International Journal for Parasitology 41 (2011) 463–469

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Activity and turnover of eosinophil and neutrophil granulocytes are altered in visceral leishmaniasis

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ARTICLE INFO

Article history:
Received 26 October 2010
Received in revised form 24 November 2010
Accepted 25 November 2010
Available online 21 December 2010

Keywords:
Eosinophil cationic protein
Myeloperoxidase
Parasite
Leishmania donovani
Eosinophil
Neutrophil

ABSTRACT

Visceral leishmaniasis (VL) is a health issue in Sudan. Our aim was to investigate the involvement of eosinophils and neutrophils in VL by serum and plasma measurements of eosinophil cationic protein (ECP) and myeloperoxidase (MPO) and some key cytokines and chemokines. Blood was collected from 125 VL patients and 181 healthy Sudanese controls from the same rural area. Results showed reduced eosinophil and neutrophil counts in the VL group ($P = 0.0001$and $P = 0.002$, respectively). Serum-ECP levels were higher in the controls ($P < 0.0001$), while plasma MPO levels were higher in the VL group ($P < 0.0001$). Levels of IL-5, granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-17 were increased among the VL group ($P < 0.0001$, $P = 0.017$ and $P = 0.03$, respectively), whereas eotaxin and IL-8 levels were reduced ($P < 0.0001$ and $P = 0.002$, respectively). Positive correlations were found between IL-8 and ECP/MPO ($P < 0.0001$). We conclude that eosinophil and neutrophil turnover and activity are increased in subjects in rural areas of Sudan. In VL the turnover was further increased, but the relatively low secretory activity of eosinophils and neutrophils in VL may relate to the reduced production and availability of the chemokines eotaxin and IL-8. The combined assay of ECP and MPO in serum and plasma provides further insight into the mechanisms of eosinophil and neutrophil involvement in disease and constitutes a novel approach to the study of disease processes.

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1. Introduction

Visceral leishmaniasis (VL) is a disease of public health importance in Sudan and many other tropical countries, and is considered to be one of the main health foci of these countries (Desjeux, 1996). VL is a disease caused by protozoa of the *Leishmania donovani* complex (*L. donovani*, *Leishmania archibaldi*, and *Leishmania infantum/chagasi*). *Leishmania*, which are introduced into their human hosts by sand flies, rapidly invade macrophages, where they multiply inside phagolysosomes. Human infections may be asymptomatic (sub-clinical) or may cause a severe visceral disease that is called kala-azar (KA) (Southgate and Manson-Bahr, 1967; Badaro et al., 1986a,b; Zijlstra et al., 1994). The clinical manifestations of VL include recurrent fever, hepatosplenomegaly, general lymphadenopathy, pancytopenia, and anaemia. Death occurs in the absence of appropriate chemotherapy (Pearson and Sousa, 1996).

Leishmania parasites exhibit an absolute dependency on macrophages (Seaman et al., 1996), within which they multiply. Macrophage activation with ensuing intracellular parasite killing is the host's primary defence against *Leishmania* infection. With *Leishmania* being recognised as an obligate intracellular parasite of macrophages, studies have demonstrated that specific immunity to VL is mediated by $CD4^+$ T-helper cells and that disease susceptibility is associated with the inability to produce a macrophage-stimulating T helper type 1 (Th1) cytokine profile including IFN-γ, IL-2 and IL-12. Conversely an elevated production of immunosuppressive cytokines such as IL-10 and IL-4 (Th2 profile) and high levels of TNF-α, may be associated with susceptibility (Cotterell et al., 2000; Zijlstra et al., 2003).

Many laboratory studies on VL patients revealed the presence of pancytopenia, elevated gamma globulins and low eosinophil counts during active disease (Beil et al., 1992; Pearson and Sousa, 1996) and some authors associate the occurrence of pancytopenia

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doi:10.1016/j.ijpara.2010.11.005

in VL with the presence of membrane-associated anti-platelet, anti-neutrophil and anti-erythrocyte IgG antibodies (Pollack et al., 1988). Others documented that VL is characterised by suppression of cell-mediated immunity, as shown by unresponsiveness to the *Leishmania* skin test (LST) or Montenegro test (Manson-Bahr, 1961).

Studies on the involvement of inflammatory cells such as neutrophil and eosinophil granulocytes in the maintenance of the Th1/Th2 balance through the secretion of different cytokines and granule proteins help to understand the nature of the cellular functions and immunological balance in VL. The roles of eosinophils and neutrophils during *Leishmania* infection in humans, however, have not been well addressed. These cells exert anti-*Leishmania* activity through phagocytosis and parasite killing, using mechanisms such as those related to oxidative burst (Chang, 1981; Pearson and Steigbigel, 1981; Pearson et al., 1987; Oliveira et al., 1998), but possibly also by the action of microbicidal proteins. The recent studies by Peters and colleagues (2008) emphasise the pivotal role of neutrophils and support the "Trojan horse" hypothesis. For eosinophils, one study showed that eosinophils represent up to 15% of the cellular infiltrate at the site of *Leishmania major* infection in mice resistant to clinical disease (C57BL/6) and was not identified in tissues of infected disease-susceptible BALB/c mice (Beil et al., 1992).

Eosinophil cationic protein (ECP) is one of the major secretory proteins of human eosinophils with potent cytotoxic activities towards parasites, bacteria and mammalian cells. ECP may be measured in serum and/or plasma as a reflection of the involvement of eosinophils in the disease process (Venge et al., 1999). Likewise myeloperoxidase (MPO) may be measured as a sign of neutrophil involvement (Venge, 2004). These proteins may, as mentioned, be measured in both serum and EDTA-plasma. The levels in plasma reflect the prevailing in vivo levels, since EDTA effectively prevents any further activity of the cells with secretion into the plasma. The levels in serum, in addition to in vivo levels, reflect the secretory activity of the cells in vitro, since the cells will continue secretion of their granule proteins until they are separated from the serum by centrifugation (Bjork et al., 2000). The levels in plasma are therefore subject to turnover of the proteins in vivo in terms of production and elimination, whereas the serum levels are less affected by elimination, but are mainly affected by production i.e. secretion. We have taken advantage of these differences in this study and measured ECP and MPO in both EDTA-plasma and serum, with the hypothesis that plasma levels mainly reflect cell turnover and serum levels mainly reflect secretory activity. Thus, the major aim of this study was to investigate the involvement of eosinophils and neutrophils as reflected by the serum/plasma levels of the two marker proteins in subjects with VL compared with non-infected subjects in the same area in Sudan. The findings were compared with serum levels of several cytokines involved in the production and activation of these cells.

2. Materials and methods

2.1. Study area

The study was carried out at Tabarakalla rural hospital in Gedarif state, Sudan. It is situated along the lower Atbara River in Gallabat Province, eastern Sudan. The area is located ~70 km southeast from Gadarif town. It is endemic for *L. donovani*, and the main leishmania vector in the area is *Phlebotomus orientalis* (Hoogstraal and Heyneman, 1969).

2.2. Patient characterisation

A detailed clinical history was obtained from each patient. Particular emphasis was made regarding previous or present symptoms of any form of leishmaniasis. Subjects were questioned about their ethnic and geographic origin and were examined for clinical manifestations of VL. A general clinical examination was conducted with particular reference to hepato-splenomegaly and the position and number of enlarged lymph nodes. Liver size was measured in the mid-clavicular line from the costal margin; spleen size was assessed by measuring the distance between the costal margin in the anterior axillary line to the tip of the spleen. Lymphadenopathy was classified as localised if found only at one site and generalised if at two or more sites. The oral and nasal mucous membranes were examined for evidence of mucosal leishmaniasis. Both thick and thin blood films were examined for malaria parasites for all individuals with fever or splenomegaly.

For the diagnosis an inguinal lymph node aspiration was performed on those clinically suspected of having VL (i.e. all individuals with any of the following clinical findings: fever for >2 months, left upper quadrant pain, lymphadenopathy, splenomegaly or wasting). All individuals with suspected clinical VL but with negative results on inguinal lymph node aspiration were subjected to bone marrow aspiration from the superior posterior iliac crest. Bone marrow smears were fixed with methanol, stained with Giemsa and examined under an oil-immersion lens for *L. donovani* bodies.

Venous blood drawn from leishmania-infected patients and healthy Sudanese was separated by centrifugation and frozen in liquid nitrogen within 2 h of sampling. For the preparation of serum, the whole blood was allowed to coagulate for 1 h at ambient temperature before centrifugation and for the preparation of plasma, whole blood with added EDTA was centrifuged within 30 min after sampling. Serum, plasma and whole blood were stored and transported frozen in liquid nitrogen to Khartoum where samples were stored at −70 °C until air transport before being analysed in Uppsala, Sweden. Material collected in Sweden was frozen and stored at −70 °C. Sera and plasma samples were collected from 125 VL patients with an average age of 25 years (18–30) with 72% females (91/125), and 181 healthy Sudanese controls with a median age of 11 years (7–21) with 51% females (97/181).

2.3. Ethical approval

Ethical approval for this study was obtained from the ethics committee of Ministry of Health, Khartoum, Sudan, the Ministry of Health, Gedarif State, and from the ethics committee of Uppsala University. Informed consent was obtained from all of the adults who participated in the study. For younger children, consent was obtained from their parents.

2.4. Eosinophil and neutrophil counts

For the estimation of blood eosinophil and neutrophil counts the concentrations of eosinophil peroxidase (EPO) and MPO were measured in whole blood extracts employing the procedure described previously (Rundstrom et al., 2007). In brief, 100 µl of whole blood was extracted by the addition of 900 µl of the cationic detergent cetyl-tri-ammonium bromide (CTAB, Sigma Chemicals, Stockholm, Sweden). After 1 h the extract was centrifuged and the supernatant harvested for the assay of EPO and MPO. A standard curve was constructed by measuring the proteins in 10 whole blood extracts with known numbers of eosinophils and neutrophils. The correlation coefficients between the content of EPO and eosinophil counts, and MPO and neutrophil counts in these extracts were linear and ranged between $r = 0.96$ and $r = 0.99$. The intra-assay coefficient of variation (CV) for duplicate samples ranged between 2% and 6%.

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