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Leishmania major: *In vitro* and *in vivo* anti-leishmanial activity of paromomycin ointment (Leshcutan) combined with the immunomodulator Imiquimod

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Abstract

Paromomycin at 25, 50 and 100 μg/ml, inhibited the growth of *Leishmania major* amastigotes by 34.5%, 61.2%, 74.9% and 85.4%, 89.9%, 95.7% on the 2nd and the 4th day of treatment in culture, respectively. Methylbenzethonium chloride at 0.1 and 0.5 μg/ml and Imiquimod at 5 and 10 μg/ml, administered separately, inhibited the parasite development by 39.5% and 65.2% and 31.5% and 47.7%, respectively. Imiquimod (5–10 μg/ml) combined with either paromomycin (25, 50 and 100 μg/ml) or methylbenzethonium chloride (0.1 and 0.5 μg/ml) showed an anti-leishmanial additive effect. A 10 day topical treatment, twice daily, with an ointment containing 15% paromomycin and 12% methylbenzethonium chloride (Leshcutan), either undiluted or diluted 1:5 in soft white paraffin combined with 5% Imiquimod cream (Aldara), was as effective as Leshcutan given alone. The present study suggests that a combination of Aldara and Leshcutan is as effective as Leshcutan given alone in the topical treatment of CL caused by *L. major*.

Index Descriptors and Abbreviations: *Leishmania major*; Promastigotes; Amastigotes; Leshcutan; Aldara; *In vitro* and *In vivo* activity; PR, Paromomycin; MBCL, Methylbenzethonium chloride; IMQ, Imiquimod; Balb/c mice, Topical treatment

1. Introduction

Cutaneous leishmaniasis (CL) is one of the most important causes of chronic ulcerative skin lesions. The disease occurs clinically in several forms: acute CL, chronic CL, recurrent CL (RCL) and diffuse CL (DCL). Several species of *Leishmania* are involved, including *L. major, L. tropica* and *L. aethiopica* in the Old World, and several species of the *L. braziliensis* and *L. mexicana* in the New World. Some forms of the disease produce only mild, self-limiting lesions, while at the other extreme are the destructive mucocutaneous forms (MCL), caused by *L. braziliensis* and *L. panamensis*. With some exceptions (Magill et al., 1993), CL infections are limited to the skin and rarely metastasize to other sites or invade the reticuloendothelial system.

Due to an effective cellular response mediated by Th1 cells, the disease generally heals within several months and is characterized by strong DTH activity and poor antibody response (Nashed et al., 2000 and Wei et al., 2004). Macrophage killing of *Leishmania* is most effectively stimulated by IFNγ, both *in vitro* and *in vivo*, and can be further enhanced by TNF (Bogdan et al., 1990; Liew et al., 1990). Of particular interest is the observation indicating the role of IL-1 and IL-12 in the development of a protective Th1 immune response and in controlling the progression of chronic leishmaniasis in the highly susceptible Balb/c mice (Heinzel et al., 1993 and von Stebut et al., 2000). The role of dentritic cells in the development of immune response against CL was recently reported (Moll, 2000; Shah et al., 2003; von Stebut et al., 2000).

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Presently there is no optimal therapy for CL, and chemotherapy generally depends upon a small group of compounds each with its own efficacy and toxicity (Bari and Rahman, 2003; Croft and Yardley, 2002 and Lee and Hasbun, 2003). A new drug—Miltefosine, given orally, was shown to be effective against American CL caused by *L. panamensis* and *L. braziliensis*, with a respective cure rate of 66% and 94% (Soto et al., 2001). Topical treatment of CL was recently summarized by Gamier and Croft (2002). ''Leshcutan'', an ointment comprising of 15% paromomycin sulphate (PR) and 12% methylbenzethonium chloride (MBCL) in soft white paraffin (Teva Pharmaceutical Comp. Jerusalem, Israel), was proven to be highly effective against a variety of leishmanial strains both in human and in several animal models (El-On et al., 1988). However, the efficacy of either Leshcutan or various reformulated paromomycin ointments showed variable results, with a cure rate of 9–90% against Old and New World CL (Arana et al., 2001).

In leishmaniasis, the immunological state of the host appears to play an important role in the clinical pattern of the disease and on the efficacy of the treatment. It was therefore suggested to administer immunochemotherapy to patients suffering from severe forms of the disease, who did not respond to chemotherapeutic treatment (Falcoff et al., 1994, Machado-Pinto et al., 2002 and Mayrink et al., 2006). However, in certain cases, only a marginal synergism was obtained with the combined agents (Arana et al., 1994). Imiquimod (IMQ, 3M Pharmaceuticals, USA), a low molecular weight imidazoquinoline, is a novel immune-response-activating agent. It was found to induce IFNγ, TNF, IL-1β, IL-1α, IL-6, IL-10, IL-1 receptor antagonist, granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte CSF (G-CSF) (Skinner, 2003 and Testerman et al., 1995). IMQ has recently been shown to act synergistically with anti-leishmanial compounds in the treatment of CL, both in humans (Arevalo et al., 2001) and in experimentally infected mice (Buates and Matlashewski, 1999). The aims of this study were therefore, (a) to examine the effect of PR and MBCL alone and in combination with IMQ on the development of *L. major* amastigotes in infected murine macrophage in culture, and (b) to determine the efficacy of a topically applied IMQ ointment combined with Leshcutan, on the development of the parasite in experimentally infected Balb/c mice.

2. Materials and methods

2.1. Parasite strains and host animals

Male C3H/HeJ, 10- to 14-weeks-old, served as the source of peritoneal macrophages for *in vitro* studies and Balb/c mice of the same age served for monitoring drug efficacy *in vivo*. Rabbits were used as blood donors for blood agar medium. *L. major* (WHO code: MHOM/IL/80/Freidlin) was applied in all the *in vivo* and the *in vitro* experiments. The parasites were maintained at 28 °C by biweekly passage on either RPMI-1640, supplemented with 10% fetal calf serum (FCS) and antibiotics (Biological Industries, Beit Haemek, Israel) or blood agar medium. Parasites were also maintained as a stabilate at −70 °C and *in vivo* in experimentally infected Balb/c mice.

2.2. Drug efficacy

2.2.1. The effect of Imiquimod combined with paromomycin/methylbenzethonium chloride on the development of L. major amastigotes in vitro

The efficacy of IMQ (3M Pharmaceuticals, MN, USA), PR (Farmitalia, Italy) and MBCL (Rhone-Poulenc, Seine, France) on *Leishmania* amastigotes was determined as previously described (El-On et al., 1991). Briefly, C3H mouse peritoneal macrophages (PM) were prepared from thioglycolate-stimulated mice. On the harvesting day, the cells were collected in RPMI-1640 medium containing 5 U of heparin, 100 μg streptomycin and 100 U of penicillin per ml. Before adding the PM to the plates, 10 × 10-mm sterile cover slips were placed into the wells of a 24-well (16 mm in diameter) microplate (Costar, USA). The cell concentration was brought to 3×10^5 cells/ml and 1 ml was placed in each well. After incubation at 37 °C for 2 h at 5% CO_2, the medium was replaced by fresh medium containing 10% FCS and 1×10^6 promastigotes per ml. After an additional 24 h, the medium was replaced again by fresh medium containing PR, MBCL, or IMQ, either alone or in combination. The development of the parasites was determined on the second and fourth day after initiation of treatment by the examination of three cover slips for each treatment, after staining with Giemsa. The effect of the drug on intracellular parasitic development was followed by counting the total number of intracellular amastigotes in 400 cells. The product of this compared with the product of the control is the parasite survival (PS):

$$\text{PS}\ (\%) = \frac{\text{Total number of intracellular amastigotes (Experimental)}}{\text{Total number of intracellular amastigotes (control)}} \times 100$$

The growth inhibitory effect is described as 100%—PS (%).

2.2.2. Analysis of combined drug effects

The isobologram method was used to evaluate the nature of interaction of PR and IMQ as described previously (Loewe, 1953 and Steel and Peckham, 1979). The concentrations of PR and IMQ required to reduce the PS by 50% (IC_{50}) on the second day of treatment were determined and plotted on the *x* and *y*-axes in a two-coordinate plot. The strait line connecting these two points is the line of additivity. The concentrations of the two drugs used in combination to provide the same effect (IC_{50}), were then placed in the identical plot. Points above this line indicate antagonism, and those below the line, indicate synergism.