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Study of Apoptosis in Skin Lesions of Leprosy in Relation to Treatment and Lepra Reactions

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ABSTRACT

In leprosy on treatment, one factor contributing to the healing of skin lesions with minimal fibrosis may be apoptosis of inflammatory cells, even though apoptosis is sparse in leprosy as compared to tuberculosis. The degree of apoptosis in skin lesions of leprosy was studied by histopathologic examination (HPE) and by DNA fragmentation and electrophoresis. The effect of various parameters on apoptosis was noted in untreated disease, during treatment at 3 and 6 months, and in lepra reactions in different parts of the spectrum of leprosy. Of the 31 patients, 13 had paucibacillary (PB) and 18 multibacillary (MB) disease. Twenty one patients were in reaction: 16 had type 1 reaction and 5 had type 2 reaction. The controls included patients with non-granulomatous skin diseases; there were no normal controls, and no separate controls for cases with reaction. Apoptosis occurred more frequently in patients with leprosy as compared to the controls. In both PB & MB lesions, apoptosis was observed to increase progressively with treatment at 3 and 6 months, and was more prominent in the MB cases at 6 months of treatment. When lesions in either type 1 or type 2 reaction were compared to lesions not in reaction, a significant increase in apoptosis ($p = 0.014$) was found only in lesions with type 2 reaction and those which were at 6 months of treatment. The type of treatment regimen, or oral steroids given for reactions, did not significantly alter the degree of apoptosis. Our observations indicate that increased apoptosis is present in leprosy lesions and that in leprosy it progressively increases with anti-leprosy treatment up to 6 months. If the process of apoptosis in skin lesions is followed up for a longer period of time, the degree of apoptosis may be expected to decline. The study of apoptosis may help to understand the mechanism of clearance of bacilli and resolution of granulomas in leprosy patients.

RÉSUMÉ

Au cours du traitement de la lèpre, un facteur contribuant à la cicatrisation des lésions cutanées en l'absence de fibrose significative pourrait être l'apoptose des cellules inflammatoires, même si l'apoptose est rapportée comme peu commune dans la lèpre, comparée à la tuberculose. Le degré d'apoptose dans les lésions cutanées de la lèpre fut étudié à l'examen histopathologique (HPE) et par examen de la fragmentation de l'ADN par électrophorèse. L'effet de divers paramètres sur l'apoptose fut noté lors de maladie non traitée, en cours de traitement à des temps de 3 et 6 mois et dans des réactions lépreuses pour différents points du spectre immunopathologique de la lèpre. Parmi les 31 patients, 13 avaient la forme paucibacillaire (PB) et 18 la forme multibacillaire (MB). Vingt-et-un patients étaient en réaction, 16 ayant une réaction de type 1 et 5 de type 2. Les contrôles étaient représentés par des patients avec des maladies cutanées non granulomateuses, sans contrôle avec peau normale, et sans contrôle séparé pour les cas avec réactions. L'apoptose était plus fréquente chez les

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patients atteints de la lèpre que chez les contrôles atteint de lésions cutanées non granulomateuses. A la fois dans les lésions PB et MB, il y eut un accroissement progressif de l'apoptose aux temps de traitement de 3 et 6 mois, qui fut le plus net chez les cas MB à 6 mois de traitement. Lorsque les lésions de type 1 ou de type 2 furent comparées à celles sans réaction, une augmentation significative de l'apoptose (p = 0,014) fut observée dans les réactions de type 2 et celles qui furent à 6 mois de traitement. Le type de traitement, ou bien les corticostéroïdes oraux administrés pendant les réactions n'ont pas altéré de façon significative le degré d'apoptose. Nos observations indiquent qu'il existe une apoptose plus importante dans les lésions de lèpre que dans celles de lésions cutanées non-granulomateuses et qu'elle augmente progressivement avec le traitement, et ce au moins jusqu'à 6 mois. Si l'apoptose dans les lésions cutanées avait été suivie au-delà de 6 mois, le degré d'apoptose aurait probablement décliné. L'étude de l'apoptose devrait pouvoir permettre de mieux comprendre les mécanismes d'élimination des bacilles de Hansen et la résolution des granulomes chez les patients souffrant de lèpre.

RESUMEN

En el tratamiento de la lepra, un factor contribuyente a la curación de las lesiones de la piel, con mínima fibrosis, puede ser la apoptosis de las células inflamatorias, aunque se piensa que la apoptosis en la lepra es rara comparada con la apoptosis en tuberculosis. En este estudio se examinó el grado de apoptosis en las lesiones de la piel en la lepra por histopatología (HP) y por fragmentación y electroforesis del DNA. Se registró el efecto de varios parámetros sobre la apoptosis en la enfermedad no tratada, en la enfermedad con 3 y 6 meses de tratamiento, y en las reacciones de la lepra en diferentes partes del espectro de la lepra. De los 31 pacientes estudiados, 13 tenían lepra paucibacilar (PB) y 18 lepra multibacilar (MB). Veintiún pacientes estaban en reacción, 16 tenían reacción leprosa tipo 1, y 5 reacción leprosa tipo 2. Los controles incluyeron pacientes con enfermedad de la piel no granulomatosa; no hubo controles sanos ni controles separados para los casos en reacción. La apoptosis ocurrió más frecuentemente en los pacientes con lepra que en los pacientes control. Tanto en los pacientes PB como en los MB la apoptosis aumentó progresivamente con el tratamiento a los 3 y 6 meses, y fue más prominente en los casos MB a los 6 meses de tratamiento. Cuando se compararon las lesiones de las reacciones tipo 1 o tipo 2 con las lesiones de la lepra no reaccional, se encontró un significante incremento en la apoptosis (p = 0.014) sólo en las lesiones de las reacciones tipo 2 y en aquellas de los pacientes con 6 meses de tratamiento. El tipo del régimen de tratamiento o los esteroides orales administrados para controlar las reacciones no alteraron significativamente el grado de apoptosis. Nuestras observaciones indican que la apoptosis está incrementada en las lesiones de la lepra y que aumenta progresivamente con el tratamiento anti-leproso administrado durante 6 meses. Se esperaría una disminución en el grado de apoptosis en las lesiones seguidas por periodos más prolongados de tiempo. El estudio de la apoptosis puede ayudar a entender el mecanismo de eliminación de bacilos y la resolución de los granulomas en los pacientes con lepra.

In leprosy, the clinical, bacteriological and histopathological manifestations are profoundly affected by the immunological status of the host, which determines the type of leprosy. The clinicopathologic bipolarity may stem from the dual response of lymphocytes, dendritic cells, monocytes and macrophages to *M. leprae* ([1, 4, 14]). Usually chronic granulomatous diseases of the skin heal with fibrosis, eg: the scarring left behind by lupus vulgaris or scrofuloderma. However, in leprosy, with proper treatment or spontaneously as in indeterminate leprosy, the majority of skin lesions heal without much fibrosis. One factor which may contribute to this is thought to be apoptosis, a mechanism of cell death which has no accompanying inflammatory component and thus may explain the relative lack of fibrosis mentioned above.

Apoptosis is an active, self-destructive cellular process and is considered an integral part of the repertoire available to the cell to respond to deleterious stimuli from within and without ([5, 7, 14]). In tuberculosis, circulating polymorphonuclear neutrophils (PMN) show increased spontaneous apoptosis, and *Mycobacterium tuberculosis*-induced activation accelerates PMN apoptosis.([2])

In the resolution of leprosy granulomas with treatment, one mechanism of cell loss is believed to be apoptosis, especially for high turn-over granulomas. The number and density of apoptosis in leprosy may be affected by factors such as bacillary load and degree of cell mediated immunity ([4, 10, 14]). Since type1 and type 2 reactions in leprosy may be associated with changes in immune status, and the type 2 reaction is an acute inflammatory state, the number and density of apoptosis is likely to vary in these states also. We have therefore studied the degree of apoptoses by two different standard laboratory techniques and observed the effect of various parameters on apoptosis in skin lesions in untreated leprosy, during treatment, and during reactions.

MATERIALS AND METHODS

The study included 34 patients with leprosy attending the leprosy clinic at our center. Untreated, histopathologically confirmed leprosy patients were enrolled in the study, including patients with or without reactions, irrespective of sex, type and duration of disease. For controls, 20 patients from the general skin out-patient department were studied. Those with granulomatous skin disorders such as cutaneous tuberculosis and sarcoidosis were excluded. No normal skin samples were taken for control purposes. Also, there were no separate controls for those with reactions. Appropriate treatment regimen recommended by WHO, i.e., multi drug therapy (MDT), either multibacillary (MB) or paucibacillary (PB) regimen [(15)] was given to the patients according to the type of the disease.

To study the apoptosis in the tissue specimens, biopsies were taken before starting treatment and at 3 and 6 months post-treatment, from the most active edge of the most prominent lesion, regardless of reactional state. All the initial biopsies were taken before the treatment started, and all subsequent biopsies were taken from the same lesion. For control purposes, biopsies were taken from 20 patients with skin diseases other than leprosy or other granulomatous disorders. From the tissue specimens, apoptosis was detected by two different methods for comparison i.e., histopathologic examination (HPE) and by DNA fragmentation and electrophoresis. All the biopsies were bisected, and the portion for HPE was fixed in formalin, embedded in paraffin, and 6μ thick tissue sections were cut and stained by hematoxylin and eosin. By HPE, apoptotic bodies were identified by the following features: nuclear condensation, round to ovoid bodies, eosinophilia of the cytoplasm and karyorrhexis/karyolysis ([7]) (Fig. 1). The number of apoptotic bodies per 10 high power fields/sample was recorded. The readings were taken by an experienced histopathologist, who was unaware of the treatment status of the patient. The fields were within the granulomas, and in biopsies where the granulomas were not prominent, the fields chosen were among the inflammatory infiltrates.

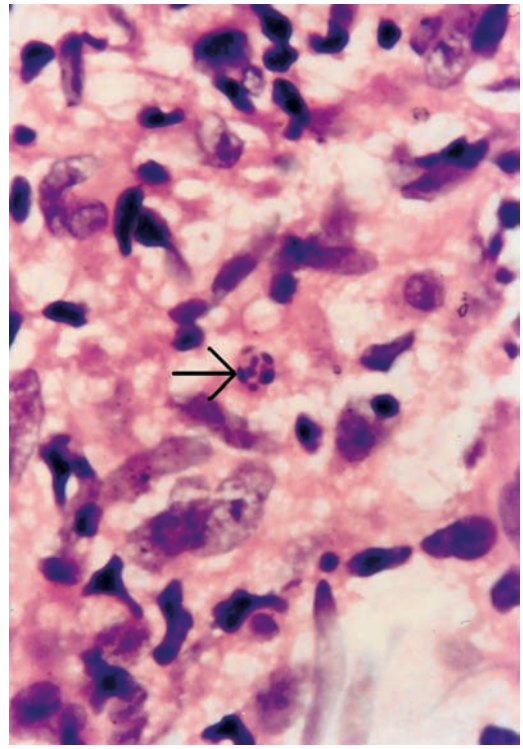

FIG. 1. Apoptotic cell depicting characteristic nuclear fragmentation (H&E stain ×550).

The other part of the bisected biopsy tissue was transported in normal saline for analysis of DNA fragmentation. DNA was isolated from all the samples by the method of Palmiter et al ([14]). Briefly, the tissue was teased and suspended in 500μl of lysis buffer containing 1% SDS and 0.01% protienase K in Tris-EDTA (TE) buffer (ph 8.0) and incubated at 55°C overnight. After phe-

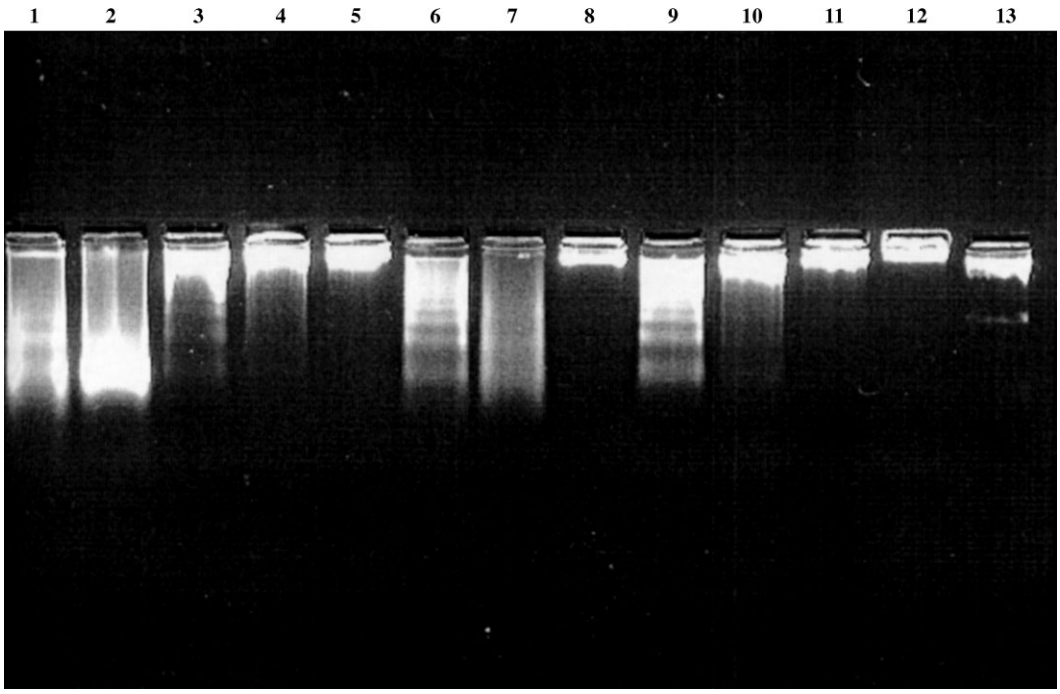

FIG. 2 1% Agarose gel electrophoresis showing DNA fragmentation pattern; Lanes 1, 2, 3, 6, 7, 9 and 10 showing the typical "ladder pattern" of apoptoses.

nol-chloroform extraction, the DNA was precipitated with chilled isopropanol, and resuspended in TE buffer. The prepared DNA was run on 1% agarose gel, stained with ethidium bromide, and the gel was DNA viewed under ultraviolet light, to identify the typical "ladder pattern" of fragmented DNA specific to apoptosis. The pictures were captured using a gel documentation system (Image master, Pharmacia Biotech). Statistical analyses were done using non-parametric Chi-square tests and McNemar's Chi-square tests.

RESULTS

Of the 34 patients, 2 patients were lost to follow up and one patient died after 2 months of treatment due to dapsone hypersensitivity syndrome. Therefore, skin biopsy results from 31 patients were available for analysis. These were from 26 males and 5 females, having a mean age of 35.6 ±1.7 years. The majority of the patients (75%) were in the 16–45 years age group followed by 46–70 years (22%) and 1 (3%) was aged 14 years. Of the total patients 13 (42%) had paucibacillary (PB) and 18 (58%) had multibacillary (MB) disease. Twenty one patients were in reaction: 16 (76%) had type 1 reaction (9 in the PB and 7 in MB the group) and 5 (24%) had type 2 reaction.

Skin biopsies were taken from the control group of 20 patients, (12 males, 8 females) who had a mean age of 34.8 ± 1.8 years. The controls selected had various non-granulomatous dermatoses including lichen planus (5), discoid lupus erythematosus (4), pemphigus (4), warts (4), leiomyoma (2) and pseudopelade (1). The results obtained with both methods were compared in the PB and MB groups and controls at the start of the study, in relation to anti leprosy treatment, in Type 1 and Type 2 reactions, and in patients receiving corticosteroids for control of reaction.

By the method of HPE, apoptotic bodies were detected in all the specimens at all the stages, whereas by DNA fragmentation and electrophoresis (Fig. 2), apoptosis was detected only in 9 (26%), 16 (52%) and 26 (87%) of patients at baseline (untreated) and at 3 and 6 months of treatment respectively. Detection of apoptosis by the method of HPE at baseline and at 3 months and 6 months after completing treatment is presented in Table 1. The detection of apoptosis by DNA fragmentation and elec-

TABLE 1. *Apoptoses by the method of HPE at baseline and at 3months and 6 months after completing treatment*

Diagnosis	Number of cases	Apoptoses (mean ± S.D.)/10hpf		
		Baseline	3 months after treatment	6 months after treatment
PB with type 1 reaction	9	2.04 ± 1.33	3 ± 1.48	5 ± 1.29
PB without reaction	4	2 ± 1.42	2.5 ± 1.34	3.5 ± 1.44
MB with type 1 reaction	7	2.12 ± 1.83	3.82 ± 1.40	6 ± 1.60
MB with type 2 reaction	5	3.8 ± 1.82	4.8 ± 2.59	9.4 ± 3.21
MB without reaction	6	2.5 ± 0.89	3.2 ± 1.3	5.17 ± 1.17

trophoresis in PB and MB disease at baseline and at 3 months and 6 months after treatment is presented in Table 2. A comparison of apoptosis by the method of HPE between the different groups is shown Table 3.

Twenty two patients received MDT-MB therapy, 7 patients received MDT-PB therapy and 2 patients received rifampicin, ofloxacin, and minocycline (ROM) therapy. Comparing the different treatment groups, apoptosis was found more frequently with treatment in all the groups. Comparing apoptosis between the groups, no significant change in the apoptosis was observed at all the 3 stages of treatment. (The values from the two patients who received ROM therapy were not compared with other groups),

Of the 31 patients, 23 (74%) patients received corticosteroids (19 for reactions and 4 for neuritis); the starting dose of prednisolone was 40 mg/day, which was then tapered over a period of 3–6 months. On comparing apoptoses between the steroid-treated and non-treated patients, both the groups showed an increase in apoptoses with the duration of treatment, but the effect of steroids on this trend was not statistically significant in any of the groups at any period ($p > 0.05$).

In summary, apoptosis was found more frequently in patients with leprosy as compared to the controls. In both PB & MB portions of the leprosy spectrum, apoptosis progressively increased with treatment at 3 and 6 months, but it was more noticeable in the MB cases, especially at 6 months of treatment. When biopsies of either type 1 or type 2 reaction were compared to those not in reaction, a significant increase in apoptoses was found in cases with type 2 reactions, also at 6 months of treatment. All of the MDT regimens showed increased degrees of apoptosis, but the process was not affected by either of the regimens. The administration of corticosteroids did not appear to significantly alter the degree of apoptosis.

DISCUSSION

Apoptosis is one form of cell death which is non-inflammatory, and it has been postulated to play a role in the resolution of leprosy granulomas as a mechanism of cell destruction. It might represent a strategy of the immune system to eliminate infected cells, but few studies have shown apoptosis to be present in leprosy lesions ([1, 4, 15]). One recent study assessing a small number of samples only from untreated patients found trends suggesting apoptosis in leprosy lesions might be more frequent in PB disease ([15]). However, sufficient information on apoptosis in various situations, such as during reactions, in different portions of the leprosy spectrum of disease (indirectly indi-

TABLE 2. *Detection of apoptosis by electrophoresis to depict DNA fragmentation at various periods in PB and MB disease.*

Disease spectrum	Number and % age of patients		
	Baseline	3 months after treatment	6 months after treatment
PB n = 13 ($p > 0.05$)	3 (23%)	6 (46%)	8 (61%)
MB n = 18 ($p < 0.05$)	6 (33%)	10 (77%)	18 (100%)

TABLE 3. *Comparison of apoptoses by HPE between the various groups at different periods.*

Disease groups	p-value		
	Baseline	3 months	6 months
PB with type 1 reaction vs. PB without reaction	0.55 (p >0.05)	0.16 (p >0.05)	0.20 (p >0.05)
PB with type 1 reaction vs. MB with type 1 reaction	0.56 (p >0.05)	0.68 (p >0.05)	0.19 (p >0.05)
MB with type 1 reaction vs. MB without reaction	1.00 (p >0.05)	0.57 (p >0.05)	0.32 (p >0.05)
MB with type 2 reaction vs. MB without reaction	0.07 (p >0.05)	0.16 (p >0.05)	0.014 (p <0.05)
MB with type 1 reaction vs. MB with type 2 reaction	0.13 (p >0.05)	0.26 (p >0.05)	0.03 (p <0.05)
PB without reaction vs. MB without reaction	0.52 (p >0.05)	0.14 (p >0.05)	0.17 (p >0.05)

cating immune status), and the effects of treatment, are not available.

Comparing two methods of apoptosis detection i.e., HPE and DNA fragmentation and electrophoresis, the HPE method was found to be more sensitive and provided a more exact quantitative difference in apoptosis at different stages. However, using the HPE method as a readout for apoptoses is not ideal because this method is subject to observer variability and incorrect interpretation if read by an observer not experienced in apoptoses ([4, 15]). The method of DNA fragmentation and electrophoresis is not subject to observer variation and the results are more reliable and probably of greater value than HPE, but in our study, which required a quantification of apoptoses, DNA fragmentation and electrophoresis was observed to be less sensitive. Using DNA fragmentation and electrophoresis, we could not detect the apoptotic process in expected numbers, but the trends in the detection of apoptoses between HPE and DNA fragmentation and electrophoresis were similar.

In comparing the frequency of apoptosis in lesions across the disease spectrum, it was found that in all subsets of disease, there was progressive increase in apoptoses from baseline to 6 months of treatment. Our findings of significantly more apoptoses in patients with leprosy compared to controls supports the similar observation in previous studies ([4, 10, 15]). Walsh, *et al*. ([15]), using a well established "TUNEL" assay found apoptosis to be more frequent in untreated lesions of PB disease in comparison with MB disease; however we observed apoptosis to be greater in lesions of MB compared to PB group. Our observation supports a previous *in vitro* study in which apoptosis increased with increasing concentration of *M. leprae* ([6]), although this was an *in vitro* study and did not involve skin lesion analysis. Generally, this suggests that apoptoses can be found in leprosy, but since it was not present in all the samples and the numbers of apoptoses were small, unlike in tuberculosis, the significance of apoptoses in leprosy remains unclear.

Regarding the effect of treatment, the number and density of apoptoses increased progressively with treatment in all the groups of patients. One previous study has shown that sulfonamides can induce apoptosis of circulating leukocytes, by their metabolites becoming attached to these cells and promoting up regulation of apoptosis inducing factors ([9]). Since sulfonamides are present in both MDT-MB and MDT-PB regimens and the effect of rifampicin and clofazimine on apoptosis is not known, an exact explanation of the role of MDT regimens in the increased apoptoses is not clear. With treatment, inflammation subsides and the bacillary load also declines. Both of these effects should cause a fall in apoptosis. But we have found increasing apoptosis with both treatment regimens at 6 months. Since treatment results in the resolution of leprosy granulomas with a modest proportion of the inflammatory cells undergoing apoptosis, this could explain the finding of increased apoptosis early in the treatment. Resolving granulomas initially show apoptoses of inflammatory cells with treatment. In fully resolved granulomas the apoptosis may even cease completely and so there may occur a fall in apoptoses. Also, the apoptotic bodies are understood to be spontaneously cleared with time by phagocytosis. Hence, we speculate that studies carried out for a longer period may show decreasing apoptosis.

In lesions of Type 2 reaction, a significant increase in apoptosis was observed at 6 months of treatment when compared to non-reactional group as well as to the type 1 reactional group. Oliveira and colleagues found apoptosis to be greatly accelerated in circulating polymorphonuclear neutrophils in patients experiencing ENL ([11]). The increased expression of pro-apoptotic members of Bcl-2 protein family and of TNF-α in type 2 reaction is likely to induce more apoptosis ([11]). Sampaio and colleagues have shown that neutrophils stimulated with *M. leprae* secrete large amounts of IL-8 and TNF-α, and drugs with anti TNF-α properties such as thalidomide given for severe ENL reaction may cause a decrease in apoptosis ([13]). Our findings support the suggestions that greater bacillary load and type 2 reaction can lead to an increase in apoptosis.

Though the number and density of apoptosis was more in the prednisolone treated group, the values were not statistically significant when compared to the non-prednisolone treated group. Though corticosteroids are among the drugs that can induce apoptosis especially in thymic and circulating lymphocytes, no significant association with corticosteroid treatment and apoptosis was found in this study. A previous study has shown differential effect of steroids on apoptosis, with apoptosis being inhibited in few specific tissues, especially glandular tissues ([3]). Also the pro-apoptotic effects of corticosteroids may have been offset by the effect of anti-leprosy treatment, with *M. leprae* induced inflammation resolving and the corticosteroids further reducing the inflammation.

Apoptosis is the end point of an energy dependent cascade of molecular events and is regulated by several genes which induce p53, c-Myc, Bcl-2, CED-3 and Fas genes ([7, 17, 18]). *M. leprae* induced apoptosis of circulating monocytes has been shown to occur *in vitro* ([6]). Studies on blood mononuclear cells in leprosy patients have shown greatly increased apoptoses of lymphocytes especially the CD8+ and CD19+ cells compared to CD4+ cells ([10]). These results demonstrated that *M. leprae* can lead to apoptosis of macrophages through a mechanism that could be at least partially related to the expression of pro-apoptotic members of the Bcl-2 protein family and of TNF-α. Apoptosis may also have a role in nerve damage in leprosy, as shown by a recent study which investigated the possibility that human Schwann cells are susceptible to cell death through the activation of Toll like receptor 2 (TLR2), a pattern recognition receptor of the innate immune system ([12]). Hence, apoptosis promoted by *M. leprae* may induce damage in the affected tissues, also may act as a defense mechanism by shedding the infected and effete macrophages and lymphocytes.

Further study of apoptosis may help to understand the method/mechanism of clearance of bacilli and resolution of granulomas in leprosy patients. It is expected that the degree of apoptosis will decrease in patients with resolving disease; however an increasing trend is likely to be seen in the initial period of therapy when immune response is being reconstituted and bacillary clearance is occurring. If the process of apoptosis in skin lesions is followed up for a longer period of time, the degree of apoptoses is expected to come down. From this study it is not possible to delineate the exact role of each of the factors like type of disease, reactions, treatment regimens and steroids, with each contributing directly or indirectly to the final result. Hence further studies with larger number of patients and prolonged follow up designed to detect apoptosis for at least up to 1 year duration are warranted.

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