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STUDIES ON THE T-CELL PHENOTYPES IN CHILDREN WITH KALA-AZAR IN FARS PROVINCE, IRAN

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ABSTRACT

The subpopulations of T-lymphocytes were studied in children with visceral leishmaniasis in the active stage of disease prior to treatment (n=28) and in controls (n=15) using specific monoclonal antibodies (Behring OKT3, OKT4 and OKT8). The percentage of total peripheral blood T-lymphocytes ($CD3^+$ T-cells) in patients were within the same range as those of normal children (71.09± 1.07 vs 68.89± 1.29). The percentage of $CD4^+$ T-lymphocytes showed a significant increase ($P<0.005$) in comparison with the control (53.92 ± 1.20 vs 45.59 ± 1.35). A decrease in the percentage of $CD8^+$ T-lymphocytes (20.81 ± 0.71 vs 23.89 ± 0.72) was observed which was significant at ($P<0.025$). The ratio of $CD4^+/CD8^+$T- lymphocytes was significantly increased at $P<0.005$ as the value of the ratio was 2.70 ± 0.17 for kala-azar patients and 1.91 ± 0.05 for the controls. The immunosuppression in kala-azar patients studied could be due to dysfunction of antigen specific T-cells and its subsequent effect on various cytokine release rather than changes in the phenotypic characters of these cells.
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INTRODUCTION

Infantile kala-azar, a systemic disease caused by *Leishmania donovani infantum* is endemic in Iran. Visceral leishmaniasis in this region is characterized by pronounced abnormalities in host immune response, including hypergammaglobulinemia, reduced delayed-type hypersensitivity response to leishmanin and other antigens, and also high levels of circulating immune complexes.[1-3] The mechanisms of depressed responsiveness remain unclear. However, the contribution of T-lymphocytes to the observed unresponsiveness has been reported.[4] Previous studies in infantile kala-azar have demonstrated a reduction in the percentage of circulating T-cells and an elevation in the percentage of B-cells.[1] In Mediterranean kala-azar in a recent investigation, a significant decrease in the number of $CD4^+$ cells was observed as the number of cytotoxic suppressor $CD8^+$ T-cells were increased.[5] In Kenyan patients with visceral leishmaniases, the total number of T-lymphocytes were within the same range as those of normal individuals, however the ratio of $CD4^+/CD8^+$ cells was inverted similar to Mediterranean kala-azar.[6]

This paper reports findings of lymphocyte phenotypes during the active phase of visceral leishmaniasis in 28 patients in Shiraz, Iran.

PATIENTS AND METHODS

Twenty-eight patients with clinical symptoms compatible with kala-azar who referred to Shiraz hospitals

over a period of 18 months were included in this study. Other criteria for diagnosis of kala-azar were IFA titers of ≥1/128; bone marrow aspirations were performed only on six patients. All blood samples were drawn prior to treatment. The patients studied responded well to glucantime treatment. Fifteen healthy children of similar age and as close as possible in socioeconomic background with the kala-azar cases, were used as control.

Determination of T-cell Phenotypes

Five mL of blood was drawn from patients and the controls, mixed with EDTA and layered on 3 mL of ficolle-Hypaque gradient using the method described by Boyum (1968).[7] The lymphocyte-rich layer was washed thrice in cold Hanks' solution and resuspended at 4×10^6/mL in cold RPMI 1640 medium. The T-cell phenotypes from patients and the controls were determined by adding specifically-defined monoclonal antibodies (OKT3, OKT4 and OKT8 monoclonal antibodies, Behring, Germany) to 100 µL of cell suspensions. These antibodies included 40 µL OKT3, 20 µL OKT4, 20 µL OKT8 and 20 µL of normal mouse serum which was used as the control. These antibodies were used to determine lymphocyte subsets, which included total T-cells ($CD3^+$) helper/inducer T-cells ($CD4^+$) and suppressor/cytotoxic T cells (CD8+), respectively. The cells were incubated at 4°C for one hour with gentle shaking after 30 minutes and then washed three times in Hanks' balanced salt solution. 20µL of fluorescein-isothiocyanate (FITC)-labelled goat anti-mouse immunoglobulin (Behring, Germany) was added to the cell pellet and incubated for another 30 minutes at 4°C with gentle shaking of the tubes after 15 minutes. After three washings, the cells were examined under the bright light for visual counting and then under UV light for membrane fluorescence and the percentage of positive cells were determined.

Skin test

All patients were tested intradermally with 0.1 mL of leishmanin (Institute Pasteur, Tehran, Iran). The dermal reaction was read 24 and 48 hours after skin test. The criteria for reactivity were erythema and induration of ≥ 6mm at the site of skin of skin test. The leishmanin used was also tested in many cases of cutaneous leishmaniasis and controls; satisfactory reactions were observed.

Indirect Fluorescent Antibody Test

IFA test was performed according to the method described previously and reciprocal titer of at least ≥1/128 with bright fluorescence was considered positive.

Statistical analysis

Results for the patients and controls are expressed as mean ± SE. The students's t-test was used to test for statistically significant differences between patients and control group.

TABLE I. Age and sex of the patients with kala-azar and the controls.

	Patients with kala-azar		Controls	
Sex	M	F	M	F
	14	14	6	6
Age ± SD (yrs)	1.5 ± 0.99		2.56 ± 1.4	

RESULTS

The age and sex of the patients and controls are presented in Table I. The antibody titers of the patients ranged from 1/128 to 1/1024. The distribution included 1/128(1), 1/256(8), 1/512(15) and 1/1024(3) respectively and the titers of control sera were <1/64. Amastigotes were present in the bone marrow smears of the six patients studied.

A pronounced leukopenia was observed in patients as compared to the controls (4.48 ± 0.27 vs 8.27 ± 0.53 × 10^3/mm^3). The results of lymphocyte subsets are presented in Fig. 1. No significant differences were found in the percentage of $CD3^+$ T-lymphocytes in patients as compared to the controls. A significant increase (P<0.005) was observed when OKT4 antibody was used for detection of $CD4^+$ T-lymphocytes of the patients and controls (53.92 ± 1.20 vs 45.59 ± 1.35). In regard to T suppressor/cytotoxic or $CD8^+$ T-lymphocytes, a significant decrease (P<0.025)

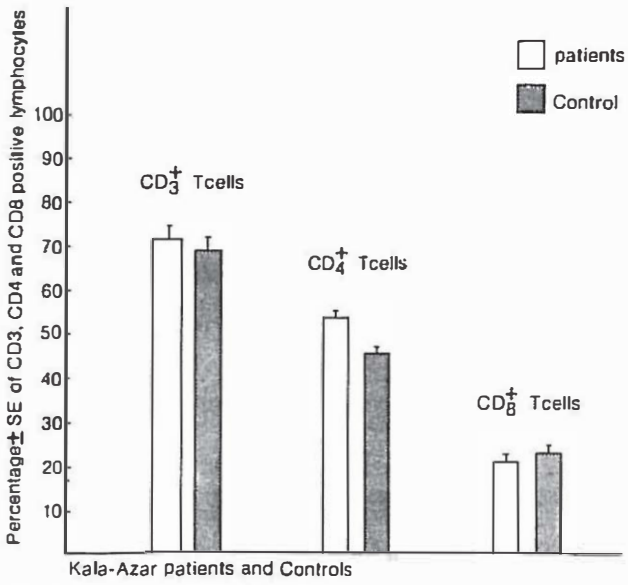

Fig. 1. Percentage ± SE of lymphocytes reactive with OKT_3, OKT_4 and OKT_8 monoclonal antibodies in patients and controls.

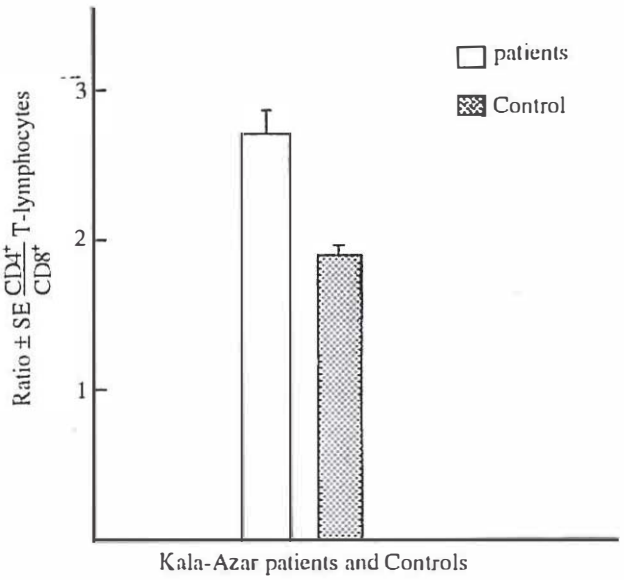

Fig. 2. Ratios ± SE of $\frac{CD4^+}{CD8^+}$ T-lymphocytes in patients with Kala-Azar and controls.

was observed (20.81 ± 0.71 vs 23.89 ± 0.72). The ratio of $CD4^+/CD8^+$ T-lymphocytes were significantly increased at $P<0.005$ as the value of this ratio was 2.70 ± 0.17 for the kala-azar patients and 1.91 ± 0.05 for the controls (Fig. 2).

All the patients studied demonstrated a negative leishmanin test.

DISCUSSION

Visceral leishmaniasis is prevalent in Iran; the causative agent is called *L. infantum* as the majority of cases are children in the pediatric age group.

Studies in man and experimental animals suggest that cellular immunity is of primary importance in limiting pathogenesis of visceral leishmaniasis.[8,9,10] Suppression of cell-mediated immunity occurs through increase in $CD8^+$ T-cell or changes in cytokine production. Cytokines are capable of activating macrophages which can eliminate the intracellular organisms including Leishmania amastigotes.

Our findings demonstrated an increase in the percentage of $CD4^+$ lymphocytes and a decrease in the percentage of $CD8^+$ T-lymphocytes. These results are in contrast with the findings of Sciotto et al.[5] who reported reduction in the level of $CD4^+$ T-cell with an increase in the level of $CD8^+$ T-cells during active phase of disease in six children with Mediterranean visceral leishmaniasis. In cases of visceral leishmaniasis from Kenya, Koech et al.[6] also reported a higher proportion of $CD8^+$ T-cells in patients than in the controls, although the total numberof T-cell in patients and the controls were similar.

The lack of T-cell response *in vitro* to specific and non-specific antigens and low levels of IL-2 and γ-interferon in acute phase of visceral leishmaniasis which has been demonstrated by other investigators indicate the absence of positive delayed-type hypersensitivity to leishmanin in these patients.[5,11] An analysis of *in vitro* T-cell response in Indian kala-azar[4] confirms the present findings as these investigators demonstrated that even depletion of $CD8^+$ T-cells did not reverse the specific unresponsiveness in these patients. The present studies indicate that immunosuppression in these patients could be possibly attributed to the lack of proper T-cell functions rather than merely changes in the phenotypic characteristic of these cells.

Although visceral leishmanisasis is also associated with hyperganmaglobulinemia and decreased complement components, it has been demonstrated that the serum components and the adherent cells do not contribute significantly to cell-mediated suppression in this disease.[2,12]

For further understanding of the nature of immune suppression in kala-azar, the functional disturbances of T-cells cannot be ignored during acute phase of infection. More studies on the level of various cytokines, particularly the changes in the level of IL4 and as a result, increase in the synthesis of IgE and selective enhancement of some of the IgG isotypes in indicated duringactive phaseof infection as these patients have a very low level of γ-interferons.[13]

As peripheral blood lymphocytes present only 2% of the total lymphocyte pool in the normal adult human body,[14] it will be interesting to study the lymphocyte suhsets in the organs mainly affected such as spleen, liver and bone marrow of kala-azar patients.

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