

COMPARATIVE CELLULAR IMMUNE HOST RESPONSE IN ACUTE VS HEALED LESIONS OF CUTANEOUS LEISHMANIASIS

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Background: Cutaneous leishmaniasis (CL) has become a major public health problem in Pakistan and research is now focused to characterize the host's immune response, its clinical correlation and subsequent implications in management in this disease. This study was done to evaluate cellular immune host response in patients with active and healed CL and its possible implications in prevention of disease in susceptibles. **Methods:** This cross sectional and comparative study was conducted in Armed Forces Institute of Pathology (AFIP) and Military Hospital (MH) Rawalpindi (1998-2000). 30 biopsies of active skin lesions and 15 biopsies from healed lesions, after processing, were studied for various immunophenotype cells by using monoclonal antibodies. Total and differential T cell counts were recorded in these skin tissues. Non parametric Kruskal-Wallis Test for one way ANOVA was used to compare the median cell counts between active, healed and normal skin and p-value <0.05 was considered significant. **Results:** The total cell counts, CD3+ cells and CD57+ (NK) cells were found statistically different ($p < 0.001$) when active forms of the disease were compared with healed lesions and normal skin tissue. The difference was not significant ($p > 0.05$) on comparing healed lesions with normal skin tissue biopsies except in case of CD3+ cell counts ($p < 0.05$). However, CD4+, CD8+ and CD19+(Plasma cells) counts were never seen significant ($p > 0.05$) on comparison. **Conclusion:** NK cells and gamma delta cells seem to be responsible for limitation of the disease and elimination of the parasite from the lesion in cases of acute cutaneous leishmaniasis.

Keywords: Leishmaniasis, Cutaneous leishmaniasis, Cellular immune host response

INTRODUCTION

The leishmania parasite is capable of producing a broad spectrum of disease in humans, ranging from asymptomatic infection to disfiguring scars or the potentially fatal visceral form. Pakistan is endemic for cutaneous leishmaniasis and the disease appears to be spreading all over the country^{1,2}. Old world cutaneous leishmaniasis (in Pakistan) caused by *Leishmania major* or *Leishmania tropica* is often a self-healing disease; however, persistent nonhealing forms are also known. The outcome of infection depends largely on the immune responsiveness of the host and the virulence of the infecting parasite strain^{3,4}. Leishmaniasis, being one of the parasitic infections, presents their host with varied immunological problems and the net outcome, as in other parasitic infections, is that the infection tends to be long and chronic with host mounting varied and sometimes unsuccessful immunological responses. Similar to other chronic infections, it is the cell mediated immune response that has critical importance in leishmaniasis^{5,6}. It is responsible for the elimination or protection of the parasite and perhaps modulation of the clinical features. Granulomatous inflammatory infiltrate forms in response to infection with leishmania parasite⁷. The nature of cells comprising this infiltrate has been an area of active investigations in human and murine models. The particular areas of interest are dissection of the T cell subsets, the antigen recognised by these cells and by the cytokines they produce. The characteristics of cellular immunity in cutaneous

leishmaniasis have been looked for in the peripheral blood as well as in skin tissue⁵⁻⁸. In Pakistan, no such study has been done either on peripheral blood or on tissue sections.

This study was therefore planned to analyse the immunocompetent cells present in the active lesions of cutaneous leishmaniasis and to compare this cellular picture with recently healed lesions of the disease and subsequently to look for possible implications of the results in planning for mass vaccination in susceptible population as this disease has become a public health problem in various regions of Pakistan.

MATERIAL AND METHODS

The patients included in the study were mostly from the province of NWFP and Punjab. Few cases were from the northern areas. The patients belonged to a heterogeneous population of both sexes and all ages. Most patients were from the military but civilian population was also included in this study. Active cases of acute cutaneous leishmaniasis were having either a single or two lesions on exposed body parts, which were not more than 2-8 weeks old. Multiple (more than three) or sporotrichoid lesions were not included. Healed cases were treated proven cases of cutaneous leishmaniasis who had the disease during last one year.

Initially, 42 patients with clinically suggestive lesions of cutaneous leishmaniasis and 15 patients with healed lesions of the disease were included. Subsequently 12 patients who did not parasitologically

confirmed diagnosis (either positive skin slit smears or skin biopsies for leishmania parasites) were excluded. Cutaneous biopsies were performed at the peripheral part of the lesion within the inflammatory active outline in active cases and from centre of the healed lesions in the form of elliptical sections after local anaesthesia. The immunophenotyping was done on 30 skin biopsied specimens on formalin- fixed, paraffin-embedded tissue sections of active skin lesions and on 15 specimens from healed lesion. 15 skin biopsies of normal healthy skin tissues were also included in the procedure as controls.

Immunophenotyping was done using the streptavidine: biotin method. Primary antibodies used were non-alcohol murine antiCD3, CD4, CD8, CD19, CD57 (ZYMED, San Fransisco, California USA). Second antibody used was Biotinylated Second antibodies (BSA). The kit used for immunohistochemistry was Streptavidine/biotin Histostain SAP kit (ZYMED, San Fransisco, California USA). Smears of separated and washed lymphocytes were used as positive controls with each batch. Immunophenotyping of 15 normal skin tissue specimens were used for comparison. A written consent was taken from all patients after explaining the project in detail. After fixing and processing, slides were made from each specimen, which were stained with routine hematoxylin and eosin stain.

The slides were then read under a light microscope (Nikon). Five slides from each specimen were stained using different monoclonal antibodies. Smears of washed and separated lymphocytes were used as positive controls with each batch. Negative controls without the primary antibody were included in each run and blocking of avidin binding and endogenous peroxidase was done prior to deposition of the first monoclonal antibody. Slides were made by using the streptavidine: biotin method and were read under a light microscope (magnification x 250 and 400). Ten randomly chosen microscopic fields in the area of the inflammatory infiltrate / granuloma were selected.

Total cells and number of positive cells were counted in each field. Percentage of cells in granulomas staining with these antibodies was obtained by dividing the number of positive cells by the total number of cells and multiplying by 100. "INSTAT" programme was used for data processing and "Kruskal-Wallis Test" for one way ANOVA was used for difference in medians of cell counts in these three unbalanced size groups. Statistical significance was derived by knowing p-values ($p < 0.05$ as significant, $p < 0.01$ as very significant and $p < 0.001$ was considered as extremely significant).

RESULTS

Our patients belonged to a heterogeneous population of both sexes, but as our study included patients from the military hospitals, the majority of patients were males. There were 57 (95%) males and 3(5 %) females. Age of the patients ranged from 15 years to 63 years. In active lesions, the duration of illness was between 2-8 weeks. Almost all the patients had lesions on the exposed parts of their bodies. The pattern of the active lesions was in the form of plaques, nodules, nodulo-ulcerative or ulcers. All 15 healed lesions were healed ulcers. These were hyperpigmented with slight scarring in the centre. Positive skin slit smears were seen in all cases of acute lesions included in the study. The skin sections of 30 acute cases stained routinely with hematoxylin and eosin, showed infiltrates composed of lymphocytes and macrophages arranged diffusely without showing well organised granulomata. Neutrophils, eosinophils, langerhan giant cells and plasma cells were also seen. This predominantly mononuclear infiltrate extended from the upper to the lower dermis, sometimes surrounding a zone of necrosis. The overlying epidermis showed moderate ulceration with hyperplasia. Leishmania trophozoite (LT) bodies were seen lying intra and extra cellularly in 17 histological sections out of 30 cases of acute CL. Histopathology of healed lesions showed a mild to moderate upper and mid dermal perivascular infiltrate composed of lymphocytes, histiocytes, plasma cells and occasional eosinophils. The overlying epidermis was mildly hyperplastic and no ulceration was seen. No LT bodies were seen in any of the sections. Histology of normal skin tissue fragments showed a mild perivascular lymphocytic infiltrate in the upper dermis. Total number of T cells and subsequent cellular subsets (CD3+,CD4 +, CD8+, NK cells and CD19 cells) seen in active lesions of CL, healed lesions and in normal skin tissues were recorded in each case (10 fields per sections x 400) and their mean counts are shown in Table-11. The number of CD45RO+ (gamma delta cells) was indirectly calculated by subtracting the sum of CD4 +and CD8+ cells from the total CD3+ cells in each group. These cells in active lesions were 11656 (97.55% of the total CD3+ cells), in healed lesion 1094 (9.08%) and in normal tissue biopsies the gamma delta were 13 (9.10%) of the total CD3+ cells. Sample photomicrographs of CD3+,CD4 +, CD8+, NK cells and CD19 cells detected on immunophenotyping of active lesions and healed lesions of CL are shown in Fig 1-5 A & B respectively. Non parametric Kruskal-Wallis Test for one way ANOVA was used to compare the median cell counts between active lesions, healed lesions and normal skin tissues. Comparative results of total cell count, CD3+ cells, CD4 + cells, CD8+ cells, CD57+ (NK) cells, CD19+ (Plasma) cells in each group have been shown in Tables-2.

Table-1: Comparative counts (means) of total T cells and different subsets of T cells in active lesions, healed lesions and normal skin tissues

Type of lesions	Total T cells (mean)	CD 3+ Cells (mean)	CD 4+ cells (mean)	CD 8+ cells (mean)	NK cells (mean)	CD 19 cells (mean)
Active lesions	1065.63	398.30	5.03	4.73	181.87	2.53
Healed lesions	222.33	86.73	8.33	5.47	33.8	2.67
Normal Skin	60.47	9.53	5	3.67	4.67	2.67

Table-2: Comparative statistical analysis of total as well as differential T cell counts in active lesions, healed lesions and normal skin tissues.

Comparisons	Total T cells	CD 3+ cells	CD 4+ cells	CD 8+ cells	NK cells	CD 19 cells
Active vs Healed	p < 0.001 (**)	p < 0.001 (**)	P > 0.05 (x)	P > 0.05 (x)	p < 0.001 (**)	p > 0.05 (x)
Active vs Normal	p < 0.001 (**)	p < 0.001 (**)	P > 0.05 (x)	P > 0.05 (x)	p < 0.001 (**)	p > 0.05 (x)
Healed vs Normal	p > 0.05 (x)	p < 0.05 (*)	P > 0.05 (x)	P > 0.05 (x)	p > 0.05 (x)	p > 0.05 (x)

(x) = Statistically not significant, (*) = Statistically significant, (**) = Statistically very significant

Fig-1 A

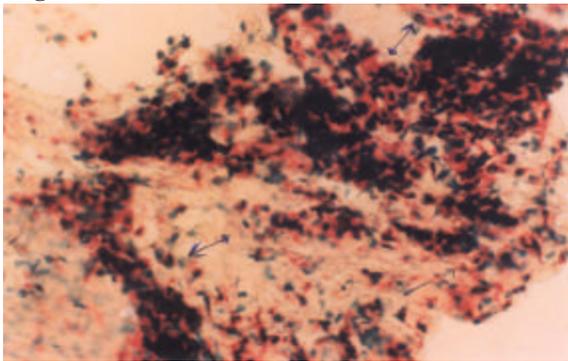


Fig-1 B

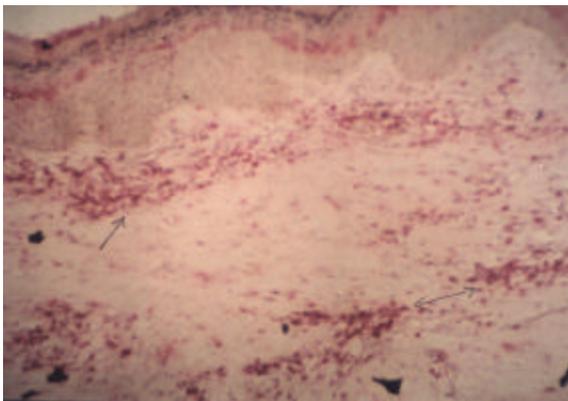


Fig-1: CD+3 Cells (arrows) in active lesions (A) and in healed lesions (B)

Fig-2 A

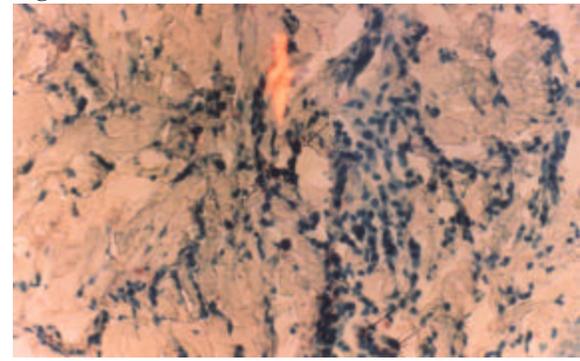


Fig-2 B

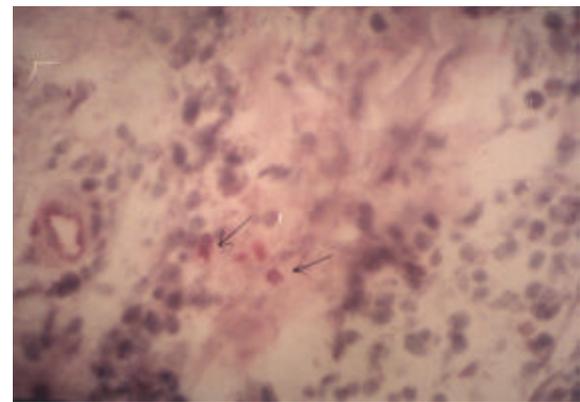


Fig-2: CD+4 Cells (arrows) in active lesions (A) and in healed lesions (B)

Fig-3 A

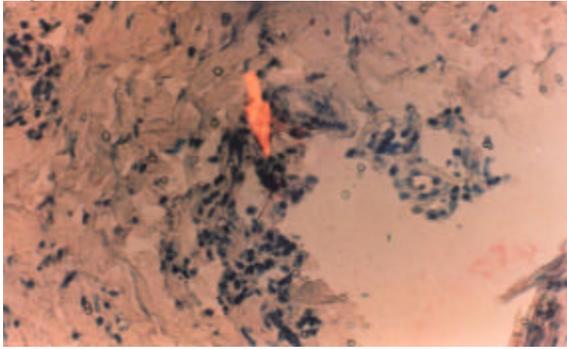


Fig-3 B

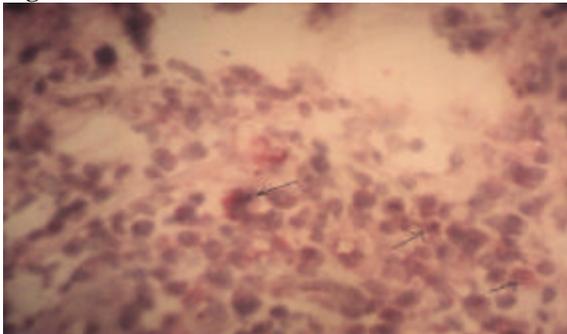


Fig-3: CD+8 Cells (arrows) in active lesions (A) and in healed lesions (B)

Fig-4.A

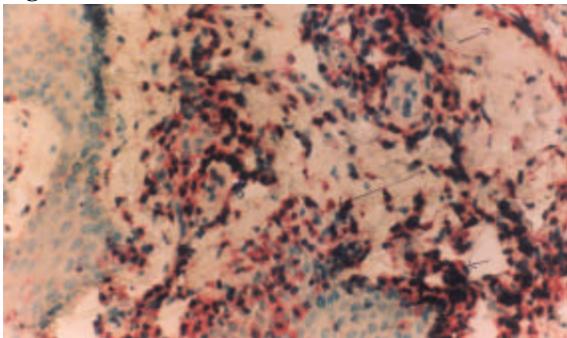


Fig-4.B

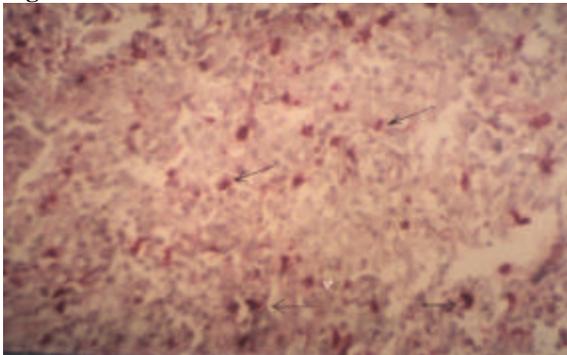


Fig-4: NK Cells (arrows) in active lesions (A) and in healed lesions (B)

Fig-5.A

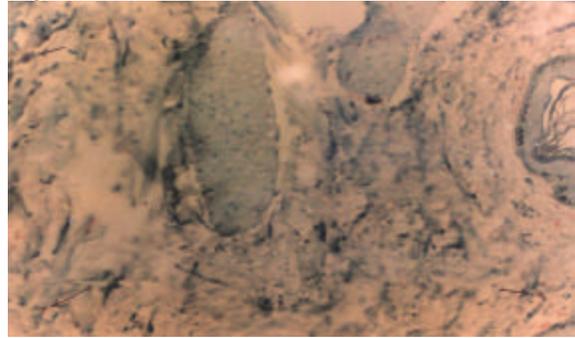


Fig-5.B

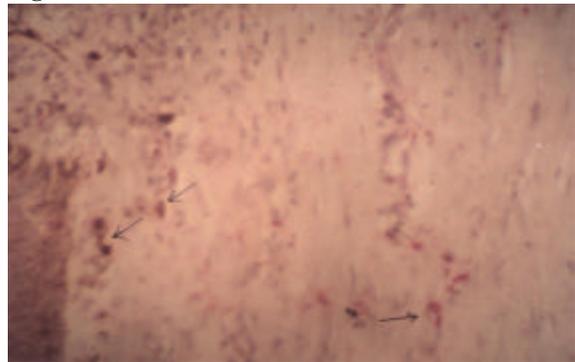


Fig-5: CD+19 Cells (arrows) in active lesions (A) and in healed lesions (B)

DISCUSSION

Th1-type cellular immune responses play a critical role in protection against infection with *Leishmania* parasites, whereas activation of Th2-type cells is thought to result in progressive disease. More over the parasite species have also an important role of in determining the pattern of immune response as infection with *L. amazonensis* induces a Th2-type immune response, whereas *L. major* induces a Th1-type response¹⁰. The immunophenotyping of cellular subsets seen in cutaneous leishmaniasis forms the basis of understanding the cellular immune response in various stages and in different variants of the disease. Some work has been done on evaluation of cellular immune host response in CL by immunophenotyping of cellular subsets of T lymphocytes in peripheral blood as well as in skin tissues, but no such study was conducted in Pakistan before. Our study showed some striking similarities and differences when compared with earlier studies. A rich T cell component amongst the macrophages and other cells was seen in all active cases and it was statistically very significant when these cases were compared with healing lesions or normal skin tissues, as described in earlier studies^{8,11}. The total number of cells of the inflammatory infiltrate was more than the total number of cells that were stained with different monoclonal antibodies.

This indicates that there may be other cells that form part of a chronic granulomatous infiltrate including the fibroblasts, histiocytes, macrophages, dermal dendritic cells and Langerhan cells⁸. The inflammatory cellular infiltrate in active lesions contained a large number of CD3+ cells but a low CD4+ and CD8+ cell count. This relatively low number of CD4+ and CD8+ cells may be an indirect indicator for the presence of the significant number of gamma delta, and/or CD45RO+ cells. However, 37.4% and 39% CD3+ T cells in active and healed lesions respectively in our study was lower than that of Modlin¹² (58%), Jaroskava¹¹ (67%), Barral¹³ (48%) and of Ridel⁴ (40-75%) but were closer to that reported by Lima¹⁵ and Esterre⁸. There was a significant difference when CD3+ T cells in active cases were compared with healed or normal skin tissue. The number of CD4+ cells and CD8+ cells in our study was markedly lower when compared with other studies^{8,11,16} and there was no significant difference in active, healed or normal skin tissues. The high median value of CD3+ cells with very low CD4+ and CD8+ cells revealed the presence of another subset of T cells (CD3+ but CD4- and CD8- T cells). These cells (gamma delta cells) are thought to play a direct role in the granuloma formation together with cytokine IL-1 and TNF alpha, possibly secreted by activated T lymphocytes^{8,15,17,18}. Lima et al²⁰ suggested that these gamma-delta subset forms about 20% of T cells in early stages of cutaneous leishmaniasis and the number of cell decreases steadily with the age of the lesion and maturation of the granuloma and these cells appear to be critical near or at the commencement of immune response to leishmania^{15,18,19}. Our findings were considered similar to those of Lima et al²⁰ and only the percentage appears to be higher. The reason for this high percentage could be the unavailability of the specific monoclonal antibodies against gamma delta cells and CD45RO+ cells. Plasma cells in our cases were almost negligible similar to previous studies²⁰. Natural killer (NK) cells formed a high percentage of the cellular infiltrate in active lesions and a marked difference was seen when acute lesions were compared with healed or normal tissue²⁰⁻²².

By comparing the immunophenotype of the active and healed lesions of CL, we were mostly able to confirm the previous reports of other researchers that the bulk of the inflammatory infiltrate is made up of T cells^{8,20,23}. The predominant cells in the inflammatory infiltrate are CD3+ cells and CD57+ (NK) cells, and a small percentage of CD4+, CD8+ cells. Also a large percentage of cells were seen in the infiltrate, which were CD3+, CD4- and CD8-, these were presumed to be gamma delta cells. There was a marked influx of NK cells and gamma delta

cells in the infiltrates of all clinical variants. These cells seem to be the effectors cells in early stage of the disease and may be critical at the commencement of the immune response to leishmania. Thus presence of NK cells may be a good prognostic marker. These results could be of primary importance in developing new strategies for management of localised lesions and in developing vaccines to exploit this preferential role of NK cells and gamma delta cells in limiting the disease.

CONCLUSION

In old world CL, it is the immune response produced by the stimulation of the Th1 limb of T cells that is helpful in limiting as well as elimination of the parasite. NK cells and gamma delta cells probably have a key role in these defence mechanisms. With this data a coherent model of immune response and the possibility of an effective vaccine may become available.

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