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Evaluation of serological markers to monitor the disease status of Indian post kala-azar dermal leishmaniasis

Debanjan Mukhopadhyay[a], Nilay Kanti Das[b], Sritama De Sarkar[a], Alak Manna[a], Dwijendra Nath Ganguly[c], Joyashree Nath Barbhuiya[b], Arup K. Maitra[a], Avijit Hazra[a], Mitali Chatterjee[a,*]

[a] Dept. of Pharmacology, Institute of Postgraduate Medical Education and Research, 244 B, Acharya J C Bose Road, Kolkata 700 020, India
[b] Dept. of Dermatology, Calcutta Medical College, Kolkata-700 073, India
[c] Dept. of Dermatology, School of Tropical Medicine, Kolkata-700 073, India

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ABSTRACT

Post kala-azar dermal leishmaniasis (PKDL), a dermal sequel of visceral leishmaniasis presents with macular or polymorphic lesions. As immunological variations between these two forms have not been delineated, we evaluated levels of antileishmanial total Ig, IgG and its subclasses, IgM, IgE, IgG avidity, cytokines IL-10, IL-4, IL-13 and expression of CD19. The levels of Ig and IgG in polymorphic PKDL were higher than macular PKDL, while significant curtailment in levels of Ig, IgM and IgG following treatment was evident only in polymorphic PKDL. With regard to IgG subclasses, IgG1 and IgG3 were significantly raised in polymorphic PKDL, whereas in macular PKDL only IgG1 was elevated; treatment decreased levels of IgG1, IgG2 and IgG3 only in polymorphic PKDL; IgE levels were raised in both groups but no marked alterations occurred following treatment. The avidity of IgG was higher in polymorphic PKDL and correlated with duration of disease. IL-10 was higher in polymorphic PKDL and decreased significantly after treatment, whereas in macular PKDL IL-4 predominated. Taken together, in PKDL the humoral immune response was greater in the polymorphic variant than the macular form suggesting that serological markers may have a role in monitoring polymorphic PKDL.

1. Introduction

Leishmaniasis is a complex vector borne disease having diverse clinical manifestations ranging from a self healing cutaneous to a fatal visceral form. The disease globally affects about 12 million people with an increasing incidence of 1.5–2 million new cases diagnosed every year and 350 million people at risk.[1] Post kala-azar dermal leishmaniasis (PKDL), a dermal sequel of visceral leishmaniasis (VL) or kala-azar is confined mainly to the Indian subcontinent and Sudan along with its adjoining areas; importantly, owing to the absence of any zoonotic reservoirs, patients with PKDL are considered as reservoirs for kala-azar in India,[2] thereby assuming greater clinical relevance from an Indian perspective, where eradication of VL is presently a national priority.[3]

Based on their clinical features, patients with PKDL in the Indian subcontinent can be categorized into two broad subgroups: macular PKDL, where the patients primarily present with hypopigmented macules and patches, and polymorphic PKDL where in addition to hypopigmented macules, the patients also have papules/plaques and/or nodules.[4] The polymorphic group is predominant, ranging from 45–85%[2] of all PKDL cases. Although considerable

* Corresponding author. Tel.: +91 33 2223 4135; fax: +91 33 2223 4135.
E-mail address: ilatim@vsnl.net (M. Chatterjee).

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work has been done regarding the histopathological variations of macular and polymorphic PKDL,[5] humoral responses of PKDL have always been studied as a single entity and studies regarding their immunological differences, if any, are notably absent.[6,7] All studies regarding humoral responses in PKDL have shown elevated levels of either antileishmanial Ig and/or IgG and its subclasses, but differences between the macular and polymorphic variants have not been explored.[6,7] It has been documented that patients with macular PKDL respond more slowly to antileishmanial drugs than the polymorphic variant.[8] Another confounding variable is the inability to measure clinical cure in patients with macular PKDL as regeneration of melanocytes usually takes 3–6 months.[8]

With a view towards identifying a potential serological marker for monitoring the disease status of polymorphic and macular PKDL, the aim of this study was to dissect the humoral response in macular and polymorphic PKDL in terms of the antileishmanial Ig, IgG and their subclasses, IgM and IgE status and to establish whether a correlation existed between cytokines responsible for immunoglobulin class switching and associated IgG subclasses.

2. Materials and Methods

2.1. Reagents

All immunological reagents (anti human CD3, anti human CD19 and ELISA kits for IL-4, IL-10, IL-13) were from Immunotools, (Friesoythe, Germany) and others were from Sigma-Aldrich (St. Louis, MO, USA), except protease inhibitors and 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS, Roche Applied Science, Penzberg, Germany), rK39 immunochromatographic test strips (In Bios International, Seattle, WA, USA), para nitro phenyl phosphate (PNPP) and urea (Sisco Research Laboratories, Mumbai, India), tetra methyl benzidine (TMB) from Genei (Bangalore, India) and foetal bovine serum (FBS) from Himedia (Mumbai, India). Polystyrene coated maxisorp strips were obtained from Nunc Immunomodules (Roskilde, Denmark).

2.2. Study population

From 2008–2011, 57 patients clinically diagnosed with PKDL were recruited from the Dermatology Outpatient Department, School of Tropical Medicine, Kolkata, India. The diagnosis of PKDL was primarily clinical and corroborated with history of prior VL or residence in an endemic zone for VL and confirmed by rK39 strip test and/or demonstration of *Leishmania donovani* bodies by Giemsa staining. Additionally, in cases where the interval between onset of VL and appearance of PKDL was short, we confirmed our diagnosis by doing an ITS1 based PCR from dermal biopsies. In cases where parasite isolates were obtained, they were typed as *L. donovani*.[9] None of the patients suffered from any other infections or had any pre-existing disease. As controls, 15 age and sex-matched healthy volunteers were recruited from non-endemic areas and were seronegative for anti-leishmanial antibodies. Patients were randomly allocated to receive either sodium antimony gluconate (SAG; 20 mg/kg body weight/day intramuscular for 4 months) or miltefosine (100 mg/day per oral for 4 months). Among them, 28 were followed up successfully and received SAG (n=14) or miltefosine (14) and were either polymorphic (22) or macular (6). Samples were collected at disease presentation and on completion of treatment.

2.3. Ethics statement

All patients provided informed written consent (in case of a minor, the parent/guardian provided the same) and the study received approval from the Institutional Ethical Committee of School of Tropical Medicine, Kolkata, India and Institute of Post Graduate Medical Education & Research, Kolkata, India.

2.4. Preparation of crude Leishmania lysate

Crude *L. donovani* antigen (LDA) lysate was prepared from a *L. donovani* strain MHOM/IN/90/GE1F8R using log phase promastigotes harvested in ice-cold phosphate buffered saline (0.02 M, pH 7.2, PBS); the resultant pellet was resuspended in lysis buffer [20 mM Tris-HCl, 40 mM NaCl (pH-7.4), 5 mM EDTA and protease inhibitor cocktail[10]]. After several freeze-thaw cycles and centrifugation (1000 rpm x 5 min), the supernatant was collected and protein concentration determined;[11] lysates were stored at -20 °C until use. The LDA was diluted in phosphate buffer (0.02 mol/L, pH 7.8) and served as the coating antigen (1 μg/well/100 μl).

2.5. ELISA for total antileishmanial immunoglobulin

LDA was added to polystyrene coated wells, incubated overnight at 4 °C and followed by three washings with PBS supplemented with 0.05% Tween-20 (PBS-T); the nonspecific binding sites were blocked by PBS supplemented with 2% FBS (PBS-FBS) for 2 h (200 μl/well); sera (diluted 1:500 in PBS-FBS; 100 μl/well) was added and incubated overnight at 4 °C. The wells were finally incubated with horse radish peroxidase (HRP) conjugated protein-A (diluted 1:5000 in PBS-FBS, 100 μl/well) at 37 °C for 30 min and after five washes with PBS-T, binding was detected using ABTS (100 μl/well), optical density (OD 405 nm) being measured on an ELISA reader (Model 680, Bio-Rad, California, USA).

2.6. Determination of antileishmanial IgG, IgM and IgE

ELISA for antileishmanial IgG, IgM and IgE was measured using LDA as the coating antigen in the polystyrene coated wells. After an overnight incubation at 4 °C followed by three washings with PBS-T, the remaining reactive sites were blocked with PBS-FBS (200 μl/well) for 2 h; sera (diluted 1:500 for IgG/IgM and 1:5 for IgE in PBS-FBS, 100 μl/well) were added and incubated overnight at 4 °C. After three washes with PBS-T, the wells were incubated with anti human HRP-IgG or IgM (diluted 1:50 000 and 1:25 000 respectively in PBS-FBS, 100 μl/well) or with alkaline phosphatase conjugated anti human IgE (diluted