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IMMUNLOGICAL SIGNATURES OF CURE IN EXPERIMENTAL VISCERAL LEISHMANIASIS FOLLOWING THE COMBINED IMMUNOCHEMOTHERAPY WITH PENTOSTAM AND TRE HALOSE DI MYCOLATE

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ABSTRACT

The purpose of this study to propose an alternative treatment to visceral leishmaniasis. Visceral leishaniasis is most neglected one of the top five parasitic diseases which is caused by Leishmanial donovani. The treatments which are available having toxic side effects and drug resistant cases also reported. To meet this purpose, Our work is based on the use of combined immunochemotherapy of tre haloes di mycolate and low dosages of pentostam and comparison the results from the higher standard dosage which has undesirable side effects .The studied was carried out by studying the immunological status of golden hamsters in infected and treated experimental hamsters by evaluating cellular and humoral immune responses. In the delayed type of hypersensitivity foot pad swelling in the infected animal was 11.6mm as compared to the treated with combined immunochemothrapy which was 8.4 mm. The results of macrophages migration inhibition test and DTH are in agreement to each other. ELISA and IHA titer values significantly reduced in in animals which were treated with combined TDM and Low dosage of pentostam as compared to the lower dosage alone.

KEYWORDS: Visceral leishmaniasis, Combinatorial therapy, Tre haloes di mycolate, pentostam, Leishmanial donovani, immono-chemptherapy.

INTRODUCTION

The term Leishmaniasis refers to a group of vector-borne diseases caused by 17 different species of protozoan parasites of the genus *Leishmania*, belonging to the family Trypanosomatida, order Kinetoplastida.[1].*Leishmania* is transmitted to humans by sand flies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World. *Leishmania* infections have been reported in 98 countries in all continents. The disease is endemic in tropical and subtropical regions. Leishmaniasis has a worldwide distribution with important foci of infection in Central and South America, southern Europe, North and East Africa, the Middle East, and the Indian subcontinent. Currently the main foci of visceral leishmaniasis (VL) are in Sudan and India.[2] Visceral leishmaniasis (VL), also known as kala azar is targeting tissue macrophages. It is among the most neglected infectious diseases.[3] Classical manifestations of VL include chronic fever, hepatosplenomegaly, and pancytopenia. Most cases can be detected through serologic and molecular testing.[4].*Leishmania* species exploit discrete mechanisms to elude the cellular immune defenses, such as inhibition of phagolysosomal fusion, and reactive nitrogen species (RNS)- and ROS-mediated macrophage microbicidal effects, dampening of cell-mediated immune response via blockade of antigenic peptide display to T cells, impaired secretion of Th1 cytokines, and infiltration of IL-10 producing T regulatory cells.[5-7].The treatment of visceral leishmaniasis (VL) is complicated because of intra-macrophagic refuge of the amastigotes, rendering the patient immunodeficient and unable to eliminate the parasites through the natural defense mechanisms.[8] The quandary of VL has been compounded due to concomitant infection in acquired immunodeficiency syndrome (AIDS) patients. Although the global burden of leishmaniasis has remained stable for several years, causing the loss of 2.4 million disability adjusted years, there are also changing patterns of disease.[9] Increasing numbers of human immuno deficiency virus (HIV) coinfections, human migration, and resettlement, especially important where leishmaniasis is zoonotic, make resurgence a possibility[10] So far, many clinical trials have been done in India to optimize the therapeutic regimens and to protect the efficacy of limited number of available anti-leishmanials.The currently use drugs is shown in figure(1)

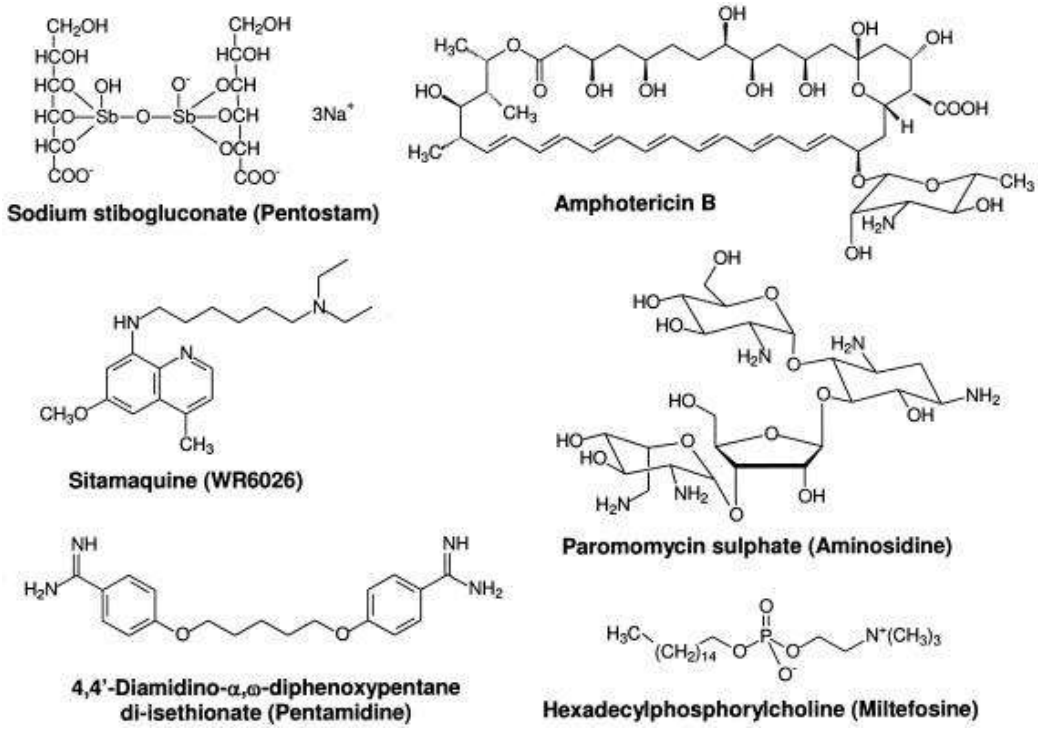

Figure:1 Drugs currently used in the treatment of Leishmaniasis

In recent years four new potential therapies have been introduced for visceral leishmaniasis (Table 1). These include an amphotericin B liposome formulation registered in the United States and Europe (AmBisome) [11, 12] oral miltefosine[13] which has been registered in India and is now in phase IV trial; a parenteral formulation of aminosidine[14]. Currently completing phase III clinical trials in India and on trial in East Africa and oral sitamaquine , which has completed phase II trials in India, Kenya, and Brazil [15,16] .

Table: 1 Current drugs for visceral leishmaniasis

Disease	Status of Drugs	Drugs
Visceral Leishmaniasis	First –line drugs	Sodium stibogluconate(pentostam),meglumine antimoniate(Glucantime),AmphotericinB(Fungizone),Liposomal amphotericinB(AmBisome),Pentamidine
	Clinical trials	Mitefosine(oral,phaseIV),paromomycin(phaseIII),Sitsmaqine(oral, phaseII),other amphoterucinB formulations

There is no vaccine available for VL; hence control of VL exclusively depends on chemotherapy. Available treatment options for VL are limited and not up to satisfactory standards due to problem relating to efficacy, adverse effects, increasing drug resistance, high cost and need for hospitalization to complete the full dose of treatment. Chemotherapeutic drugs presently in use may exert severe toxic reactions in treated individuals. Common side

effects during treatment are vomiting and giddiness, jaundice, hematuria, pneumonia,ECG abnormalities and anaphylactic shock which may further complicate the treatment. Furthermore, the above drugs do not result in the complete cure.[17]

For drug treatment to be successful, the cooperation of the immune mechanism of the host is often required.[18] One of the strategies for the treatment of leishmaniasis is to use specific or nonspecific immuno stimulation with microbial products or synthetic polymers Cord factorThe tre halose dimycolate, is a glycolipid molecule found in the cell wall of Mycobacterium tuberculosis and similar species. It is the primary lipid found on the exterior of M. tuberculosis cells.[19] TDM has been observed to influence immune responses, induce the formation of granulomas, and inhibit tumor growth.[20]

In this paper a combined immunochemothereupeutic approach is used to treat the visceral leishmaniasis by using tre haloes di mycolte and low dosage of pentostam. Our aim in this study to evaluate the efficacy of combinatorial immunochemotherapy of TDM and low dosage of pentostam and compared with the standard high dosage treatment which is having toxic effects when use alone. Immunological status in experimental leishmaniasis in golden hamsters was studied to evaluate the efficacy combinatorial immune chemo therapeutic approach.

MATERIAL AND METHODS

Laboratory culture

Leishmania donovani parasites(NICD-CII strain) were cultured on brain heart infusion agar with inactivated rabbit blood (BHMARB).Dehydrated Brain heart infusion powder and bacto-agar from Difco laboratories ;Detroit,Michigan ,USA were used for culturing the parasites.Hanks balanced salt soluton(HBSS) was prepared according to the prescribed procedure.

Animals

Healthy male golden hamsters(Meso criticus auretus) each weighing around 60-100 grams were purchased from the animal house facility of the central drug research nstitute(CDRI), Luckhnow, India. Hamsters were routinely used for in vivo maintenance of Leishmania parasites and for experimental purposes.

Immunoadjuvants

An aqueous suspension of tre haloes di mycolate(TDM) ,1mg/ml prepared from the mycobacterium bovis strain AN% was supplied by the kind courtesy of late Prof. Edgar leadeder,laboratories de Bochemie,Central national de la Racherche Scientifique,9119 Gif-sur-Yvette France.

Drug

Sodium stibogluconate (pentostam) equilvalent to 0.3 mg/Sbmg^{-1} was obtained from the welcome foundation,U.K.

In vitro cultivation of parasites

The promastigotes of L donovani (NICD:CII strain) were maintained on a biphasic medium containing brain heart infusion agar medium with rabbit blood and HBSS as overlay according to the method of Jalees et al (1982).[21]

Animal and Infection

The axenic culture of L.donovani was maintained on BHMARB medium containing HBSS as an overlay. The primary inoculum was subculture after 5 days. Promastigotes were harvested at 4^{o}C after successive washing in sterile normal saline containing gentamycin.Hamsters were infected peritoneal using an inoculum of about 1x10^{7} promastigotes/0.1 ml per hamster .Two weeks after the inoculation animals were split into six groups. Each group contains 5 animals.

Drug and TDM Treatment

Sodium stibogluconate (pentostam) equivalent to 0.3 mg/sb mg^{-1} was obtained from welcome foundation, UK on post inoculation day 30, the animals were given pentostam at a dose of 10 mg and 20 mg /kg of body weight for 5 days. An aqueous suspension of TDM (1mg/ml) was taken for inoculation.

Haematological Studies

Total leukocyte count and Differential count were made from normal, infected and treated animals according to the method described by Dacie and Lewis (1970).[22]

Immunological studies

The delayed type skin hypersensitivity (DTH) and macrophages inhibition test (MMIT) were employed for the assessment of CMI response

Table 2: DTH Response to L.donovani Antigens in Footpad Swelling

Animal Group	Mean Diameter+_SD**(mm) in weeks after challenge	P value
Normal	0	-
Normal+TDM	0	-
Infected	11.56+_0.64	P<0.001
Infected + TDM	12.02+_0.68	P<0.10
Infected +Drug(10mg)	10.58+_0.56	P<0.01
Infected+Drug(10mg)+TDM	8.4+_0.39	P<0.001
Infected+Drug(20mg)	9.36+_0.13	P<0.001

* Each group contained 5 animals

**Each value is the arithmetic mean of 5 values

- All values having p<0.05 are significant
- Significance was calculated by student‘t’ test

Table 3: Macrophage Migration Inhibition Test in L. donovani infected and treated hamsters

Animal Group	% Migration	% Migration inhibition +_SD	Statistical Significance
Normal	**92.5**	**91.0+_0.71**	-
Infected	60.0	4.08+_0.3	p<0.001
Infected+TDM	59.14	5.52+_2.07	p<0.01
Infected +Drug(10mg)	7.42	20.34+_1.42	p<0.001
Infected+Drug(10 mg)+TDM	83.39	59.75+_4.55	p<0.001
Inected+Drug(20 mg)	87.38	62.8+_3.27	p<0.001

- Each experimental group was compound with its respective control
- Each value is the arthemetic mean of 5 values
- Each value of p<0.01 are significant
- Signiicance was calculated by student‘t’ test

A. Skin Test(Delayed Type Hypersensitivity)

The test was carried out according to the method Crook and Holbrook (1983) [23].The delayed type of hypersensitivity response (DTH) was monitored by giving foot pad injections of washed promastigotes suspended in 0.5%phenol-saline (0.25ml,2.0x10^8 ml.The thickness of inoculated foot-pad was measured by using dial calipers(IME England) at24 hours following injections.

B. Macrophages Migration Inhibition Test (MMIT)

Following infection, animals showing positive DTH reaction from each experimental group (with or without TDM) were injected intraperitoneally with 10 ml of sterile paraffin oil.After

48 hours, the peritoneal fluid was collected into a separating funnel and allow to stand for one hour. The supernatant was separated by centrifugation at 1200 rpm for 10 minutes at 4^{o}C. The supernatant was separated and the cells were pooled, washed thrice with HBSS and suspended in RPMI 1640 medium containing penicillin (100 units/ml) and Streptomycin (100ug/ml). The peritoneal exudate cells were adjusted at a concentration of 3x10^{6} cells/ml and Capillary migration inhibition test was performed by modified method of Coyle et al.,[24]

Detection of Humoral Immune Response

The sera samples obtained from different groups of infected and treated animals were tested for the presence of specific antiL.donovani antibodies by means of IHA and ELISA.

A. Indirect Haemagglutination

This test was done according to the method of Mathew et.al. (1975).[25] A 3% suspension of sheep RBC were tanned with tannic acid91:20,000) for 30 minutes at 4^{0}C.The tanned cells were added to two fold dilutions of the serum. The highest dilution of serum showing the reaction was recorded as the titration end point.

B. Enzyme –Linked Immunosorbant Assay (ELISA)

About 2 mg of anti-hamster IgG in 0.1 ml of PBS was added to 5 mg of alkaline phosphatase and mixed at room temperature. The mixture was dialyzed extensively PBS at 4^{o}C.later glutaraldehyde was added to give a final concentration of 0.2% of the above mixture and incubated for 102 hours at room temperature. After incubation it was first dialyzed against PBS at 4^{o}C and then 0.05 M Tris (hydroxyl methyl amino methane) buffer, pH8.0 at 4^{o}C .To remove the insoluble materials the solution was centrifuged at 5000 rpm for 10 minutes and the test was performed according to the method of Lin et al(1981).[26]

Table 4: Indirect Haemaglutination test in L. donovani infected and treated hamsters

Animal Group	IHA Titre(mean+_SD)
Normal	Negative
Infected	2231+_735.67(915-2560)
Infected+Drug(10 mg)	1920+_905.09(640-2560)
Infected+Drug(10 mg)+TDM	70.4+_21.47(32-80)
Infected+Drug(20 mg)	108.4+_42.93(32-128)

- Each group contained 5 hamsters
- Figures in parenthesis represent the range of these values
- IHA Titer less than 32 was taken as negative

Table 5: ELISA Titer in infected and Treated Experimental hamster

Animal Group	ELISA Titer(Mean+_SD)
Normal	Negative
Infected	1024+_724(512-2048)
Infected+Drug(10 mg)	256+_181(128-512)
Infected+Drug910 mg)+TDM	32+_11.31(16-64)
Infected+Drug(20mg)	32+_11.31(16-64)

- Each group contained 5 hamsters
- Figures in parenthesis represent the range of these values

RESULT AND DISCUSSION

Visceral leishmaniasis or Kala azar is a disorder produced by a flagellated protozoan, leishmanial donovani. The disease is of major health importance leading to severe morbidity and mortality.[27] The importance of Leishmnial infection has been fully realized as in accordance with the goals of WHO ‘s tropical disease programme , visceral leismaniasis has been given a high priority next to malaria. Till date no effective nontoxic therapy for L donovani infection is available. Common side effects during the treatment are vomiting, giddiness, jaundice; hematuria, pneumonia and cardiograph abnormalities and anaphylactic shock which may further complicate the treatment. Furthermore, the above drugs often do not cause complete cure. For drug treatment to be successful, the cooperation of the immune mechanism of the host is often required. In the case of visceral leishmaniasis, the T cell mediated arm of the immune response is of paramount importance in containing and eventually eliminating the parasites from infected macrophages.Our study provides the evidence that a combined treatment of lower concentration of drug dose and adjuvant tre – halose-di mycolate (TDM) is equally or more effective than the currently employed standard procedures.Tre haloes di mycolate (6-6’diester’s of trehalose) synthesized by Mycobacteria,Cornybacteria and Nacariae as a part of theis cell wall has been shown to possess several immunopotentiating properties.[28,29].Kierszenbaum et al (1984) have reported that aqueous TDM suspension modulates mouse macrophages function,invitro, by augmenting both internalization and intra cellular destruction of Trypanoma cruzi[30].

The cellular immune response was studied by foot pad swelling, a measure of delayed type hypersensitivity response (DTH), due to administration of phenol suspended promastigotes was negative in normal animals and the animals treated with TDM. In vitro the CM responses were detected by the development of delayed type hypersensitivity response (DTH) following intradermal antigen inoculation. The test has been widely used in studies on the epidemiology

of kala azar in Kenya and of mucocutaneous leishmniasis in South African countries.[31] Foot Pad swelling in response to animal challenges by phenol suspension of promastigotes was taken as a measure of DTH responsiveness. The DTH response becomes positive only after 6-8 weeks of infection and gradually subsides when treated with the combination of TDM and lower dosage of drug (Table2). The result of macrophages migration inhibition is shown in Table 3.Infected animals group showed maximum migration inhibition. The lower drug doses did not appear to affect this situation, while the use of combined therapy significantly affects the macrophages migration inhibition values. On the Basis of various reports there are sufficient evidences to believe that the nonspecific protection in hamsters following the TDM administration .is mainly achieved due to macrophages activation a few days after its intra peritoneal inoculation of TDM in hamsters. The importance of macrophages is well documented for host protection against intracellular parasites. A parallelism exists between the cytotoxic activities of tre haloes di ester mediated macrophage sand their ability to release H_2O_2upon pharmacological triggering.[32,33]. The humoral immune response against leishmaniasis in golden hamsters was estimated by using Indirect haemaglutination(IHA) and Enzyme-linked immune absorbent assay(ELISA) tests. Serum samples from the animals of infected group was 2231 and after the treatment with low dosage alone the titer value was 1920 where as in the animal group treated with the same amount of low dosage of pentostam combined with pentostam the titer value obtained was 108.4(Table 4). Serum samples obtained from animals of different groups showed variation in the ELISA antibody titer values .Infected and lower dosage treated animal groups showed the rise in the antibody titer where as in the group of the combined therapy of drug and TDM ,the titer was 32(Table 5).

The combinatorial use of lower dosage of drugs which was not effective alone when combined with tre haloes di mycolate effectively reduce the parasitic load and can be used effectively to treat the visceral leishmaniasis.

CONCLUSION

In the absence of effective vaccine and vector control measures, control of VL almost exclusively depends on chemotherapy. The available drugs are costly and may require hospitalization that needs monitoring which cause substantial loss of income for affected families. Emergence of drug resistance further complicates the treatment of disease. Multidrug regimens for VL hold much promise, however, more studies are required on

treatment of HIV-VL co-infections as they serve as silent reservoir in endemic areas.. We have proposed the immunochemotherupic approach to treat the visceral leishmaniasi by using an immunomodulator tre halose di mycolate along with low dosage of pentostam which was significantly effective in combinatorial used instead of alone to reduce the toxic side effects.

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