CYTOKINE MEASUREMENT IN LYMPHOCYTE CULTURE SUPERNATANT OF INACTIVE LEPROMATOUS LEPROSY PATIENTS

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Abstract

The aim of the present study was to determine the effects of stimulation of sonicated Mycobacterium leprae (MLS) extract and phorbol myristate acetate (PMA) on the pattern of cytokine production in peripheral blood mononuclear cells (PBMC) and to find out whether there is any difference between stimulation of MLS extract and PMA. Blood samples were collected and PBMC isolated from 43 inactive lepromatous leprosy patients. After culture for 24 hours, lymphocytes were stimulated with MLS extract and PMA. In the culture supernatant, IL-2, 4, 6, 8, TNF-alpha and TGF-beta levels were measured by using ELISA. M. leprae stimulated group IL-6, IL-8, TNF-alpha and TGF-beta levels were found significantly higher than PMA stimulated group (P<0.05). However, there was no difference between the two groups for IL-4. Only IL-2 levels were higher in PMA stimulated group than M. leprae stimulated group. Sonicated M. leprae extract have a strong effect on cytokine levels in vitro. Our results suggest that antigens with varying specificities favour the production of distinct cytokine patterns following in vitro restimulation.

Key words: Cytokines, leprosy, peripheral blood mononuclear cells

The cell-mediated immune response is an important aspect of host resistance to mycobacterial infection and is thought to be tightly regulated by a balance between the type 1 cytokines including interleukin-2 (IL-2), interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and IL-12 and the type 2 counterparts such as IL-4, IL-6 and IL-10. TNF-α is capable of many pro inflammatory activities including macrophages activation. The classically described Th1 and Th2 cells and the cytokines they produce have been demonstrated in murine models of bacterial and parasitic diseases, to be pivotal for protection or immunopathology. Leprosy is an infectious disease caused by Mycobacterium leprae in which the clinical manifestations correlate with the cell mediated immunity (CMI). The leprosy spectrum, which is composed of well established chronic polar and borderline forms, has been studied as a model in which clinical manifestations correlate with local cytokine patterns and the nature of T cell responses. At one extreme of the immunological spectrum, lepromatous patients are characterized by multibacillary disseminated disease and a lack of specific T cell responses to M. leprae and their lesions express a type 2 cytokine profiles that promotes a humoral immune response and progressive disease. Activation of T lymphocytes by antigen or mitogen can be separated into two intracellular signals. One of them involves the activation of protein kinase C, which can be stimulated directly by phorbol esters such as phorbol 12-myristate 13-acetate. In the present study, we examined the effects of stimulation by sonicated M. leprae (MLS) extract and phorbol myristate acetate (PMA) on the pattern of cytokine production in peripheral blood mononuclear cells culture (PBMC).

Materials and Methods

Prior to start of the study the protocol was approved by the local ethics committee. Informed consent was obtained from all individuals included in the study. Forty-three cases (29 male, 14 female) with inactive lepromatous leprosy with mean age 60.05 ± 2.28 years and diagnosis years of mean 30.66 ± 2.93 were included in the study. In all patients, the main components of the clinical assessment were the history, skin examination, nerve palpation, nerve function assessment (VMT-ST) and eye examination. Wiping firmly in the nasal passage, nasal mucous membrane smears were taken, using a sterile cotton wool bud and a smear was prepared. The results of nasal smears were reported as the number of AFB (acid fast bacilli) seen in the oil immersion (100x) and the logarithmic bacterial index (BI) were evaluated. The morphological index (MI) were determined according to AFB appearing as solid (viable), fragmented or non-solid (non-viable).

Preparing M. leprae extract

Lyophilized extract of M. leprae was kindly gifted by Dr. Patrick Brennan (Colorado State University). It was dissolved in PBS (phosphate-buffered saline) and vortexed for 2-3 minute and centrifuged at 2000xg for 10 minutes. The suspension of bacteria was sonicated (Ultra Turrax T 25 IKA Labortechnik) for 20 minutes with five minutes intervals to disrupt clumps. The supernatant was digested with 10 µg/mL DNAase and RNAase per mL for 1 hour at +4°C. The
supernatant was centrifuged at 32000 g for 40 minutes. The final pellet was stained using Ehrlich-Ziehl-Neelsen (EZN) method to demonstrate bacterial fragment by light microscope.

Separation of PBMC and culture

Heparinized venous blood (2 mL) from the individuals diagnosed as leprosy were obtained into tubes with EDTA. Peripheral blood mononuclear cells from blood were isolated by density gradient separation with 1.077 Biocoll separating solution. PBMC were cultured in 24-well plate (TPP-Tissue culture cell plate, Switzerland) in 2 mL of complete medium (RPMI 1640 with L-Glutamine, 10% fetal calf serum), penicillin 100 U/mL and added 20 µL M. leprae extract. After incubation for 24 hours these supernatants were collected from lymphocytes under MLS extract, phorbol myristate acetate (PMA) effect and from controls. All supernatants were assayed for IL-2, IL-4, IL-6, IL-8, TNF-α and TGF-β levels by ELISA (Biosource California, USA).

Statistical analysis

Results are reported as mean ± SE and data were analyzed statistically by the Student t-test and paired t-test, with the level of significance set at P<0.05.

Results

Forty of our patients were “0” and only three of them were “1+” with respect to bacillary index, but none of these bacilli were solid. All patients were enrolled as inactive lepromatous leprosy. IL-4, IL-6, IL-8, TNF-α, TGF-β levels were elevated in MLS and PMA groups compared to controls (P<0.001). IL-6, IL-8, TNF-α, TGF-β levels of MLS stimulated group were found significantly higher than PMA stimulated group (P<0.05). However, there was no difference between the two groups for IL-4. Only IL-2 levels were higher in PMA stimulated group than MLS stimulated group (Table).

Discussion

In human infectious disease, the varying immunological response to an invading pathogen determines the clinical manifestations of the infection and the outcome to the host. No where is this more evident than in leprosy, where a clear dichotomy exists between the T cell cytokine pattern and the clinical form of the disease. The type I T cell cytokine pattern predominates in self-curing tuberculoid patients and, in contrast, the type 2 T cell cytokine pattern characterizes the progressive disease of lepromatous patients. Lepromatous patients are characterized by multibacillary disseminated disease and a lack of specific T cell response to M. leprae and their lesions express a type 2 cytokine profile that promotes a humoral immune response and progressive disease. Sharma et al have reported that in lepromatous leprosy patients, PBMC IL-4 and IL-6 levels increased in response to MLS and PMA stimulation. Also, it has been reported that primary stimulation was required for IL-4 production, followed by restimulation with a mitogen. In our study, the induction of IL-4 and IL-6 by MLS extract and PMA in lymphocytes of inactive lepromatous leprosy patients shows that cells are predominantly of the Th1 type. It has been proposed that M. leprae also has suppressive activity on IL-2 production when PBMC are stimulated with the specific antigen, PPD and mitogens PHA-P.

It has been shown earlier that the use of PMA in cultures of mitogen-activated T lymphocytes from LL patients induced the expression of the IL-2 gene. On the contrary, we found that the use of PMA and MLS extract in cultures of PBMC from lepromatous leprosy patients did not induce the expression of the IL-2.

TNF-α is a cytokine that plays a role both in antimycobacterial defense and in leprosy immunopathology. Antas et al have also reported that percentage of TNF-α staining following in vitro antigen stimulation of PBMC from leprosy patients significantly increased in comparison to unstimulated culture medium. On the other hand, increased production of TGF-β in mycobacterial infections has been observed and could contribute to the inability of macrophages and T cells to control mycobacterial infections. In this study, lepromatous leprosy monocytes stimulated with MLS and PMA spontaneously expressed high levels of IL-8, TGF-β and TNF-α.

Several factors have been implicated in the regulation of cytokine production patterns by T-helper subsets. The previous commitment of the T-lineage, the existing lymphokine composition, the nature of the antigen presenting cell (APC) involved, MHC class II ligand density, costimulatory signals and the antigenic composition of the stimulus have all been considered to be involved in the evolution of distinct cytokine patterns which ultimately determine the outcome of an immune response. The ultimate mechanism and factors determining the cytokine profile of the immune response have been the subject of much debate and controversy. Antas et al have found that kinetics of IL-4 production in M. leprae stimulated

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<tr>
<th>Cytokine (pg/mL)</th>
<th>Unstimulated</th>
<th>Antigen stimulated</th>
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<tr>
<td></td>
<td>controls</td>
<td>MLS</td>
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<tr>
<td>IL-2</td>
<td>0.9 ± 0.2</td>
<td>0.7 ± 0.1</td>
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<tr>
<td>IL-4</td>
<td>0.8 ± 0.1</td>
<td>34.3 ± 1.7</td>
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<td>IL-6</td>
<td>29.9 ± 8.4</td>
<td>210.9 ± 102.7</td>
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<tr>
<td>IL-8</td>
<td>12.2 ± 0.8</td>
<td>2034.7 ± 395.5</td>
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<tr>
<td>TNF-α</td>
<td>4.1 ± 0.9</td>
<td>55.3 ± 47.1</td>
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<td>TGF-β</td>
<td>98.2 ± 11.4</td>
<td>438.5 ± 85.2</td>
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*P <0.001; **P <0.05; MLS – sonicated M. leprae; PMA – phorbol myristate acetate
PBMC of leprosy patients were higher than in vitro stimulation with phytohemagglutinin (PHA).\textsuperscript{14}

We observed that IL-4 concentration in culture supernatant of sonicated \textit{M. leprae} stimulated group was similar to that from PMA stimulated group (MLS: 34.3±1.7 pg/mL, PMA: 31.04±0.6 pg/mL). The IL-6, IL-8, TNF-\(\alpha\) and TGF-\(\beta\) concentrations of MLS stimulated group was higher than those from PMA stimulated group. Our results suggest that antigens with varying specificities favour the production of distinct cytokine patterns following \textit{in vitro} restimulation.

References


