

Protein phosphorylation pattern in the immune cells of leprosy affected individuals

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Abstract

Background: Leprosy is an infectious disease in which the susceptibility to the pathogen *Mycobacterium leprae* and the clinical manifestations are attributed to host immune cell response. Receptor mediated events and signalling in the immune cells are mediated by protein phosphorylation. The main signalling pathways and protein kinases known to be involved in the regulation of immune cells are cAMP dependent kinases, calcium/calmodulin dependent kinases, protein kinase C and mitogen activated protein kinases. The cumulative consequence of alterations in signalling pathways can be evaluated by intrinsic cellular protein phosphorylation by γ -P32 ATP. The present study was designed to assess the protein phosphorylation in the immune cells of leprosy patients as compared with normal individuals.

Methodology: Lymphocyte protein phosphorylation was conducted in 15 leprosy patients and 9 normal individuals. Protein phosphorylation of lymphocytes was carried out in the presence/absence of protein kinase modulators. The phosphorylation patterns were documented and analysed consequent to SDS-PAGE, staining, destaining, drying and autoradiography.

Results: The major phosphorylated proteins in lymphocytes were of molecular weights 20-22, 24-29, 30-35, 43, 46-50 and 66-68 kDa. In general, the major phosphorylated proteins were similar in the controls and in the patients. The phosphorylatability of these proteins varied with different modulators. Variations in the phosphorylation pattern were observed in 25% of the leprosy patients where there was a decrease of the 66kDa protein and a decrease of 20-22kDa protein phosphorylation.

Conclusion: The observed alterations in the protein phosphorylation pattern could be due to alteration in kinases and/or their substrates or due to the effect of *M. leprae* on immune cells.

Key Words: Leprosy, lymphocyte, protein phosphorylation.

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Introduction

Leprosy is a chronic disease caused by the infectious agent *Mycobacterium leprae*. Susceptibility to *M. leprae* infection has been attributed to a cell mediated immune (CMI) defect in the host. Leprosy manifests clinically as a spectrum ranging from the extensive form of disease, lepromatous leprosy (LL) where CMI is decreased, to the limited form, tuberculoid leprosy (TT) where the CMI is elevated [3,6]. The spectrum of immune response is reflected as differential expression of various pro, anti and regulatory cytokines such as TNF- α , IL-2, IFN- γ , IL-4, IL-8, IL-10, IL-12 [1,9,15] and chemokines CC, CXC types [11], suggesting that the host immune response to *M. leprae* is a complex

cascade of biochemical and immunological events. The steps beginning from antigen presentation, signalling and then to expression of immune modulators is mediated by cell signalling pathways. Earlier studies have suggested that biochemical events of T-cell activation could be altered in leprosy [18, 19, 22]. *In vitro* studies on intracellular signals triggered during *M. leprae* association with monocytes suggest the involvement of tyrosine kinases, protein kinase C, and PI3 kinases [16]. The defective antigen processing in LL patients was also postulated to be due to a signalling defect which is mediated by protein phosphorylation [20]. Hence, the present study of protein phosphorylation in the immune cells is a protein based approach to identify the

immune defect. This approach could elucidate the cumulative consequence of any alterations occurring in the signalling pathway which could be evaluated by biochemical reaction protein phosphorylation. The highly sensitive $\gamma^{32}\text{P}$ ATP signaling pathway evaluation method, where the labelled terminal phosphate of ATP molecule is transferred to the cellular substrates by intrinsic protein kinases, was used in the present study .

Materials and Methods

Materials

Histopaque-1077 (Ficoll), RPMI-1640, phytohaemagglutinin (PHA), lipopolysaccharide (LPS), Trishydroxymethylaminomethane, MgCl_2 , cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP) and Phosphatidyl inositol (PI) were obtained from Sigma Chemical Company, St Louis, USA. *M. leprae* soluble antigen (MLSA) was a gift from Dr. Brennan, Colorado State University, USA. Human peripheral nerve glycoprotein (PNGP) was extracted as previously described [23]. Methyl prednisolone (MP, Pharmacia, Belgium) was obtained as a tablet and dissolved in RPMI-1640 and added to the cells at a concentration of 20 $\mu\text{g}/\text{ml}$. $\gamma^{32}\text{P}$ ATP 5' Triphosphate Triethyl ammonium salt (5000 Ci/mmol) was obtained from the Jonaki Labelled Biomolecules laboratory, Centre for Cellular and Molecular Biology, Hyderabad.

Patients and controls

This study was approved by the Blue Peter Research Centre, ethical committee review board. After informed consent was obtained, 5 ml of heparinised venous blood was drawn from 15 clinically confirmed leprosy patients attending the Blue Peter Research Centre, LEPRO India, Cherlapally. The study included a total of 15 patients, 10 of whom were undergoing treatment. The classification of these patients was as follows: four borderline tuberculoid, two pure neural and four lepromatous leprosy cases. The other five were old leprosy patients released from treatment (RFT). Control samples were taken from 9 individuals who were healthy laboratory personnel with no history or signs and symptoms of the disease.

Isolation of lymphocytes

Lymphocytes were isolated according to the method described by Boyum [2]. In brief, heparinised venous blood was diluted at 1:2 ratio with RPMI-1640 medium and layered on Histopaque (Sigma Chemical Company) and centrifuged at 108xg for 15 minutes. The interface was aspirated and suspended in RPMI-1640 medium. These cells were pelleted and resuspended twice in RPMI-1640. The viability of the cells was checked by 0.01% trypan blue exclusion method. The cell suspensions were further used either for 2-hour incubation with modulators at 37°C or Magnetic associated cell separation (MACS) or protein phosphorylation.

Lymphocyte Protein Phosphorylation

From the time of lysis of the lymphocytes until protein phosphorylation, the procedure was carried out at 4°C. The lymphocytes were quickly spun down to remove the RPMI and suspended in homogenisation buffer consisting of 20 mM Tris HCl pH 7.6; 1 $\mu\text{g}/\text{ml}$ Benzamidine; 0.1mM Sodium Orthovanadate; 0.1% Triton X-100 (v/v); and 200 μm Phenyl Methyl Sulphonyl Fluoride (PMSF). Each lymphocyte suspension was gently mixed with pellet pestles and sonicated at an amplitude of 40, pulse 4 for 4 minutes. This suspension was centrifuged at 108xg for 10 minutes at 4°C to remove particulate matter and the supernatants were used for the phosphorylation assay. Protein was measured according to *Lowry et al.* [17].

Stimulation assay

The Histopaque isolated lymphocytes were incubated in a 24-well culture plate for 2 hours at 37°C in a CO₂ incubator (Forma Scientific, USA) with or without modulators. The modulators used were PHA, LPS, PNGP, MLSA and MP. 2x10⁶ cells were added to each well. After incubation the cells were harvested and lysed for the phosphorylation assay.

Magnetic Associated Cell Separation (MACS)

CD3 microbeads were used for the positive selection of T-cells [8, 21]. The microbeads were added to the cell suspension and mixed gently. The suspension was loaded on Mini MACS LS magnetic separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany). The unbound cells were washed off and bound cells (T lymphocytes)

were eluted and lysed for the phosphorylation assay.

Phosphorylation assay

Protein phosphorylation on lymphocytes was standardized for optimal phosphorylation patterns and a number of consecutive experiments were conducted for reproducibility. Briefly, 100µl total reaction mixture for protein phosphorylation consisted of 20mM Tris-HCl buffer pH 7.5, 10-20mM MgCl₂ with or without modulators and with an equal amount 100µg of patient or normal lymphocyte protein. To each sample, 1µCi of ³²P-ATP was added [24]. The reaction was terminated by addition of Laemmli's dissociation buffer and heated at 100°C for 3 minutes. The proteins were separated on a 12% SDS-PAGE [14] at a voltage of 100V and 60mA on a Biorad (USA)/ Genei (Bangalore, India) vertical electrophoretic units. The gels were stained with commassie blue and destained, dried on a Biorad gel dryer and kept in contact with Kodak X-ray film at -70°C for 7 to 10 days for autoradiography as previously described [23]. The molecular weight markers used were phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and trypsin inhibitor (20.1 kDa). Autoradiograms were analyzed based on the observational differences between samples which ranged from 10% to 40% band density differences and this was considered significant in analysis [24]. A representative autoradiogram for each set of experiments is presented.

Results

Protein phosphorylation and autoradiography analysis of lymphocytes revealed ³²P-labelled protein bands in the range of 20 kDa to 200 kDa. These phosphorylated proteins were present in patients as well as in controls. The representative autoradiograms of each experiment are presented in Figures 1-3.

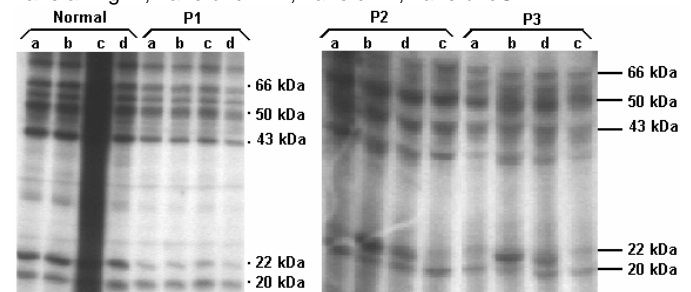
Protein Phosphorylation of normal and leprosy lymphocyte proteins

Autoradiograms of reaction mixtures of lymphocyte protein extract incubated with ³²P-ATP in the presence or absence of modulators under normal assay conditions showed four major ³²P-labelled protein bands that corresponded to

20-22, 43, 50-55, 66-70 kDa proteins (Fig.1). The 20-22 kDa proteins appeared as a double band in figure 1, whereas it was a single diffused broadband in figures 2 and 3. There were other minor bands phosphorylated between 22-43 kDa in normal controls (figure 1 lanes a, b, d) which were decreased (figure 1 P1 lanes - a, b, c, d) or absent (figure 1 P2 and P3 lanes- a, b, c, d) in leprosy patients. When compared to the control, the 20-22 kDa phosphorylated set of proteins showed a significant decrease in leprosy patients in the presence of cAMP, cGMP and PI (figure 1 lanes b,c,d of P1, P2 and P3). The 43, 50-55 and 66-70 kDa protein phosphorylation was not significantly altered in leprosy patients as compared to controls (figure 1 lanes a, b, c, d of P1, P2 and P3).

Figure 1. Autoradiogram showing lymphocyte protein phosphorylation of normal and leprosy patients P1 (LL), P2 (PNL), P3 (BL) in the presence of Mg²⁺ with or without different modulators.

Lane a: Mg²⁺, Lane b: cAMP, Lane c: PI, Lane d: cGMP.



Protein Phosphorylation of stimulated leprosy and normal lymphocytes

Figure 2 shows phosphorylation patterns in short-term cultured lymphocytes of leprosy patients and normal controls in the presence and absence of modulators PHA, LPS, PNGP MLSA and methyl prednisolone, or a combination of these. The phosphorylation pattern of leprosy patients' lymphocytes stimulated with various modulators did not significantly differ from the control, except in LPS and LPS containing reaction mixtures where there was a decrease in 66-70kDa protein phosphorylation (figure 2 P1- a,b, P2 - a,d,h, P3- a,b,c).

Protein Phosphorylation of MACS purified T-cells

Figure 3 shows the protein phosphorylation pattern in purified T-cells of normal controls and leprosy patients in the presence of cAMP, cGMP

and PI. The overall phosphorylation pattern was identical for most of the proteins except for 50-55 kDa in leprosy lymphocytes. Phosphorylation of the 66-70 kDa protein in leprosy patients was decreased as compared to that of the controls (Fig 3 lanes 5, 6, 7, 8).

Figure 2. Autoradiogram showing protein phosphorylation of lymphocytes of normal and leprosy patients P1 (LL), P2 (RFT), P3 (RFT/LL) stimulated by different modulators.

Lane a: Mg2+, lane b: PHA, lane c: LPS, lane d: PHA+LPS, lane e: PHA+LPS+PNG, lane f: PHA+MP, lane g: LPS+MP, lane h: PHA+LPS+MLSA.

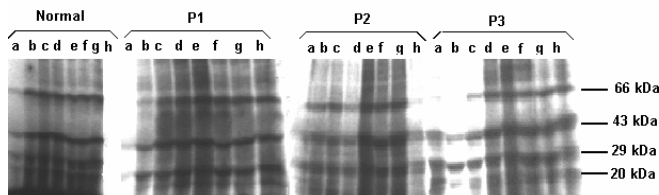
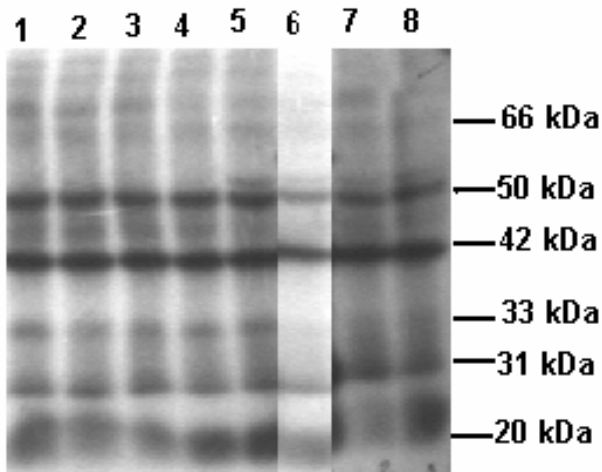


Figure 3. Autoradiogram showing protein phosphorylation of MACS isolated T-lymphocytes in the presence of Mg2+ with or without different modulators.

Lane 1: PI, lane 2: cGMP, Lane 3: cAMP, lane 4: Mg2+ correspond to normal lymphocytes and lane 5: PI, lane 6: cGMP, lane 7: cAMP, lane 8: Mg2+ correspond to leprosy patients lymphocytes.



Discussion

Protein phosphorylation has a fundamental importance in cell regulation by signal transduction where the cell responds to extra cellular stimuli. The biochemical event of this signalling mechanism is a secondary modification protein phosphorylation, which constitutes 90% of the posttranslational modifications of the cell. Extra cellular signals are known to produce diverse physiological effects by regulating the state of

phosphorylation of the specific phosphoprotein in the target cells. One third of all mammalian proteins are known to be phosphorylated and there are about 500 protein kinases encoded by the human genome. Protein kinases are broadly characterized as serine, threonine and tyrosine kinases, which phosphorylate the substrate protein on their respective residues [7]. In leprosy, host cell interaction to *M. leprae* could be regulated by phosphorylated proteins. A protein phosphorylation study using radio labelled ATP is a sensitive method to evaluate secondary modifications of proteins in lymphocytes of leprosy patients.

Various approaches have been used to analyse signal transduction molecules in leprosy [22,24]. Decreased expression of TCR zeta chain, p56lck tyrosine kinase, and transcription factor NFkβ p65 were found to be frequently associated with lepromatous (LL) leprosy and not with tuberculoid (TT) leprosy patients. Decreased expression levels of TCR- zeta chain and p56lck were not observed exclusively in leprosy but also in other diseases, such as rheumatoid arthritis, cancers and acquired immuno deficiency syndrome (AIDS) [4]. Polymorphisms in Toll-like receptors on T-cells, [5,12] signal transduction via Stat 4 in response to IL-12 [10] and PI, calcium and tyrosine kinase signalling pathways are known to be altered in multibacillary leprosy patients as compared to paucibacillary patients [22]. These alterations in signalling molecules could reflect in altered protein phosphorylation in leprosy lymphocytes.

More than one protein kinases are responsible for phosphorylation of lymphocyte proteins. These proteins include cytosolic and membrane proteins as they are extracted in the presence of Triton X-100 containing buffer. The identification of these phosphorylated proteins was not carried out in the present study; however, based on molecular weight, these may include some of the well characterized proteins such as ZAP-70 (70 kDa), Shc protein (46-52 kDa), [13], TCR-Zeta chain (21-23 kDa), p56lck tyrosine kinase and NFkβ p65 [4]. In all experiments, i.e. those using Histopaque isolated, stimulated and MACS purified cells, the results showed identical phosphorylation patterns for the major bands with some minor band differences.

There was a significant alteration of 20-22 kDa (Fig.1) protein phosphorylation in the presence or

absence of modulators suggesting that there is a concerted action of kinases on lymphocyte substrate proteins in the network of phosphorylation events that connect the antigen receptor to the intracellular signalling pathways [13].

Incubations with LPS and PHA showed a significant difference in phosphorylation patterns in two out of three patient lymphocytes. Human peripheral nerve glycoprotein and methyl prednisolone did not have any significant effect at the concentrations used in the study. Experiment with MACS isolated T-lymphocytes from six leprosy patients and three controls (figure 3) showed a decrease in the 66 kDa protein in patients. These results suggest that there may not be common alterations in protein phosphorylation in all leprosy. The overall phosphorylation patterns were also not significantly altered and this may be explained as a signal integration process in which a signal bypasses any minor defect/s in the pathway to accomplish the end event, protein phosphorylation. It is known that in cell signalling pathways, when one pathway is blocked or altered, there is a compensation mechanism so that the signal bypasses the block to the downstream cascades by alternate pathways mediated by other kinases [13]. Such a mechanism could also occur in the immune cells of leprosy.

In conclusion, the results of the present study suggest that there could be a number of kinases and substrate proteins which could phosphorylate in lymphocytes and hence there was no significant difference observed in the phosphorylation pattern of major lymphocyte proteins in leprosy patients. This occurrence could be due to signal bypass mechanisms in operation. Further work involving 2D gel electrophoresis and mass spectrophotometry of these major and minor phosphorylated proteins could identify the regulatory proteins which contribute in *M. leprae*-host cell interaction and in the progression of leprosy.

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