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Research brief

Leishmania donovani: Effect of therapy on expression of CD2 antigen and secretion of macrophage migration inhibition factor by T-cells in patients with visceral leishmaniasis

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Abstract

Visceral leishmaniasis (VL) commonly known as Kala-azar in India is one of the several clinically important infections, where Th1 sub-population of $CD4^+$ T-cells, despite a pre-requisite, fails to express macrophage migration inhibition factor (MIF) and interferon-γ which both activate the macrophage and coordinate the immune response to intra-cellular *Leishmania* sp. Expression of CD2 receptors before and after antileishmanial therapy on $CD4^+$ T-cells of VL patients and their corresponding effect on MIF were examined. Before treatment the number of T-cells expressing CD2 was low which incorporated insignificant MIF response. The immunological reconstitution was, however, observed after treatment as manifested through upregulation of $CD2^+$ T-cells with pronounced MIF generation response. The study, therefore, identifies a possible role of CD2 antigen in immunity to VL.

Keywords: Visceral leishmaniasis; CD2; Macrophage migration inhibition factor

The initial encounter of antigen specific T-cell receptors and CD4 co-receptors on the surface of *Leishmania* infected macrophages is important for activation of Th1 cells for clonal expansion due to IL2 synthesis in the presence of co-stimulation provided by macrophages (Rudd, 1996). The crucial step to initiate these events appears to be the transient binding of T-cells to the antigen-presenting cell. Cell surface adhesion molecules of the immunoglobulin super-family—lymphocyte function associated antigen-1 (LFA-1), CD2, and intracellular activation motif (ICAM-3)—can mediate T-cell adhesive interactions to enable an immune response to occur (Dustin and Springer, 1989; Hogg and Landis, 1993). Many reports suggested the role of CD2 in up-regulation of protein kinase C (PKC) mediated phosphorylation in CD4 cells, which results in increased IL2 production and its receptor expression (Cantrell et al., 1987; Droge, 1986). The present study was undertaken to explore the role of CD2 antigen co-receptors in T-cell non-responsiveness to *L. donovani* antigen in visceral leishmaniasis (VL) patients.

The experiment was designed to evaluate the level of the $CD2^+$ T-cell sub-population and its effect on the percentage release of microphage migration inhibition factor (MIF) from T-cell of active VL patients and VL patients cured after "successful chemotherapy," i.e., those with regressed spleen, disappearance of fever, and a negative parasitological status as determined by microscopical examination of splenic aspirate smears for *Leishmania* parasites. Thirty subjects of both sexes and from all age groups were studied, of which 10 represented a target

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group of active VL cases where the effect of antihuman CD2 mAb in modulation of T-cell response for the release of MIF was monitored. In addition, 10 clinically cured VL patients' cases were included as comparable group together with 10 healthy controls. All the active VL patients presented characteristic signs and symptoms of the disease and the diagnosis was confirmed by the presence of *L. donovani* in Giemsa stained bone marrow aspirate and positive serology (direct agglutination test, DAT). At the time of this study, the duration of illness ranged from 1 to 6 months and patients were untreated. Cured cases had been treated with one or more courses of pentavalent antimony and were without hepatosplenomegaly and fever after completion of more than one course of sodium antimony gluconate (SAG) treatment. Each of the control subjects had no apparent history of VL and they did not reside in VL endemic areas.

Peripheral blood mononuclear cells (PBMNC) from patients and controls were isolated by density gradient centrifugation over Ficoll–Paque (Amersham Bio-sciences, Sweden). For CD2 cell counts, freshly isolated PBMNC were washed in phosphate-buffered saline (PBS) and 1×10^7 ml^{-1} cells suspended in PBS containing 56 °C heat inactivated 2% fetal calf serum (FCS) and 0.09% sodium azide (Sigma). Cells were double stained with fluorescein isothiocyanate (FITC) conjugated mouse antihuman CD2 antibody (Clone: RPA-2.10 Becton Dickinson, USA) and PE conjugated CD3 antibody (Clone: UCHTI, Becton Dickinson, USA) for 30 min at room temperature according to manufacturer's instructions. Cells were washed twice with wash buffer (PBS containing 2% FCS, pH 7.2). Then, each sample was resuspended in 450 μl stain buffer (buffered lysing solution containing less than 50% diethylene glycol and less than 15% formaldehyde, Becton Dickinson, San Diego, USA) for examination of fluorescence in a FACS-Calibur Flow-Cytometer (Becton Dickinson, San Diego, USA.). Flow data on T-cells ($CD3^+$) positive for CD2 were evaluated on Cell Quest software. Negative control samples were incubated with irrelevant isotype matched antibodies (FITC and PE labeled IgG) in parallel with all experimental samples.

Subsequent experiments were performed to evaluate the reactivity of T-lymphocytes for the release of MIF after in vitro stimulation with anti CD2 antibody. Cells were cultured in RPMI-1640 medium (Hi-media, India) supplemented with 2 mM L-glutamine (Hi-media, India), 10% fetal calf serum (Life Technologies, Gaithersburg, USA), 5×10^{-2} mM of 2-mercaptoethanol (Sigma) and 50μg/ml gentamycin, 100 U/ml penicillin, 100 g/ml streptomycin, and then adjusted to pH 7.4 with 2 N HCl. Cells (5×10^6/ml^{-1}) pulsed with *L. donovani* promastigotes in responder (PBMNC) to stimulator (*L. donovani* parasite) ratio of 100:1 for 2 h at 37 °C were subsequently cultured in 96-well round bottomed plates pre-coated with anti-CD2 (LFA-2) antibodies (4 μg/ml).

Control cultures were set up in medium alone or medium containing phytohaem agglutinin (PHA) 20 μg/ml for 48 h. All cultures were maintained at 37 °C in a water saturated air atmosphere containing 5% CO_2. MIF was measured in all subjects as an index of cell mediated immunity (Clausen, 1971). In brief, wells were cut in a preparation of agarose in a petri dish (15×90 mm) for a reaction of cultured lymphocyte cell suspension in the presence and absence of mouse antihuman CD2 antibody. After 24 h incubation at 5% CO_2 in air at 37 °C, further incubation of MIF plates was stopped and the T-cell reactivity was assessed by measuring MIF, a glycoprotein lymphokine in percentage value, after fixation and staining in 0.37% formaldehyde, and gentian violet suspension. The diameter of the migration areas was measured by ocular meter and the percentage of migration inhibition was calculated as follows:

$$\text{Migration inhibition} = 1 - \frac{\text{mean migration diameter of cells in the presence of antigen}}{\text{mean migration diameter of cells in the absence of antigen}},$$

$$\text{Percentage migration inhibition} = \text{Migration inhibition} \times 100.$$

There was an absence of *Leishmania* specific response in T-lymphocytes in the peripheral blood during active VL (Sacks et al., 1987). To investigate the cause of in vitro T-cell non-responsiveness, the distribution pattern of CD2 antigen co-receptors was evaluated and correlated to activities of MIF generation by T-cells in active and cured VL patients and healthy controls. The number of T-cells ($CD3^+$) expressing CD2 antigen was low and it was accompanied by an insignificant secretion

Table 1
CD2 association during visceral leishmaniasis infection

Category	$CD2^+$ T-cells (μl)	MIF (%)		
		Unstimulated	CD2 stimulated	PHA stimulated
Untreated VL ($n = 10$)	654 ± 175.87	16.88 ± 4.56	37.62 ± 3.26	37.31 ± 6.89
Cured VL ($n = 10$)	1211 ± 206	40.43 ± 15	44.43 ± 15.8	40.96 ± 14.81
Healthy control ($n = 10$)	1877 ± 823.58	25.57 ± 3.36	33.63 ± 6.51	35.63 ± 6.80

Table showing low level of $CD2^+$ T-cells during active VL infection associated with poor T-helper cell response for MIF release, which increased when MNC were stimulated with anti-CD2 antibodies.

of MIF by T-cells in the active stage of the disease (Table 1). After successful chemotherapy, the number of T-cell expressing CD2 antigen increased progressively by about twofold with corresponding increase in MIF secretion by these cells. It was also apparent that low number of cells expressed CD2 antigen, so they were unable to trigger activation events in T-lymphocyte for active cytokine response to *L. donovani* antigen. Previously, Ghosh et al. (1996) reported a reduction in CD2 sub-population of T-cells in Indian patients. As a result, T-cells were not stimulated and MIF response was very low. Earlier studies have also demonstrated that stimulation via CD2 facilitated signal transduction pathways (Gouvello et al., 1998; Monostori et al., 1990), which served to augment IL12 responsiveness of activated T-cells (Gollob et al., 1996) and reversed T-cell anergy (Boussiotis et al., 1994).

However, the number of T-cells positive for CD2 started increasing after treatment, so the CD2 antigens were able to stimulate T-cells. As a result, MIF level increased. The expression or disappearance of CD2 antigen on the T-cells in immune mediated disorders such as VL can therefore be determined to monitor the response of patients to therapeutic agents.

To further determine the role of CD2 antigen, we examined the production of MIF by exposing the T-cells of patients in vitro to agnostic anti-CD2 mAb. We preferred MIF on account of its simplicity, ease of use in laboratories with limited facilities and its relative sensitivity when compared to other cytokine based detection assays such as interferon-γ (IFN-γ) evaluation, which are generally less affordable in remote rural areas where modern methods cannot be used. It was observed that patients with active infection potentially generated more MIF, which was secreted about twofold less in absence of anti-CD2 exposure. It was also observed that cells of active cases responded as well to anti-CD2 antibody as did cells from cured cases.

CD2 antigen has shown to be important in initiating phosphorylation in proteins of CD4 molecules. In these studies, the effect of CD2 antigen is obtained through regulation of diacylglycerol (DAG), which seems to be the intracellular activator of PKC (Pantaleo et al., 1987). It is appreciated that stimulation of PKC through CD2 antigen would lead to production of Th1 cytokines, which are important for resistance to intracellular pathogens. However, stimulation through CD2 can also affect expression of CD28 which can further enhance T-cell response. In addition, the CD2 pathway can also lead to direct stimulation of T-cells and may have a role in the generation of effector cells. This study identifies a crucial role for the CD2 antigen in immune responses to *Leishmania*.

References

Boussiotis, V.A., Freeman, G.J., Griffin, J.D., Gray, G.S., Gribben, J.G., Nadler, L.M., 1994. CD2 is involved in maintenance and reversal of human alloantigen-specific clonal anergy. J. Exp. Med. 180, 1665–1673.

Cantrell, D.A., Davice, A.A., Londei, M., Feldman, M., Feldman, M.J., Crumpton, M.J., 1987. Association of phosphoregulation of T_3 antigen with immune activation of T-lymphocytes. Nature 325, 540–542.

Clausen, J.E., 1971. Tuberculine induced migration inhibition of human peripheral leukocytes in agarose medium. Acta Allergol. 26, 56–80.

Droge, W., 1986. Protein kinase C in T-cell regulation. Immunol. Today 11, 341.

Dustin, M.L., Springer, T.A., 1989. T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. Nature 341, 619–624.

Ghosh, M.K., Ghosh, A.K., Addy, M., et al., 1996. Subpopulations of T lymphocytes in the peripheral blood and lymph nodes of Indian Kala-azar patients. Med. Microbiol. Immunol. 185, 183–187.

Gollob, J.A., Li, J., Kawasaki, H., Daley, J.F., Groves, C., Reinherz, E.L., Ritz, J., 1996. Molecular interaction between CD58 and CD2 counter-receptor mediates the ability of monocytes to augment T cell activation by IL-12. J. Immunol. 157, 1886–1893.

Gouvello, S.L., Manceau, V., Sobel, A., 1998. Serine 16 of stathmin as a cytosolic target for Ca_{2+}/calmodulin dependent kinase II after CD2 triggering of human T lymphocytes. J. Immunol. 161, 1113–1122.

Hogg, N., Landis, R.C., 1993. Adhesion molecules in cell interactions. Curr. Opin. Immunol. 5, 383–390.

Monostori, E., Desai, D., Brown, M.H., Cantrell, D.A., Crumpton, M.J., 1990. Activation of human T-lymphocytes via the CD2 antigen results in tyrosine phosphorylation of T-cell antigen receptor zeta-chains. J. Immunol. 144.3, 1010–1014.

Pantaleo, G.D., Oliv, D., Poggi, A., Kozumbo, W.J., Moretta, L., Moretta, A., 1987. Transmembrane signaling via the T11-dependent pathway of human T cell activation. Evidence for the involvement of 1,2-diacylglycerol and inositol phosphates. Eur. J. Immunol. 17, 55–60.

Rudd, C.E., 1996. Upstream downstream: CD28 co-signaling pathways and T cell function. Immunity 4, 527–534.

Sacks, D.L., Lal, S., Shrivastava, S., Blackwell, J., Neva, F.A., 1987. An analysis of T-cell responsiveness in Indian Kala-azar. J. Immunol. 138.3, 908–913.