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Spontaneous apoptosis in peripheral blood mononuclear cells of leprosy patients: role of cytokines

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Abstract

Peripheral blood mononuclear cells from leprosy patients underwent spontaneous apoptosis upon culture for 24 h. The apoptosis was inhibited by anti-TNFα antibodies and to a certain extent by anti-IL-1α and IL-6, thus showing that T_H2-type cytokines (mainly TNFα) are responsible for inducing apoptosis. This cytokine-mediated apoptosis could be inhibited by ionomycin and zinc, thereby suggesting that these metal ions can be used to decrease the levels of these inflammatory cytokines in various diseases.

Keywords: Apoptosis; Ionomycin; Zinc; Cytokine; Co-stimulatory molecule

1. Introduction

Apoptosis plays a crucial role in the pathogenesis of disease [1,2] and can be induced in the cells by activation of certain death molecules such as TNFα or *fas* [3,4]. In diseased conditions, it can occur due to interference of the pathogens with the intracellular signaling pathway [5]. This may lead to apoptosis in response to TCR-mediated expression of CD95 [6–8], or making T-cells sensitive to CD95 ligation.

Anergy is thought to be closely linked to apoptosis [9]. Leprosy, like other intracellular pathogens, is associated with impairment of CMI response leading to unresponsiveness or anergy in lepromatous leprosy patients [10,11]. The anergic state also occurs due to an imbalance in signaling, i.e. when T-cells are stimulated via TCR complex alone and activation through co-stimulatory molecules (i.e. CD28) does not occur [12]. Leprosy exhibits a clear-cut immunological polarity between tuberculoid and lepromatous leprosy showing a T_H1- and T_H2-type response, respectively [11,13]. The T_H2-type cytokines are responsible for inducing/maintaining anergic state in the cells. IL-10 is well known to be a down-regulator of co-stimulatory molecules, e.g. B-7 [14,15]. The antibodies to IL-4 and IL-10 can restore

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proliferation in HIV and other human infections showing that these cytokines exert suppressive effect on the cell proliferation [16–18]. It is speculated that the activation of these anergic cells may lead to an imbalance in signaling resulting in their apoptosis.

High levels of these cytokines have also been shown to induce apoptosis in PBMCs of HIV patients [19,20]. As the levels of T_H2 cytokines (IL-4, IL-6, IL-10) and certain proinflammatory cytokines such as TNFα, IL-1 and IL-6 are known to increase in reactional states in leprosy [11,21], so an attempt was made to study the role of these cytokines in inducing apoptosis in PBMCs of these patients.

2. Materials and methods

Untreated patients visiting the leprosy clinic of Nehru Hospital attached to the Postgraduate Institute of Medical Education and Research, Chandigarh were enroled in the study. Patients were classified according to the Ridley and Jopling [22] classification for leprosy. Slit skin smear and skin biopsy was carried out in all patients to confirm the diagnosis. Controls were healthy laboratory personnel on no type of medication.

2.1. Isolation of PBMCs

Venous blood (5 ml) was withdrawn from patients in vials containing heparin after obtaining informed consent. The peripheral blood mononuclear cells were isolated by layering the blood on ficoll-paque [23]. After centrifugation at $400\times g$ for 30 min, the buffy coat was taken out washed twice with RPMI 1640 and cells were suspended in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 units ml^{-1} benzylpenicillin, 100 μg ml^{-1} streptomycin and containing 10% fetal calf serum. The cells were cultured in 96-well flat- bottom culture plates in the humidified CO_2 chamber at 37°C.

2.2. Quantitation of apoptosis

Apoptosis was quantitated by staining nuclei with propidium iodide (PI) and analyzing fluorescence with FACScan (Becton Dickinson, USA) as described by Gouegeon et al. [24]. Briefly, following culture for 24 h, cells were collected after centrifugation at $200\times g$ for 10 min. The pellet was gently suspended in 0.5 ml of hypotonic fluorochrome solution (0.1% sodium citrate with 0.1% Triton X-100) containing 20 μg per ml PI for 20 min. RNase A at a concentration of 10 μg ml^{-1} was added and cells were further incubated for 10 min at 4°C. The suspension was analyzed by flow cytometry to determine PI fluorescence of individual nuclei. Apoptotic nuclei appeared as a broad hypodiploid DNA peak that was easily discriminated from narrow peak of nuclei with normal (diploid) DNA content. The percentage of cells undergoing apoptosis was taken for calculation of mean ± S.E.M. Student's *t*-test was used for statistical analysis of data.

Anti-TNFα, IL-4, IL-6, IL-1α cytokine antibodies (Amersham, UK) at a concentration of 5 μg ml^{-1}, ionomycin (Sigma, USA) at a concentration of 200 ng ml^{-1}, zinc (5 mM) and leupeptin (50 μM) were added to the cells and checked for apoptosis after 24 h. The concentrations used were not toxic for normal cells as measured by apoptosis and Trypan blue staining. The apoptosis was also measured by incubating the cells with TNFα (500 pg ml^{-1}), IL-6 (50 pg ml^{-1}), rIL-2 (40 units ml^{-1}) (a gift from NCI, Bethesda, MD), concanavalin A (10 μg ml^{-1}) and anti-TCR α/β antibodies (1 μg), which were coated on the 96-well culture plates.

2.3. Expression of co-stimulatory molecules

Cells were stained with monoclonal antibodies obtained from Becton Dickinson, Monoclonal Center, California, USA. The monoclonal antibodies were directed against the cell surface antigens CD28, ICAM-1 and B-7. CD28 was conjugated to phycoerythrin while for ICAM-1 and B-7, the cells were first stained with primary monoclonal antibody followed by secondary antibody conjugate to FITC.

Briefly, 100 μl of whole blood was incubated with 10 μl of PE- conjugated or unconjugated primary antibody for 20–30 min at room temperature. This was followed by addition of FACS lysing solution (Becton Dickinson) and further incubated for 10 min. For B-7 and ICAM-1, cells were further incubated with secondary FITC conjugate after washing with PBS. Appropriate isotype controls were also used.

Table 1
Expression of co-stimulatory molecules on the surface of PBMCs of leprosy patients

Patients	CD28	B-7	ICAM-1
Control (n=10)	362.46 ± 46.62	415.62 ± 62.21	222.40 ± 52.24
BT/TT (n=10)	251.20 ± 62.40[a,*]	387.40 ± 58.24[a,***]	182.20 ± 20.48[a,*]
BL/LL (n=10)	102.71 ± 38.71[b,**]	220.39 ± 13.93[b,**]	60.00 ± 40.20[b,**]

Values represent mean ± S.E.M. of relative fluorescence.
[a]BT/TT vs. control.
[b]BL/LL vs. control.
*$P < 0.02$.
**$P < 0.001$.
***$P < 0.05$.

3. Results

PBMCs of leprosy patients showed a decrease in expression of co-stimulatory molecules CD28 and B-7. The mean fluorescence intensity for CD28 was 102.71 ± 38.71 and 220.39 ± 13.93 for B-7 in BL/LL patients ($P < 0.001$) (Table 1) and was ∼2-fold less when compared to healthy controls thus suggesting that lack of co-stimulatory molecules leads to an anergic state in the T-cells of these patients.

To study the mechanism of anergy, the cells were cultured in the presence of anti-TCR α/β antibodies coated onto 96-well plates, and another mitogen, concanavalin A. However, we found that cells underwent spontaneous apoptosis in in vitro conditions and the apoptosis was enhanced on incubation with Con A (BT/TT, 41.16 ± 8.05; BL/LL, 47.5 ± 7.03) and anti-TCR α/β (BT/TT, 38.2 ± 6.38; BL/LL, 47.78 ± 3.68) antibodies (Table 2).

In order to understand the mechanism of apoptosis, the cells were incubated with a number of known inhibitors of apoptosis like leupeptin, ionomycin, zinc, *N*-ethyl-maleimide and cyclosporin A. The ionomycin, zinc and leupeptin were able to inhibit apoptosis by ∼45% and zinc was more efficient in blocking apoptosis, especially in patients with reaction and inhibition was almost ∼55% in case of BL/LL without reaction (16.77 ± 4.92; $P < 0.01$) and by ∼60% in patients with reaction (22.75 ± 13.47; $P < 0.02$) (Table 3).

Leupeptin (which is a non-specific inhibitor of cysteine proteases) was used to inhibit apoptosis resulting through certain cysteine proteases called caspases. Leupeptin, being non-specific, could inhibit apoptosis up to ∼60% ($P < 0.001$), thus showing the involvement of cysteine proteases. It has already been reported that leupeptin can inhibit apoptosis only by ∼60% in cells.

Since, apoptosis was inhibited significantly in ENL patients, and these patients have high levels of IL-1α, IL-4, IL-6 and TNFα, so the antibodies against these cytokines were used to study whether these T_H2-type cytokines play any role in inducing apoptosis. After 24 h of culture in presence of these antibodies, the TNFα could inhibit apoptosis in ENL patients by 65% ($P < 0.001$ for patients with reaction; $P < 0.01$ for patients without reaction), whereas IL-1α, IL-4 and IL-6 inhibited apoptosis in ENL

Table 2
Percent apoptosis in PBMCs of leprosy patients in the presence of mitogens

Patients	Spontaneous apoptosis	Con A	Anti-TCR α/β
Control (n = 8)	8.25 ± 2.88	8.00 ± 3.25	9.75 ± 2.68
BT/TT (n = 6)	25.00 ± 5.81[a,*]	41.16 ± 8.05[a,**]	38.20 ± 6.38[a,*]
BL/LL (n = 14)	37.78 ± 7.6[a,**]	47.50 ± 7.03[a,**]	47.78 ± 3.68[a,**]

Values represent mean ± S.E.M.
[a]vs. control (spontaneous apoptosis of the same patient group.
*$P < 0.05$.
**$P < 0.02$.

Table 3
Percent apoptosis in PBMCs of leprosy patients in the presence of various inhibitors

Patients	Spontaneous	Ionomycin apoptosis	Zinc	Leupeptin
Controls ($n=8$)	7.47 ± 1.48	8.00 ± 1.24	7.90 ± 0.88	7.20 ± 0.78
BT/TT ($n=6$)	27.66 ± 8.88	19.66 ± 6.76	17.42 ± 5.72[b,*]	16.28 ± 4.54
BT/TT in reaction ($n=5$)	36.54 ± 3.60[a,*]	17.42 ± 4.9[b,*]	14.23 ± 5.47[b,**]	15.42 ± 5.20[b,**]
BL/LL ($n=9$)	33.66 ± 9.09[a,***]	19.55 ± 5.46[b,***]	16.77 ± 4.92[b,***]	18.42 ± 2.40[b,§]
BL/LL (type II reaction; $n=5$)	48.50 ± 14.68[a,§]	37.50 ± 9.9[b,*]	22.75 ± 13.47[b,**]	24.28 ± 3.70[b,§]

Values represent mean ± S.E.M.
[a]vs. control (spontaneous).
[b]vs. spontaneous apoptosis of the same group.
*$P<0.05$.
**$P<0.02$.
***$P<0.01$.
§$P<0.001$.

patients only (Fig. 1) and this inhibition was about ~30% ($P<0.01$), thus suggesting that T_H2-type cytokines lead to induction of apoptosis in these patients.

The incubation of cells with IL-1α, IL-4, IL-6 and TNFα led to an enhanced cell death in PBMCs of these patients (Table 4). Since, IL-2 is known to have protective effect against apoptosis and anergic cells do not produce IL-2, so the cells were incubated in the presence of rIL-2 and it inhibited apoptosis by

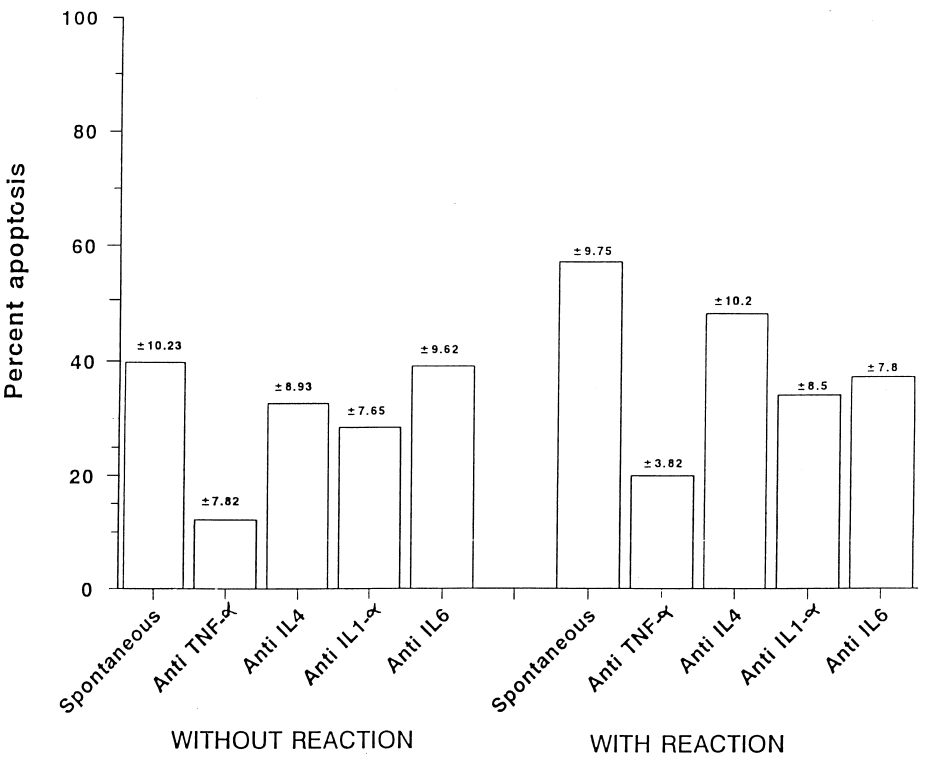

Fig. 1. Effect of various anticytokine antibodies on inhibition of apoptosis in leprosy patients without reaction (left) and with reaction (right). Values represent mean ± S.E.M. ($n=5$).

Table 4
Percent apoptosis in PBMCs of leprosy patients in the presence of various cytokines

Patients	Spontaneous	rIL-2	rIL-4	rIL-6	rIL-1α	TNFα
Control ($n=7$)	8.24 ± 2.88	7.84 ± 3.25	8.41 ± 2.5	7.25 ± 3.99	6.40 ± 2.17	7.20 ± 2.68
BT/TT ($n=8$)	25.00 ± 5.81	19.00 ± 4.15	21.50 ± 3.41	25.42 ± 5.22	22.80 ± 4.37	33.00 ± 2.45
BL/LL ($n=8$)	37.20 ± 9.4	15.74 ± 2.47[a,*]	58.88 ± 4.52[a,**]	52.54 ± 3.56[a,**]	49.20 ± 2.78[a,*]	60.24 ± 3.89[a,***]
BL/LL with reaction ($n=5$)	46.88 ± 10.4	25.66 ± 8.2[a,*]	70.60 ± 9.4[a,**]	68.00 ± 8.35[a,**]	65.23 ± 10.45[a,**]	80.00 ± 4.58[a,***]

Values represent mean ± S.E.M.
[a]vs. spontaneous apoptosis of the same group.
*$P < 0.02$.
**$P < 0.01$.
***$P < 0.001$.

~45% ($P < 0.02$), suggesting that apoptosis in these patients occurs due to an increase in T_H2 cytokines and a decrease in T_H1 cytokines.

4. Discussion

Two signals are required for normal proliferation of cells. One is mediated through TCR and another through co-stimulatory molecules such as CD28. If there is an imbalance in these signals, then the cells undergo anergy and/or apoptosis [25]. Our results show that in leprosy patients, the co-stimulatory molecules such as CD28, B-7 and adhesion molecules (ICAM-1) which play an important role in activation of T-cells, are down regulated. As a result, when cells are stimulated (with various mitogens), in the absence of co-stimulatory signals, they undergo apoptosis, which could be the cause of anergy in leprosy. In acute herpes virus infection (AHVI) and chronic HIV patients it has been shown that $CD28^-$ population fail to respond normally to activation signals through TCR and are prone to activation induced cell death (AICD). CD28 is required for IL-2 production and enhances the levels of bcl_{xL} and bcl-2 to prevent apoptosis [26].

Besides co-stimulatory molecules, the PBMCs of leprosy patients are also defective in calcium mobilization as the ionomycin could inhibit the apoptosis in these cells. Our previous findings have also shown that cells of leprosy patients are defective in proximal signal transduction pathway [27]. A perturbation of intracellular calcium homeostasis is well known to trigger apoptosis and it may occur either due to a sustained increase in cytosolic free calcium levels or a depletion of intracellular calcium stores [25,28]. Further a recent report has shown that T_H2-type cells fail to use calcium signaling pathway [29]. Since, calcium plays an important role in synthesis of IL-2, thus ionomycin might be increasing the availability of calcium for initiation of distal TCR-mediated signal transduction pathway.

The apoptosis in PBMCs of leprosy patients was also inhibited by zinc (which is known to block Ca^{2+}-dependent apoptosis by blocking calcium/magnesium endonuclease activity) [30] and was more effective than ionomycin, especially in cells from ENL patients. Zinc has been shown to inhibit apoptosis by lowering TNFα levels and increasing IL-2 in case of ENL patients (Gupta et al. 1998, communicated), although the exact mechanism of action is not known as yet. This is supported by the earlier reports which show that the serum of leprosy patients is deficient in calcium, iron, magnesium and zinc [31–34] and oral zinc therapy decreases the chances of reactions in these patients.

Thus, our study shows that anergy in leprosy patients is due to multiple defects in the TCR-mediated signaling i.e., defective calcium mobilization, zinc deficiency and lack of expression of co-stimulatory molecules. The absence of these signals could be responsible for an established T_H2-type response in lepromatous leprosy patients. These T_H2-type cytokines may lead to an increase in susceptibility of cells to undergo apoptosis. In the case of patients with reaction, anti-cytokine antibodies blocked apoptosis, further confirming that these cytokines are responsible for inducing apoptosis in these cells. rIL-2 was also able to block apoptosis indicating that cells are deficient in IL-2 and this incapability to produce IL-

2 (due to defective/insufficient signaling) may lead to anergy and/or apoptosis. Since, TNFα is well known to activate cysteine proteases (caspases), so the inhibition of apoptosis by leupeptin (a non-specific cysteine protease inhibitor) also shows the mechanism of action of TNFα could be through cysteine proteases.

To summarize, our results clearly show that cells of leprosy patients are anergic and defective in proximal TCR-mediated signal transduction pathway, leading to a perturbation in the intracellular second messengers. This leads to an imbalance in signal transduction pathway which may lead to a transition from T_H1- to T_H2-type phenotype and the overproduction of the T_H2-type and certain proinflammatory cytokines, thereby causing apoptosis in these cells. This TNFα-mediated effect can be blocked, to a certain extent, by ionomycin and zinc, thus showing that these metal ions can help in improving the CMI response in these patients especially in the case of ENL patients and in certain other inflammatory diseases.

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