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Immunological defect in leprosy patients: altered T-lymphocyte signals

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Abstract

The early events of activation were studied in paucibacillary (TT/BT) and multibacillary (BL/LL) leprosy patients by stimulation of their lymphocytes with mitogenic agents (calcium ionophore A23187/PMA) and *Micobacterium leprae* antigen (PGL-1). Maximum proliferation in response to PMA/A23187 and PGL-1 was observed in the BT/TT patients and the control group, respectively. Inositol triphosphate (IP_3) and calcium were constitutively elevated in BT/TT and LL/BL patients. PMA/A23187 caused an increase in both IP_3 and $[Ca^{2+}]_i$ in BT/TT patients and controls. PGL-1 marginally increased IP_3 levels in BT/TT patients. In the LL/BL patients, although PMA/A23187 increased IP_3 levels, but no change was seen in $[Ca^{2+}]_i$, PGL-1 had no effect. Protein kinase C levels were seen to be associated with particulate fractions in BT/TT patients and were found to increase further in response to PMA/A23187. PGL-1 did not increase translocation of protein kinase C in controls or LL/BL patients. A preactivated and sensitised state of T-lymphocytes was observed in BT/TT patients, responsive to antigen and mitogens, whereas the cells of LL/BL patients were unresponsive to PGL-1. The altered signal transduction events characterised in the MB patients thus correlate well with the anergic state of their cells.

Keywords: Leprosy; Intracellular calcium; T-lymphocyte; Inositol triphosphate; Protein kinase C

1. Introduction

Leprosy is characterised by the two polar forms, lepromatous leprosy (LL) and tuberculoid leprosy (TT). The spectrum between the polar forms embraces a number of immunologically unstable states. The most striking contrast between the two poles is the profound *Micobacterium leprae*-specific unresponsiveness of T-cells in LL as opposed to TT, which show strong T-cell proliferative responses [1,2]. There is much debate as to the nature of the T-cell anergic state and its induction in leprosy, although several emergent models appear to be gaining support [3].

Activation of T-lymphocytes by antigen or mitogen can be separated into two intracellular signals

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[4,5]. One involves the activation of protein kinase C (PKC), which can be stimulated directly by phorbol esters, such as phorbol 12-myristate, 13-acetate (PMA) [6]. The other is a calcium-dependent signal demonstrated by calcium ionophores, such as A23187 [7], associated with the production of interleukin-2 (IL-2).

$CD4^+$ T-cells require TCR engagement by an immunogenic peptide bound to MHC II molecules as well as a co-stimulus, for successful activation leading to clonal expansion [8,9]. The induction of tolerance in T-lymphocytes is thought to occur at two levels, either during maturation in the thymus by clonal deletion [10] or later functional silencing by antigen, a phenomenon termed clonal anergy [11,12]. The defect in anergic cells that renders them unresponsive and unable to produce IL-2 is not clearly defined.

2. Materials and methods

2.1. Leprosy patients and control subjects

Heparinised venous blood was drawn from untreated paucibacillary (PB or BT/TT) and multibacillary (MB or LL/BL) patients ($n = 15$ each), attending the leprosy clinic, at Nehru Hospital, PGIMER, Chandigarh, after being given prior consent. Patients were classified according to the Ridley–Jopling 5-group classification of leprosy [13]. Slit skin smears from five sites and skin biopsy for histopathological examination were carried out in all patients to confirm the diagnosis.

Ten controls were healthy laboratory personnel with no prior history of disease.

2.2. Isolation of T-lymphocytes

Lymphocytes were isolated according to the method of Boyum [14]. Lymphocytes collected from the interface after centrifugation were suspended in RPMI 1640 (Sigma) supplemented with 10% foetal calf serum, 2 mM L-glutamine and antibiotics (100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin). The adherent cells were separated by incubation in plastic Petri dishes for 90 min at 37°C in a humidified CO_2 incubator. The supernatant was further enriched for T-lymphocytes [15] by incubation in a sterile column of nylon wool (600 mg) previously washed and incubated in supplemented RPMI 1640 medium (pH 7.2). After sequential steps of incubation for 1 h at 37°C, cells were eluted with 20 ml supplemented RPMI 1640 medium. The first eluted cells were collected and concentrated by centrifugation at $200 \times g$ for 10 min at 4°C.

2.3. Stimulation reagents

Calcium ionophore A23187 (Sigma) was dissolved in DMSO and used in concentration of 0.6 μM. Phorbol-12-myristate, 13-acetate (Sigma) was dissolved in optimal concentration of 0.5 μM in DMSO. Phenolic glycolipid 1 (a gift from Dr. U. Sengupta, JALMA, Agra in a lyophilised form) was suspended in chloroform/methanol 2:1 (v/v) and aliquots were blown to dryness under nitrogen in glass tubes. Just before use, a suspension was made by sonication of the lipid at 10 Hz in 5 short bursts of 10 s each. PGL-1 was used at a concentration of 10 μg 10 μl^{-1} in all stimulation assays.

2.4. Lymphocyte proliferation assay

The isolated T-cells were cultured in 96-well microtitre plates at a density of 10^6 cells ml^{-1}. 10^4 UV-irradiated autologous adherent cells were also added and stimulated to proliferate by using reagents mentioned above. [^{3}H]Thymidine (5 μCi ml^{-1}) was added to wells 18 h before harvesting [16].

Cells were collected on glass fibre filter discs using a multiharvester apparatus (PHD cell harvester). Radioactivity was determined in a Reckbeta scintillation counter. Results are expressed as counts per minute (cpm) per 10^6 cells. The levels in unstimulated cells have been taken as basal levels.

2.5. Inositol triphosphate turnover

IP_3 turnover in cells was measured by the method of Oldham [17]. Cells (2×10^6 ml^{-1} MEM) were incubated with PMA/A23187 and PGL-1, for 30 min at 37°C in 5% CO_2 atmosphere. The cells were then treated with 1 ml lithium chloride (LiCl 10 mM) for 30 min and incubated with 0.5 μCi [^{3}H]myoinositol for 30 min at 37°C. Labelled cells were washed in

Hanks balanced salt solution (pH 7.4) to remove unbound labelled inositol and finally suspended in 1 ml HBSS. The suspension was exposed to 0.2-ml volumes of ice cold 20% perchloric acid and kept on ice for 20 min to remove proteins. Siliconised glassware was used throughout to minimise loss of inositol phosphates. Supernatant was titrated to pH 7.5 with ice-cold 1.0 N KOH and kept in ice. Precipitated $KClO_4$ was removed by centrifugation at $2000 \times g$ for 20 min, and supernatants were applied to Amprep mini columns (SAX 100 mg, Amersham), preconditioned with 5 ml of 1.0 M $KHCO_3$ and 15 ml distilled water. Elution was carried out with: (a) 5 ml water; (b) 5 ml 0.05 M $KHCO_3$ (for elution of IP_1); (c) 5 ml 0.01 M $KHCO_3$ (for elution of IP_2); and (d) 5 ml 0.17 M $KHCO_3$ (for elution of IP_3).

Results have been expressed as % recovery of IP_3 calculated as:

$$\% \text{ recovery} = \frac{\text{counts recovered in fraction}}{\text{total counts applied on column}} \times 100.$$

2.6. Intracellular calcium concentration

$[Ca^{2+}]_i$ levels in the cells were estimated by the method of McCarthy [18].

Isolated cells (2×10^6 ml^{-1}) were suspended in buffer A (containing 138 mM NaCl, 2.5 mM, KCl, 1 mM $MgCl_2$, 0.5 mM, Na_2HPO_4, 0.5 mM $MgSO_4$, 2.5 mM glucose and 5 mM HEPES pH 7.4) supplemented with 1 mM $CaCl_2$ and 1% BSA and then incubated with PMA/A23187 or PGL-1 for 20 min at 37°C in 5% CO_2 atmosphere. The suspension was then centrifuged and the cells suspended in 1 ml of buffer A.

Cells were loaded with 0.3 μM Fura-2/AM for 45 min at 37°C, washed with buffer A to remove excess and then centrifuged at $250 \times g$ for 10 min. After two further washings, the cells were suspended in 2 ml buffer A and fluorescence (F) was recorded at pre-equilibrated temperature of 37°C with 340 nm as excitation and 500 nm as emission wavelengths. Maximum fluorescence (F_{max}) was recorded after adding 0.1% Triton × 100 to the sample. The minimum fluorescence (F_{min}) was recorded by adding 1 mM EGTA. Results are expressed as nmol of $[Ca^{2+}]_i$ per million cells cytosolic calcium was calculated as:

$$[Ca^{2+}]_i = K_d \times \frac{F - F_{min}}{F_{max} - F}$$

where $K_d = 224$ nM and is the dissociation constant for the Fura-2–Ca^{2+} complex.

2.7. Protein kinase C estimation

Membrane and cytosolic fractions were isolated from T-lymphocytes for the assay of PKC according to the method of Yamiuchi et al. [19]. Aliquots (100 μl) of both fractions were incubated for 15 min at 37°C either with an equal volume of reaction mixture A containing histone IIIs 10 μg, phosphatidylserine 5 μg, DTT 5 mM leupeptin 2 μg, diolein 2.5 μg, $CaCl_2$ 0.5 mM, $MgCl_2$ 20 mM, Tris HCl 2 mM pH 7.2 and ATP 10 μM) or reaction mixture B (where phosphatidylserine was omitted) and 1.0 μCi of [γ-^{32}P]ATP. Reactions were stopped by the addition of 0.8 ml of ice-cold trichloroacetic acid (10%, w/v) and filtered onto Whatman's filter paper (0.45 μm pore size). Filters were washed with 5 ml 5% TCA (w/v) and suspended in 7 ml of scintillation fluid. Counts of samples incubated with reaction mixture A were subtracted from counts of samples treated with reaction mixture B, to determine PKC activity, which is expressed as nmol of Pi transferred to histone per mg protein per min. Protein was estimated according to the procedure of Lowry [20].

Statistical analysis was done on SYSTAT, software package.

3. Results

3.1. Lymphocyte proliferation assays

The cells of the control group proliferated on addition of PMA/A23187. Addition of PMA/A23187 enhanced proliferation in both PB ($P < 0.01$) and MB ($P < 0.01$), but the proliferation was significantly higher in the cells of the PB patients ($P < 0.001$) compared to those of the MB patients (Fig. 1).

PGL-1 did not stimulate either MB or control group cells, but caused increased proliferation in PB cells ($P < 0.01$).

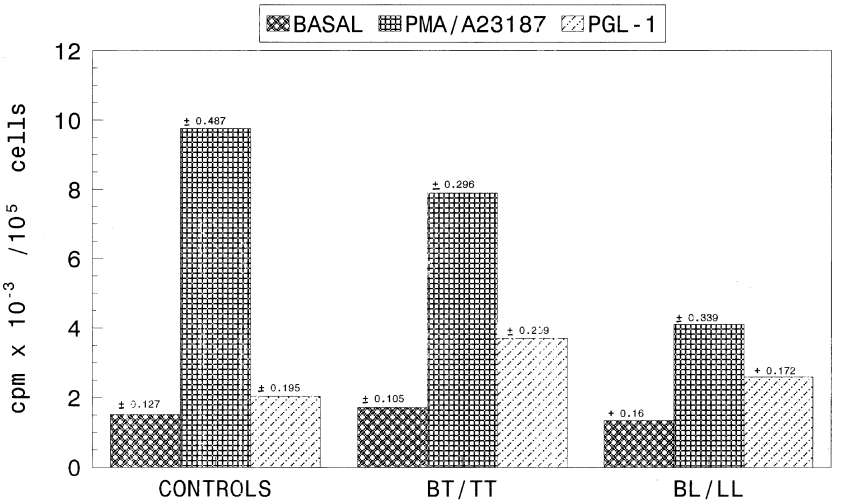

Fig. 1. ^{3}H-Incorporation in T-lymphocytes of leprosy patients ($n=15$) on incubation with PMA/A23187 and PGL-1. Data represent mean ± S.E.M.

3.2. Intracellular calcium

As an initial assessment of TCR-mediated signalling, elevation of intracellular calcium $[Ca^{2+}]_i$ was determined. Control cells demonstrated a readily detectable increase in $[Ca^{2+}]_i$ in response to stimulation with PMA/A23187. However, PGL-1 had no effect on $[Ca^{2+}]_i$.

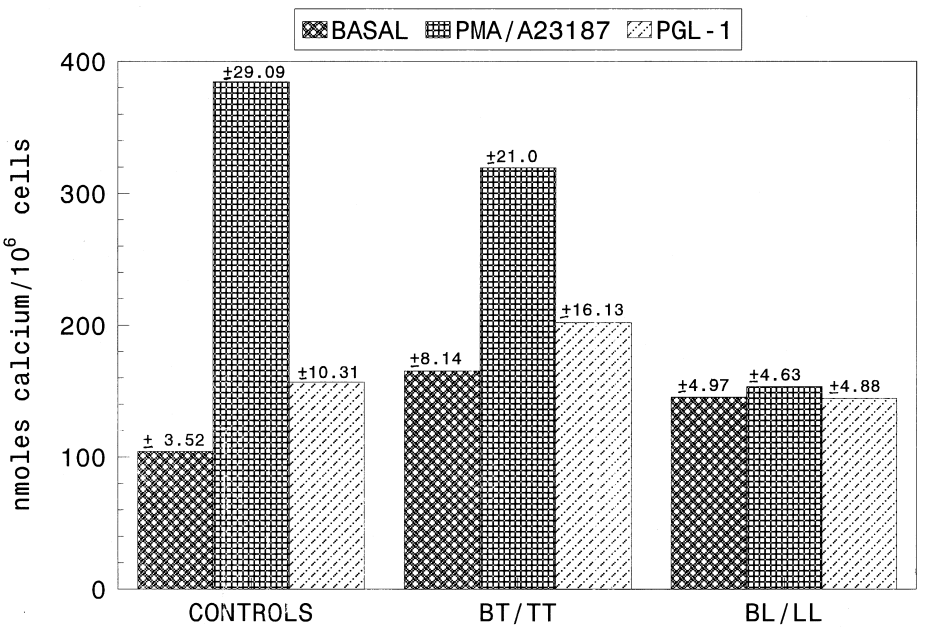

Fig. 2. Intracellular calcium in T-lymphocytes from healthy controls ($n=10$), BT/TT ($n=15$) and LL/BL ($n=15$). Data represent mean ± S.E.M.

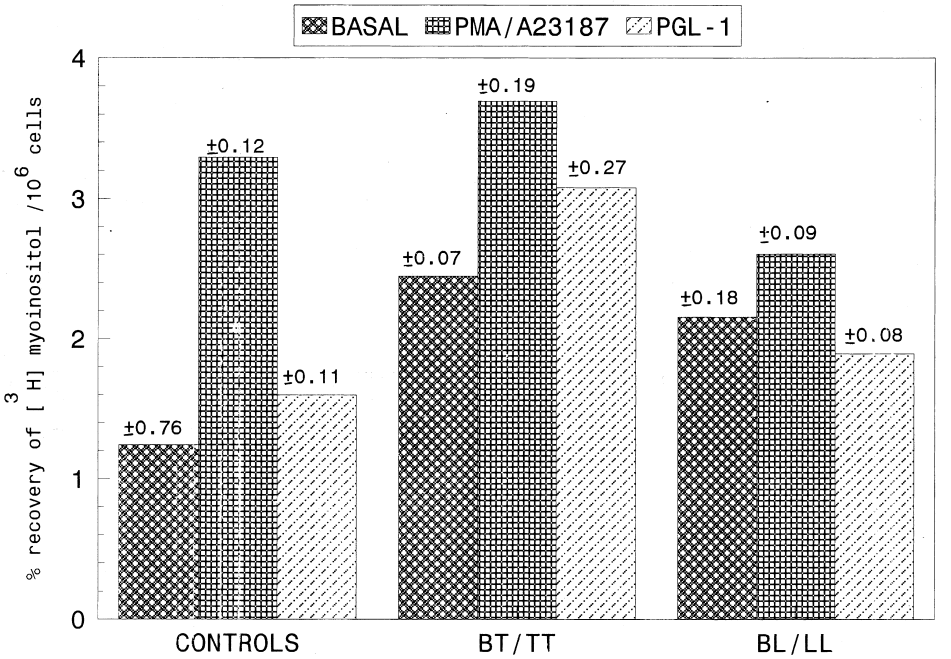

Fig. 3. Percentage recovery of [^{3}H]myoinositol from cells of leprosy patients ($n = 15$) to study IP_3 turnover on stimulation with PMA/A23187 and PGL-1. Data represent mean ± S.E.M.

The PB and MB groups showed higher basal constitutive levels when compared to the control group ($P < 0.001$), indicative of a state of activation. In the PB group, to PMA/A23187 caused an increase in $[Ca^{2+}]_i$ ($P < 0.001$) and PGL-1 could also mildly enhance $[Ca^{2+}]_i$ (Fig. 2).

The MB cells had higher $[Ca^{2+}]_i$ levels when compared to the control cells ($P < 0.01$), but there was insignificant increase in the $[Ca^{2+}]_i$ after addition of either PMA/A23187 or PGL-1.

3.3. Inositol phosphate (IP_3)

Elevated $[Ca^{2+}]_i$ is thought to result from IP_3 generated by the activity of PLC, and consequently total cellular IP_3 was also measured after restimulation of control or patient cells.

Interestingly, cells from PB patients showed higher levels of IP_3 than cells of the other groups on stimulation with PMA/A23187 and PGL-1 ($P < 0.01$) showed further increase.

The T-cells of in MB patients had higher levels of IP_3 and when compared to cells of the controls ($P < 0.001$), but were not significantly stimulated by either PMA/A23187 or PGL-1 (Fig. 3).

3.4. PKC translocation

The translocation of PKC activity from the cytosolic fraction to the membrane fraction has been considered to be an indication of PKC activation. The ability of PMA to induce translocation of PKC of T-cells has been well documented.

Incubation of the cells with PMA/A23187 induced translocation of PKC in lymphocytes from normal controls (Fig. 4). The basal levels in PB group were higher in the particulate (membrane fraction ($P < 0.001$). The PKC translocation increased further ($P < 0.001$) in response to PMA/A23187 stimulation. PGL-1 stimulation did not cause any further increase (Fig. 4).

The MB group showed almost similar PKC activity in cytosolic and particulate fractions in unstimulated cells in response to PMA/A23187 marginal increase ($P < 0.05$) was observed ($P < 0.05$); however, PGL-1 stimulation caused no further translocation of PKC.

4. Discussion

In this report, the early biochemical events of T-cell activation have been studied and the results show that cells of PB patients are in a constitutive state of activation and a near normal response on stimulation with PMA/A23187 as demonstrated by an increase in second messengers of the T-cell signal pathway. However, the cells from MB patients do not respond to these mitogens and show no change in intracellular levels of these second messengers.

It has been well established that for intracellular pathogens, CMI response is important. In the case of leprosy, high CMI localises the disease in TT form, whereas in case of LL patients, this CMI response is impaired [21–23] and the T-lymphocyte proliferation does not occur. For the activation of T-cells, the earliest biochemical events involved are the hydrolysis of phosphatidylinositol bisphosphate to inositol triphosphate (IP_3) and 1,2-diacylglycerol [24,25]. IP_3 induces increase in cytosolic free calcium [26,27], while 1,2-diacylglycerol directly activates protein kinase C. Both of these products act as second messengers in the transmission of signals for synthesis of IL-2 in T-cells.

The present study shows that cells from MB patients had an enhanced intracellular calcium and IP_3 turnover, however they are still unable to proliferate in response to *M. leprae* antigen, which suggests that TCR receptor-mediated pathway may be defective in these patients. However, even bypassing TCR receptor-mediated activation by PMA (which directly stimulates PKC) and A23187 (which increases intracellular calcium) [28–30] was unable to induce the cells to proliferate, thereby showing that signalling events downstream of or at the level of PKC and

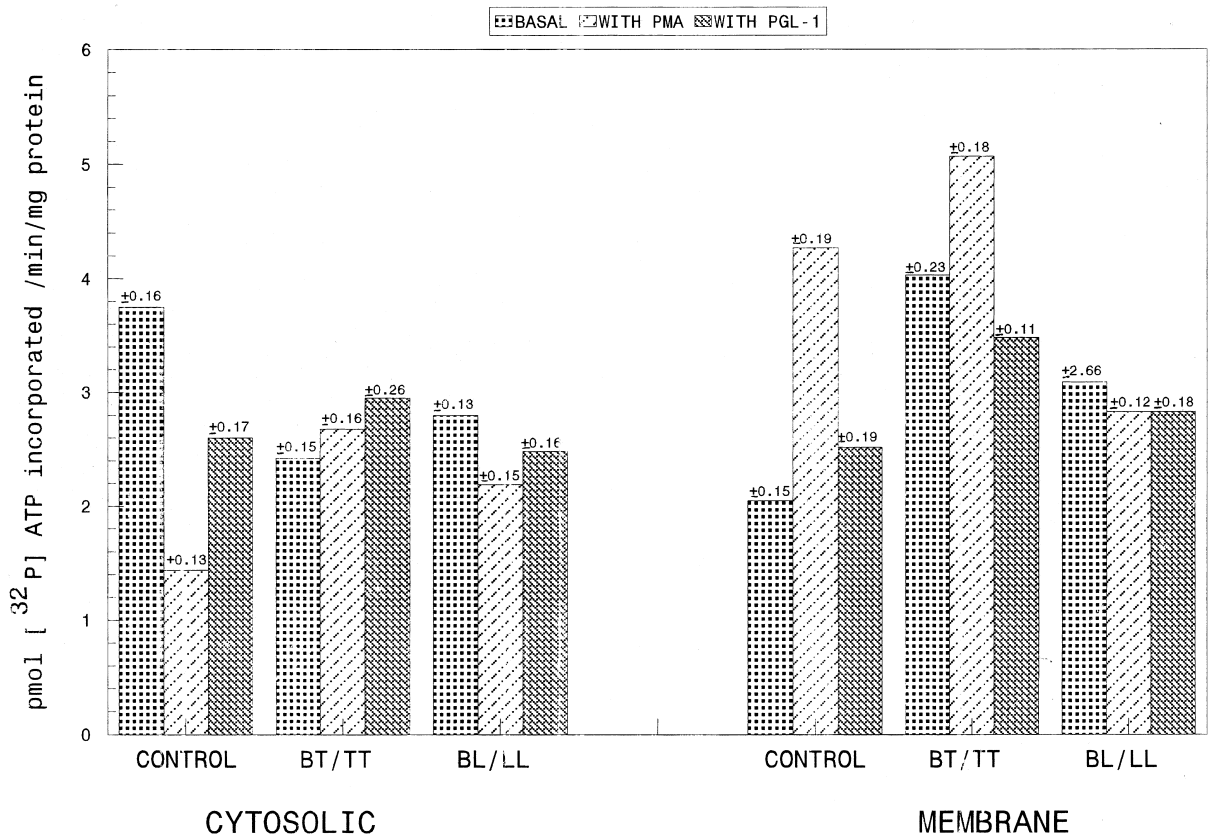

Fig. 4. PKC levels in cytosolic and membrane fractions of leprosy patients ($n = 15$). Results have been expressed as pmol [^{32}P]ATP incorporated min^{-1} mg $protein^{-1}$. Data represent mean ± S.E.M.

calcium are defective. These mitogens also failed to translocate PKC from cytosol to membrane, thus suggesting that blocking occurs at the level of PKC generation and IP_3 turnover and/or increase in intracellular calcium. As these signals are essential for proliferation of T-lymphocytes the inability of cells to activate these signals shows that cells are defective in proximal TCR-mediated signal transduction. As a result, cells of MB patients fail to respond to various mitogens, hence leading to an anergic state of the cells. These results correlate well with earlier reports of Gajewski [31] who has shown that anergic T-lymphocyte clones have altered IP_3, calcium and tyrosine kinase signalling events. Recently, Lancaster et al. [32] have shown that the anergic cells are defective in calcium mobilisation.

Keeping this in mind, we propose that the altered signal transduction events characterised in this study well establish that the anergic state of the cells may be due a defect in proximal TCR-mediated signal transduction.

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