels of NO in MΦs[36]. A combination of SAG and IFN-γ is also known to be therapeutically much more effective than SAG alone in the treatment of visceral leishmaniasis[49]. We have further observed that SAG triggers production of IL12 in both uninfected as well as infected MΦ. IL12 is known to induce Th cells to produce IFN-γ which in turn activates MΦs to produce TNFa and subsequently, NO.

SAG upregulates class I MHC molecules and enhances antigen presentation : SAG was also shown to upregulate expression of specifically MHC I molecules on the MΦ surface and enhanced class I mediated antigen presentation but not MHC class II mediated presentation in murine MΦ (Fig. 1). This may be a mechanism by which



Meglumine antimoniate (Glucantime™).

Fig. 1 Structure of Meglumine antimonite (Glucantine™).

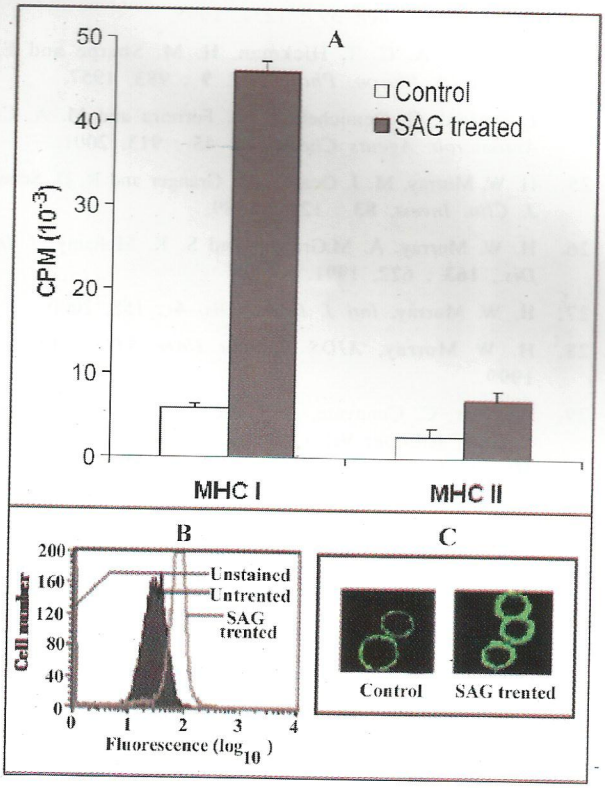


Fig. 2 SAG enhances MHC class I mediated antigen presentation and expression of MHC class I. MΦs of BALB/c and C57BL/6 mice were cultured in presence or absence of SAG for 24h. A) To study the antigen presenting function, peritoneal MΦs from BALB/c and C57BL/6 mice were either kept untreated or treated with SAG for 24 h and used as antigen presenting cells to drive the T–cell hybridoma in presence of appropriate peptide. The resulting IL-2 was tested on IL-2 dependent cell line (HT-2). The growth of HT-2 was studied using ^{3}H-Thymidine incorporation. Our studies showed that class I but not class II restricted presentation was significantly ($P<0.001$) enhanced upon SAG treatment both in normal and infected MF. B) To study the expression of MHC I molecules untreated and SAG treated MΦs from BALB/c mice were stained with FITC labeled anti-D^d (BD Pharmingen) according to the manufacturer's instruction and either analyzed on flow cytometer or examined under a confocal laser scanning microscope. The studies showed that class I expression was significantly ($P<0.001$) enhanced upon SAG treatment. Antigen presentation assay was performed at least thrice and the results are presented as mean ± SD. For flow cytometry and confocal microscopy, representative data of 3 similar experiments is presented here.

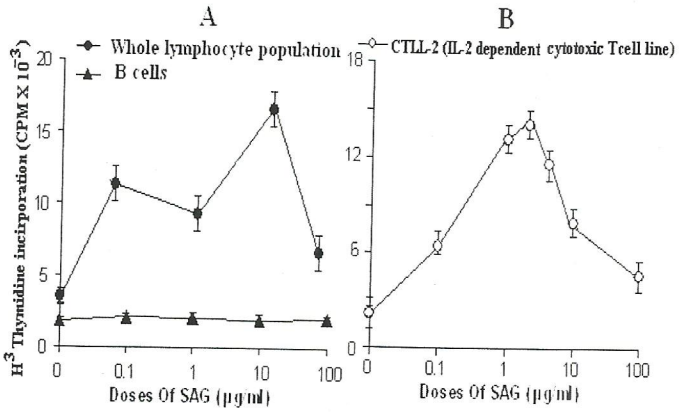


Fig. 3 SAG directly stimulates proliferation of T cells. 10^5 lymphocytes, (either whole lymphocytes or B cells from normal BALB/c mice) (A) and $5X10^4$ IL-2 dependent $CD8^+$ cytotoxic T cell line (CTLL-2) were plated in each well and were kept either untreated or treated in vitro with various concentrations of SAG. Proliferation of each types of cells was monitored by ^{3}H thymidine incorporation. Each experiment was performed at least thrice and results are presented as mean ± SD.

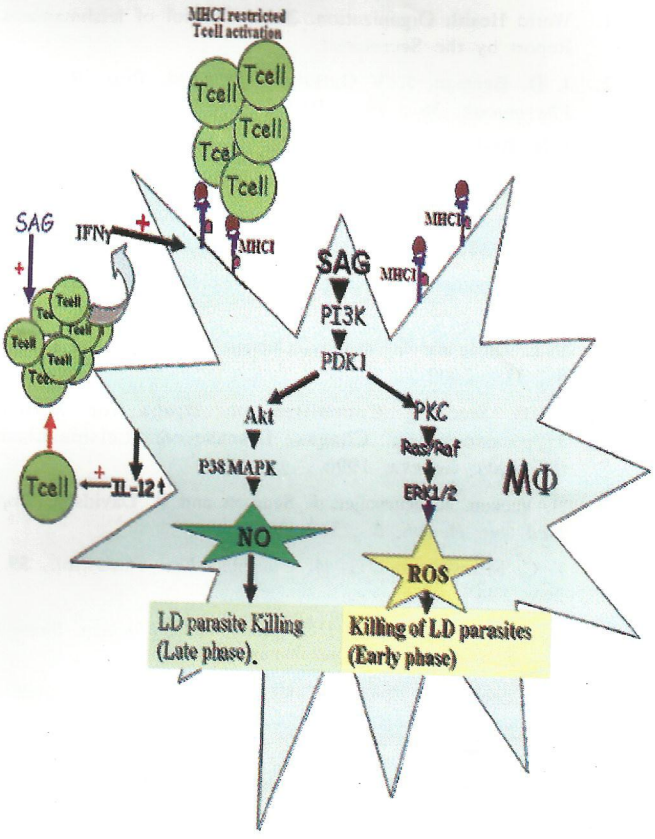


Fig: 4 SAG activates both innate and cellular arms of the immune system to eliminate LD parasite.

SAG can enhance anti-leishmanial cytotoxic T lymphocyte (CTL) response. There is indeed a report that CTLs can kill intracellular parasites [50].

Interestingly stimulation of spleen cells derived from either *Leishmania* infected or uninfected mice induced IFN-γ generation (Mookerjee Basu, unpublished data). Carter *et al.* showed that SAG treatment of infected mice imparted resistance to reinfection while SAG treatment prior to infection imparted partial resistance to *Leishmania* infection.

SAG induces proliferation of T-cells but not of B cells (Fig. 2) even in absence of antigen presenting cells (Mookerjee Basu, unpublished observation). Interestingly SAG mediated proliferation of T cells does not require IL-2 (Fig. 2).

Thus on the one hand SAG could activate T cell compartment (in both MHC independent and dependent manner), and on the other could directly activate MΦs to induce generation of microbicidal effector molecules (NO), which in concert help to potentiate both innate and cellular arms of the immune system to eliminate LD parasites.

References

1. World Health Organization. 2006, Control of leishmaniasis: Report by the Secretariat.
2. J. D. Berman, J. V Gallalee and J. M. Best, *Biochem. Pharmacol.*, **36** : 197, 1987.
3. U.N. Brahmachari, *Ind. J. Med. Res.*, **10** : 492, 1922.
4. U.N. Brahmachari, *Ind. J. Med. Gaz.*, **57** : 125, 1922.
5. W. Kikuth and H. Schmidt, *Chinese Medical Journal*, **52** : 425, 1937.
6. L. G. Goodwin, *Trans. R. Soc. Trop. Med. Hyg.*, **89** : 339, 1995.
7. S. L. Croft and V. Yardley, *Current Pharmaceutical Design*, **8** : 319, 2002.
8. WHO Steering Committee on Drugs for African Trypanosomiasis, Chagas Disease & Leishmaniasis (ICHEM): Geneva, 1996.
9. H. Veeken, K. Ritmeijer, J. Seaman and R. Davidson, *Trop. Med. Int. Health*, **5** : 312, 2000.
10. J. C. Mottram and G. H. Coombs, *Exp. Parasitol.*, **59** : 265, 1985.
11. A. K. Chakraborty and H. K. Majumder, *Biochem. Biophys. Res. Commun.*, **152** : 605, 1988.
12. A. Lucumi, S. Robledo, V. Gama and N. G. Saravia, *Antimicrob. Agents Chemother.* **42** : 1990, 1998.
13. D. Legare, B. Papadopoulou, G. Roy, R. Mukhopadhyay, A. Haimeur, S. Dey, K. Grondin, C. Brochu, B. P. Rosen and M. Ouellette, *Exp. Parasitol.* **87** : 275, 1997.
14. R.Mukhopadhyay, S. Dey, N. Xu, D. Gage, J. Lightbody, M. Ouellette and B. P. Rosen, *Proc. Natl. Acad. Sci. USA.* **93** :10383, 1996.
15. J. D. Berman, N. Edwards, M. King and M. Grogl, *Am. J. Trop. Med. Hyg.* **40** : 159, 1989.
16. W. L. Roberts, W. J. McMurray and P. M. Rainey, *Antimicrob. Agents Chemother.* **42** : 1076, 1998.
17. J. D. Berman and M. Grogl, *Exp. Parasitol.* **67** : 96, 1988.
18. D. Sereno and J. L. Lemesre, *Antimicrob. Agents Chemother.* **41** : 972, 1997.
19. J. D. Berman, *Am. J. Trop. Med. Hyg.* **30** : 566, 1981.
20. S. L. Croft, K. D. Neame and C. A. Homewood, *Comp. Biochem. Physiol.* **68C** : 95, 1981.
21. P. Shaked-Mishan, N. Ulrich, M. Ephros, and D. Zilberstein *J. Biol. Chem.* **276** : 3971, 2001.
22. W. L. Roberts, J. D. Berman and P. M. Rainey, *Antimicrob. Agents Chemother.* **39** : 1234, 1995.
23. J. Green, A. C. T. Hickman, H. M. Sharpe and E. G. Tomich, *J. Pharm. Pharmacol.* **9** : 983, 1957.
24. F. Frezard, C. Demicheli, C. S. Ferreira and M. A. Costa, *Antimicrob. Agents Chemother.* **45** : 913, 2001.
25. H. W. Murray, M. J. Oca, A. M. Granger and R. D. Schreiber *J. Clin. Invest.* **83** : 1253, 1989.
26. H. W. Murray, A. M.Granger and S. K. Mohanty, *J. Infect. Dis.*, **163** : 622, 1991.
27. H. W. Murray, *Intl J. Infect. Dis.* **4** : 158, 2000.
28. H. W Murray, *AIDS Patient Care STDS.* **13** : 459, 1999.
29. J. Alvar, C. Canavate, B. Gutierrez.Solar, M. Jimenez, F. Laguna, R.Lopez.Velez, R. Molina and J. Moreno, *Clin. Microbiol. Rev.* **10** : 298, 1997.
30. G. De Jimenez, *N. Ercoli. Exp. Parasitol.* **17** : 302, 1965.
31. H. W. Murray, G. D. Miralles, M. Y. Stoeckle and D. F. McDermott, *J. Immunol.* **151** : 929, 1993.
32. J. Alexer, K. C. Carter, N. Al.Fasi, A. Satoskar and F. Brombacher, *Eur. J. Mmunol.* **30** : 2935, 2000.
33. G. S. Nabors and J. P. Farrell, *Infect. Immun.* **62** : 5498, 1994.
34. H. W. Murray, C. Montelibano, R. Peterson and J. P. Sypek, *J. Infect. Dis.* **182** : 1497, 2000.
35. H. W. Murray and C. F. Nathan, *J Infect Dis.* **158** : 1372, 1988.
36. S. Rais, A. Perianin, M. Lenoir, A. Sadak, D. Rivollet, M. Paul and M. Deniau, *Antimicrob. Agents Chemother.* **44** : 2406, 2000.
37. I. Vouldoukis, J. C. Drapier, A. K. Nussler, Y. Tselentis, O. A. Da Silva, M. Gentilini, D. M. Mossalayi, L. Monjour and B. Dugas, *Antimicrob. Agents Chemother.* **40** : 253, 1996.
38. H. W Murray and C. F. Nathan, *J. Exp. Med.*, **189**: 741-746, 1999.
39. F.Y Liew, S. Millott, C. Parkinson, R. M. Palmer and S. Moncada, *J Immunol.* **144** : 4794-4797, 1990.
40. X. Q Wei, I. G. Charles, A. Smith, J. Ure, G. J. Feng, F. P. Huang, D. Xu, W. Muller, S. Moncada and F. Y. Liew, *Nature.* **375** : 408-411, 1995.
41. J. Mookerjee Basu, A. Mookerjee, P. Sen, S. Bhaumik, P. Sen, S. Banerjee, K. Naskar, S. K Choudhuri, B. Saha, S.

Raha and S. Roy, *Antimicrob Agents Chemother.* **50** : 1788, 2006.

42. J. Blanchette, N. Racette, R. Faure, K. A. Siminovitch and M. Olivier, *Eur. J. Immunol.* **29** : 3737-3744, 1999.

43. D. Nandan and N. E. Reiner, *Clin. Immunol.* **114** : 266-277, 2005.

44. D. Nandan, T. Yi, M. Lopez, C. Lai and N. E. Reiner, *J. Biol. Chem.* **277** : 50190-50197, 2002.

45. T. Yi, M. K. Pathak, D. J. Lindner, M. E. Ketterer, C. Farver and E. C Borden, *J. Immunol.* **169** : 5978-5985, 2002.

46. M. Olivier, D. J. Gregory and G. Forget, *Clin. Microbiol Rev.* **18** : 293-305, 2005.

47. B. Dasgupta, K. Roychoudhury, S. Ganguly, P. K. Sinha, S. Vimal, P. Das and S. Roy, *Ann. Trop. Med. Parasitol.* **97** : 245, 2003.

48. H. W. Murray and S. Delph.Etienne, *Infect. Immun.* **68**: 288, 2000.

49. H. W. Murray, J. D. Berman and S. D. Wright, *J. Infect. Dis.* **157** : 973, 1988.

50. L. E. Smith, M. Rodrigues and D. G. Russell, *J Exp Med.* **174**: 499, 1991.