



Some diagnostic aspects, using CD markers, in young Iraqi children with visceral leishmaniasis

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Abstract

The lymphocytic phenotypes involved in the pathogenesis of visceral leishmaniasis (VL) in Iraqi children have recently been investigated, in a study based on cluster-differentiation (CD) markers. Each case of VL investigated was confirmed serologically by the immunochromatography with rk – 39 antigen strip test (dipstick assay). A significant decrease was found in number of CD3 and CD4 cells in VL patients compared to the healthy control group, while the results showed that there was a significant increase in CD8 lymphocytes with VL patients in comparison to the control group. The CD4/CD8 ratio was lower in VL patients than that of the control group. There was a relative increase in CD22 of VL patients compared to the control group, but was not significant. There was no significant difference in all types of CD cells percentage of VL in relation to some clinical parameters such as gender and age. Follow up of the patients showed there was a significant increase in the percentage of CD3, CD4, and CD4/CD8 ratio at the end of therapy. A relative decrease in CD22 but still more than control group.

Key words : visceral leishmaniasis and CD – markers .

Introduction

Leishmania donovani occurs primarily in young children in North and East Africa, Asia, the Mediterranean area and South America. In India and Bangladesh, the disease is found primarily in adult (1). Cluster – of – differentiation (CD) markers are glycoprotein expressed by T- and B- lymphocytes and Natural – killer cells. Most T-helper cells express CD4, most T-cytotoxic cells express CD8 (2) and B-cells, CD22 (3). Counts of peripheral – blood lymphocytes expressing CD4 and CD8 (i . e . CD4+ and CD8+ cells) are used in the immunological follow up of diseases that cause immune dysfunction and patients who have had bone-marrow transplant. The CD4+ / CD8+ ratio relates to the immunological balance between T-helper cells and T-cytotoxic cells in the immune system ; the higher ratio is, the lower the cytotoxic activity and higher the other forms of cell-mediated immunity and humoral immunity. There have been several studies in which the CD4+ and CD8+ cells in the peripheral blood of patients with visceral leishmaniasis (VL) have been counted or the serum concentration of soluble CD4 and CD8 in such patients have been determined. For example; (4) found that, compared with healthy controls, untreated VL cases had significantly more soluble CD4 and soluble CD8 in their sera. Again compared with the corresponding values for apparently healthy controls. It was reported that CD4+ cell counts were depressed in both acute and chronic VL whereas CD8+ cell counts were only depressed in chronic VL (being within the normal range in acute cases) (5). In the later study, the counts of CD4 cells in the VL cases (but not those of CD8+ cells) showed some recovery following treatment. The main of the present study was to explore the subtypes of lymphocytes that appear to be involved in the pathogenesis of VL in young Iraqi children, both before and after treatment with sodium stibogluconate.

Patients and Method

Subject Selection

Between July and December 2013, 5 ml blood samples were collected, into heparinized tubes, from children aged < 6 years as they attend the children's hospitals in AL-Furat Al-Awsat district of Iraq. All samples were collected with permission of the Iraqi Ministry of



Health's center for the control of communicable disease and the informed consent of the parents / guardians of each children investigated . As controls, samples were collected from 10 apparently healthy children who had never lived in an area know to be endemic for VL . Another 60 samples came, pretreatment, from children with splenomegaly and VL that had been serologically confirmed (in a dipstick assay) . Further blood samples were collected from 50 of these confirmed cases of the confirmed VL, at the end of 28 days of treatment (6) with sodium stibogluconate (PentostamTM ; Glaxo Smithkline Brentford, U.K.) at 20 mg/kg . day .

Lymphocyte Separation and Analysis

The lymphocytes in each blood sample were separated using the Isopaque-Ficol centerifugation method (7) and a comercial lymphocyte-separation medium (Mediatech Manassas, VA) . The final cell pellet was resuspended in 1 ml 0.1 M phosphate-buffered saline at PH 7.2 (PBS) and then the cells in a 0.2 ml subsample of this suspension were stained with Trypan Blue so that the viable cells could be counted in a haemocytometer . The cell count allowed the remaining cell suspension to be diluted with more PBS to give first 2×10^6 viable lymphocytes / ml for use in IFAT .

IFAT Method

IFAT test, a 45 μ l sample of a lymphocyte suspension containing 2×10^6 cells / ml was transferred to a tube, mixed well with 5 μ l monoclonal, mouse antihuman CD3 CD4 CD8 and CD22 (Beckman Coulter, Paris) and refrigerated, at 2-8 °c, for 30 min . The lymphocytes in the suspension were then washed twice in PBS, resuspended in 1ml PBS containing 1% (W/V) bovine serum albumin (PBS-BSA), mixed with 50 μ l of a 1:80 dilution of fluorescein-conjugated, goat antimouse-IgG (Sanofi Diagnostic Pasteur, Marnes La-Coquette, France) in PBS-BSA, and refrigerated for another 30 min at 2-8 °c, in the dark . The lymphocytes were washed twice in PBS before being finally resuspended in 200 μ l PBS-BSA . A drop of this final suspension was placed on a microscope slide, covered with a coverslip, and examined, at $\times 400$, under a fluorescence microscope . For each such sample, 200 cell were scored positive (showing apple-green fluorescence) or negative for the CD marker being investigated .

Results

Total (CD3+) T- lymphocytes

The percentage of cells scored CD3+ in the IFAT was significantly lower for the untreated cases of VL than for the controls, with means of 48.0% and 72.5% respectively ($P < 0.05$; Table 1) . A mean of 65.3% of lymphocytes collected from the treated cases of VL were CD3+, indicating that treatment had led to a significant increase in this variable ($P < 0.05$), but this value was still significantly lower than the control value ($P < 0.05$; Table 1) .

Helper / inducer (CD4+) T-lymphocytes

The percentage of lymphocytes scored CD4+ was also significantly lower for the untreated cases of VL than for the controls, with mean of 48.2% and 54.6% respectively ($P < 0.05$; Table 1) . As with the CD3+, there was also evidence of partial, post-treatment recovery in the percentage of lymphocytes scored CD4+, with a mean value (52.2%) that was significantly higher than recorded in the untreated cases but still significantly lower than the corresponding control value ($P < 0.05$ for each; Table 1) .

Cytotoxic / Suppressor (CD8+) T-lymphocytes

The percentage of lymphocytes scored CD8+ was significantly higher for the untreated cases of VL than for the controls, with means of 63.0% and 42.3% respectively ($P < 0.05$; Table 1) . Again the mean value for the treated cases of VL was intermediate (50.2%), this



time being significantly higher than the control value and significantly lower than the value for the untreated cases ($P < 0.05$ for each; Table 1).

CD4+ / CD8+ Ratio

The mean CD4+ / CD8+ ratio was lowest in the untreated VL cases (0.76), highest in the controls (1.29), and intermediate (1.03) in the treated VL cases (Table 1).

B – lymphocytes (CD22+)

Although the percentage of lymphocytes scored CD22+ was significantly higher for the untreated cases of VL than for the controls, with means of 15.6% and 12.0 % respectively, this difference was not statistically significant (Table 1). The corresponding value for the treated cases of VL (12.0%) was again intermediate but not significantly different from the values for the controls of untreated cases.

Effects of Patient Age and Gender

Among the untreated cases of VL, neither age nor gender appeared to have significant effect on the percentages of lymphocytes scored CD3+, CD4+, CD8+, or CD22+ (Table 2).

TABLE 1. The results of the IFAT-based phenotyping of the lymphocytes from the healthy controls and cases of visceral leishmaniasis.

Mean percentage, and (S.E) of lymphocytes scored :						
Group	No. of subjects	CD3+	CD4+	CD8+	CD22+	CD4+/CD8+ ratio
Controls	10	72.5(4.2)	54.6(2.1)	42.3(0.4)	12.0(2.4)	1.29
Untreated cases	60	48.0(1.6)	48.2(3.5)	63.0(2.0)	15.6(0.5)	0.76
Treated cases	50	65.3(2.4)	52.2(0.2)	50.2(0.2)	12.0(1.3)	1.03

TABLE 2. The results of the IFAT-based phenotyping of the lymphocytes from the 60 untreated cases of visceral leishmaniasis, split by case gender and age.

Mean percentage, and (S.E) of lymphocytes from :			P	Mean percentage, and (S.E) of lymphocytes from subjects aged :			P
Markers	Boys	Girls		< 1 year	1 year	> 1 year	
CD3+	47.6(2.4)	47.8(1.6)	>0.05	46.3(2.3)	45.2(3.1)	44.2(2.5)	>0.05
CD4+	53.7(2.3)	51.3(2.1)	>0.05	52.4(2.5)	56.2(1.5)	57.0(1.8)	>0.05
CD8+	61.3(0.6)	62.6(3.6)	>0.05	63.5(1.8)	60.7(0.8)	60.5(3.5)	>0.05
CD22+	11.4(0.9)	14.0(0.2)	>0.05	13.1(4.2)	13.8(1.3)	14.0(2.5)	>0.05



DISCUSSION

The phenotyping of peripheral blood lymphocytes can give an idea of the immunological status of patients, including those with VL. It is clear that the CD values in IFAT give more positive cells, for each CD marker investigated than immuno-peroxidase staining based on the same primary antibodies (8). The difference may indicate the (lower) loss or blocking of markers during the preparation of lymphocytes for IFAT, and is quicker and easier to perform than immunoperoxidase staining and can give results that appear at least as good, although neither method allows CD4⁺ monocytes or CD8⁺ natural-killer cells to be distinguished from T-lymphocytes carrying the same markers (9). The present results indicate that T-lymphocytes (i.e CD3⁺ cells) are depressed in children with VL, perhaps explaining why the proliferated responses of Kenyan patients with VL, to both heterogenous and *Leishmania* antigens, were found to be severely suppressed (10). Such suppression may be mediated by macrophages, either by defective antigen processing and presentation or by the elaboration of suppressive mediators such as interleukin 10, tumor growth factor- β and prostaglandin E₂ (11). A VL – associated, significant increase in the percentage of lymphocytes expressing CD8⁺ was observed in the present study and by (12). However, (5) reported that counts of CD8⁺ cell in the peripheral blood were normal in acute cases of VL and uniformly low in chronic cases. A role of CD8⁺ T-cells in protection against *Leishmania* infection was suggested by (13), who reported that such cells were responsible for the conversion of BALB/c mice that were susceptible to *L.major* infection into a resistant phenotype (after depletion of the rodents' CD4⁺ T-cells). This observation was made despite the fact that CD8⁺ T-cells also produce interferon – γ on activation and can directly destroy macrophages harbouring leishmanial amastigotes (14). In the present study, a relatively low percentage of lymphocytes from untreated VL cases was found to be expressing CD4. Also (5) found CD4⁺ cells to be relatively rare among the lymphocytes of both acute and chronic cases of VL. In VL, CD4⁺ cells may be lost by apoptosis, since such cells from *Leishmania* – infected, susceptible mice undergo rapid apoptosis, produce relatively little interleukin-2 and interferon – γ , and fail to mediate delayed – type hypersensitivity reactions (15). The relatively low CD4⁺ / CD8⁺ ratio seen in the children with untreated VL is an indicator of immune suppression in such patients. Also (15) reported low CD4⁺ / CD8⁺ ratios in VL cases (at the time their disease was diagnosed), caused by both a suppression of CD4⁺ cells and an elevation of CD8⁺ cells. Although in the present study, the percentage of lymphocytes identified as B-lymphocytes (because they expressed CD22) appeared slightly elevated in the untreated cases, the difference from the controls values was not statistically significant. Increase in the number of B-cells can result from the polyclonal activation of such cells (16). It was suggested by (17) that B-cells can inhibit natural resistance against *L.donovani* infection in mice, with B-cell – deficient mice clearing parasites more rapidly from their livers and never developing splenic infection. Encouraging in the present study, a standard course of treatment with sodium stibogluconate resulted in some normalization of all the parameters measured. It seems likely that this normalization continues after the course of treatment is complete, since the CD4⁺ / CD8⁺ ratios recorded by (16), 3 months after the post – treatment recovery of VL cases, appeared completely normal. Also (18) suggested that B-lymphocyte counts made during and after chemotherapy may be useful in monitoring the clinical improvement of VL cases. In the present study, however, there was no significant change in the percentage of lymphocytes identified as B-cells either as a result of VL or of the treatment of the VL.

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