

α 4 Integrins and Sialyl Lewis x Modulation in Chronic Chagas Disease: Further Evidence of Persistent Immune Activation

S. A. LAUCELLA, A. RIARTE, N. PRADO, J. ZAPATA & E. L. SEGURA

Instituto Nacional de Parasitología 'Dr Mario Fatała Chabén' and Administración Nacional de Laboratorios e Institutos de Salud 'Dr Carlos G. Malbrán', Ministerio de Salud y Acción Social, Buenos Aires, Argentina

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We have previously shown that titers of soluble platelet selectin (s-P-selectin) and soluble vascular cell adhesion molecule-1 (s-VCAM-1) were increased in sera of patients with chronic *Trypanosoma cruzi* infection. In this study, we analyzed the expression of CD49d-integrins, that bind to VCAM-1, and sialyl Lewis x (SLe^x), which binds selectins, in peripheral blood lymphocytes of 27 patients with Chagas' disease at different levels of disease severity. Patients with a mild form of Chagas' disease showed a lower number of CD49d⁺ cells, in comparison with those with severe chronic cardiopathy. Decreased levels of CD49d⁺ cells were detected in CD3⁺ cell populations. Conversely, SLe^x expression was found to be decreased in patients with severe Chagas' disease. Levels of soluble platelet endothelial cell adhesion molecule-1 (s-PECAM-1) were significantly increased in the plasma of patients with severe Chagas' disease while unaltered levels of MCP-1 were recorded. These data show that VCAM-1 and P-Selectin ligands are differentially expressed during the chronic phase of the *Trypanosoma cruzi* infection. These findings also reinforce a role of the P-selectin/SLe^x adhesion pathway rather than very late antigen-4 (VLA-4)/VCAM-1, in the pathogenesis of Chagas' disease.

Dr S. Laucella, Av. Paseo Colón 568 (1063), Buenos Aires, Argentina. E-mail: slaucella@yahoo.com

INTRODUCTION

Chagas' disease, a protozoan infection caused by *Trypanosoma cruzi*, is one of the most important public health problems in Latin America [1].

The most serious long-term pathological feature of the *T. cruzi* infection is a persistent inflammatory cardiomyopathy that may lead to congestive heart failure and death [2]. Different mechanisms have been put forward to participate in the pathogenesis of Chagas' disease including denervation of the sympathetic system [3], microvascular injury [4] and autoimmune mechanisms [5, 6] triggered by the presence of the parasite [7, 8].

Although associated with the protection against *T. cruzi* [9–11], the immune response is likely to be involved in the pathogenesis of Chagas' disease [5, 12]. An effective host immune response to pathogens requires a focal accumulation of leukocytes, but excessive accumulation can lead to inflammatory disease and tissue pathology [13].

The recruitment of leukocytes into sites of inflammation is recognized to involve a cascade of sequential events in which cell adhesion molecules (CAMs), cytokines and chemoattractants work in a highly regulated manner to direct leukocytes from the vascular lumen, across the endothelium, to the inflammatory stimulus. Endothelial CAMs belonging to the selectin family (platelet- and endothelial-selectin) mediate early and reversible events involving leukocyte rolling and margination along the luminal surface of postcapillary venules. Selectins mediate cell–cell contact by binding to carbohydrate-containing molecules, including multivalent forms of fucosylated, sialylated, and sometimes sulfated lactosaminoglycans, such as sialyl Lewis x (SLe^x) and P-selectin glycoprotein ligand-1 (PSGL-1) which also displays SLe^x containing O-glycan [14].

Vascular cell adhesion molecule-1 (VCAM-1) and intracellular cell adhesion molecule-1 (ICAM-1), members of the immunoglobulin (Ig) superfamily, regulate later and irreversible steps, leading to the firm attachment to the vascular endothelium

and subsequent diapedesis of leukocytes [15]. The counter-ligands of VCAM-1 are heterodimeric $\alpha 4$ (CD49d)-bearing integrins [16] with a noncovalent linkage to either a $\beta 1$ or $\beta 7$ chain. Integrin $\alpha 4\beta 1$ (CD49d/CD29 or very late antigen-4, VLA-4) is constitutively expressed in lymphocytes, monocytes, basophils and eosinophils but is absent in neutrophils [16]. Integrin $\alpha 4\beta 7$ (CD49d/ $\beta 7$), recognizes VCAM-1 and is particularly critical in lymphocyte homing because it interacts selectively with mucosal addressin cell adhesion molecule (MAdCAM-1) [16]. Both integrins are receptors for fibronectin, one of the most important extracellular matrix proteins at the inflammatory sites.

Soluble forms of CAMs (s-CAMs) have been reported in the circulation; interestingly, their release from cells is affected by the same stimuli, such as cytokines and other inflammatory mediators, that cause their increased expression on the cell surface [17].

We have previously shown that patients at different stages of *T. cruzi* infection or displaying different clinical forms of Chagas' disease had a characteristic pattern of circulating cell adhesion molecules [18]. Titers of s-VCAM-1 and s-P-selectin were found to be increased in sera of patients with chronic Chagas' disease. Moreover, high levels of s-P-Selectin were associated with disease severity [18].

In the study presented here, we measured the expression of $\alpha 4$ -bearing integrins and SLe^x, -the ligands of VCAM-1 and P-selectin, respectively, in lymphocytes of patients with Chagas' disease at different levels of disease severity. In order to achieve further characterization of the inflammatory process during chronic Chagas' disease, other inflammatory mediators involved in leukocyte recruitment-like soluble platelet-endothelial cell adhesion molecule-1 (s-PECAM-1) [19, 20], and monocyte chemoattractant protein-1 (MCP-1) [21], were also determined and correlated with disease severity. Our data suggest the involvement of differentially expressed cell adhesion molecules in the pathogenesis of chronic chagasic myocarditis.

PATIENTS AND METHODS

Patients. Sera were obtained from individuals aged 23–72 years who lived in *T. cruzi* endemic areas in Argentina. Anti-*T. cruzi* antibodies were assessed in sera by enzyme-linked immunosorbent assay (ELISA) using whole homogenates of the epimastigote form of the parasite as antigen, by indirect haemagglutination, and indirect immunofluorescence assays. An individual was considered infected if at least two of the three tests were positive. Individuals underwent clinical and cardiological examination including at least a chest radiography, electrocardiographic (ECG) studies and echocardiography when necessary. After serological and medical examinations, the subjects were divided into groups with asymptomatic or borderline heart disease (mild disease), patients with myocardial dysfunction (severe disease), and those considered healthy controls (controls). The mild disease group ($n = 16$; mean \pm SD age = 35 ± 9 years) comprises categories 0 and I of the Kuschnir classification [22] Table 1. Patients with mild disease were either asymptomatic or had some electrocardiographic abnormality, but normal cardiothoracic radiological relation (Table 1). The severe Chagas disease group ($n = 11$; 49 ± 13 years) included categories II and III of the Kuschnir classification. Patients with severe Chagas disease had all the

clinical findings present in the group with mild disease, as well as myocardial dysfunction, as measured by clinical or radiological signs of cardiomegaly and/or heart failure (Table 1). Healthy seronegative controls ($n = 10$) (45 ± 8 years) and patients with Chagas' disease showed none of the following conditions: hypertension, vascular, ischaemic, or congenital heart disease, cancer, syphilis, diabetes, arthritis, or allergy. An ethical committee approved the protocol for blood sampling and informed consent was obtained from all the subjects studied.

Monoclonal antibodies (MoAbs). Anti-human CD49d-integrin (the α chain of VLA-4) and anti-human SLe^x labelled with fluorescein isothiocyanate (FITC) were obtained from Pharmingen (San Diego, CA, USA). The anti-CD3-phycoerythrin (PE) was obtained from Dako Corporation (Carpinteria, CA, USA), whereas IgM-FITC, IgG2a-FITC and IgG2a-FITC/IgG2a-PE isotype controls were purchased from Caltag Laboratories (Burlingame, USA).

Isolation and immunofluorescence of peripheral blood mononuclear cells (PBMC). Human PBMC were obtained by gradient centrifugation of heparinized venous blood on lymphocyte separation media. One hundred μ l of RPMI medium containing 1×10^6 cells were incubated with anti-CD49d-FITC or anti-SLe^x for 30 min at room temperature. Staining with non-related IgM or IgG2a showed the non specific fluorescence. For double-labelling studies, MoAbs directed to CD49d or SLe^x molecules were mixed with the MoAb recognizing CD3 cell surface antigen. Cells were fixed with 1% formaldehyde, 1% sodium cacodylate-NaCl for at least 15 min and 10^4 cells per tube were analyzed with a Beckton-Dickinson FACScan flow cytometer. Data were analyzed by using the FACScan program (Becton Dickinson, Mountain View, CA, USA). The percentage of CD49d⁺ or SLe^{x+} cells as well as the mean cell expression of these molecules (defined as the mean fluorescence intensity) were calculated in all patients in all groups.

Determination of s-CAMs and MCP-1 levels in plasma. Peripheral venous blood samples were collected into heparin tubes and centrifuged immediately at $300 \times g$ for 10 min at 4 °C. Plasma aliquots were frozen at -70 °C until needed. Plasma levels of s-PECAM-1 and MCP-1 were measured, in duplicate, by a two-site ELISA according to the instructions of the manufacturer. The s-PECAM-1 ELISA was obtained from R & D System (London, UK), whereas the MCP-1 ELISA was obtained from Endogen (Woburn, USA).

Statistical analysis. Results are expressed as the mean \pm SD. Group comparisons were made by use of an analysis of variance followed by Bonferroni (SAS software) *T*-test to determine differences between groups. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Expression of CD49d integrins and SLe^x in peripheral blood lymphocytes from patients with different clinical status of chronic Chagas' disease

The expression of $\alpha 4$ -bearing integrins and SLe^x in lymphocytes of patients with Chagas' disease were evaluated in order to better understand the adhesion pathways involving VCAM-1 and P-selectin.

The relative number of T cells in the lymphocyte-gated cell population was similar in all groups studied (Table 2).

Patients with mild Chagas' disease showed a lower number of CD49d⁺ cells compared with those with severe chronic cardiopathy ($P < 0.05$) (Table 2). However, the mean CD49d

Table 1. Clinical classification of subjects studied

Characteristics	Group 0	Group 1	Group 2	Group 3	HS
Serology for <i>T. cruzi</i>	positive	positive	positive	positive	negative
Cardiothoracic ratio	0.50	0.50	0.51–0.54	≥ 0.55	0.50
ECG abnormalities					
Intraventricular block	none	frequently	almost always	almost always	none
Atrioventricular block		frequently	almost always	almost always	
Sinus node dysfunction		frequently	almost always	almost always	
Ventricular arrhythmias		almost always	almost always	always	
Atrial arrhythmias				frequently	
Primary T wave changes		frequently	frequently	frequently	
Abnormal Q waves				frequently	
Echocardiography	normal	normal or slightly abnormal	abnormal	abnormal	normal
Radionuclide angiography ejection fraction	0.50	0.40	0.31–0.40	≤ 0.30	0.50

ECG: electrocardiographic; HS: healthy subjects.

cell expression (defined as green fluorescence intensity) was unchanged in the different groups evaluated (data not shown).

Double-labelling studies were performed to analyze whether the decrease in CD49d⁺ cells occurred differentially in T cells. A lower number of CD49d⁺ cells in CD3⁺ cell populations was recorded in patients with mild disease in comparison with patients with severe Chagas' disease ($P < 0.05$) (Table 2).

We also evaluated the expression of SLe^x, the counter-ligand of P-selectin. As shown in Fig. 1, the mean SLe^x cell expression is decreased in patients with severe Chagas' disease (mean \pm SD = 174.82 ± 84.03 log. fluorescence intensity), compared with those with a mild form of the disease (282.01 ± 132.33 ; $P < 0.05$) or uninfected controls (279.20 ± 74.38 ; $P < 0.05$) whereas the number of SLe^{x+} cells was similar among the different groups studied (data not shown).

Levels of s-PECAM-1 and MCP-1 in the plasma of patients with chronic Chagas' disease and healthy controls

To achieve further characterization of the inflammatory process

during chronic Chagas' disease, MCP-1, a potent chemo-attractant for memory T cells, *in vitro*, as well as a known stimuli for IL-4 secretion [21] and s-PECAM-1, a molecule involved in diapedesis [19, 20], were also determined.

s-PECAM-1 levels were significantly increased in the severe Chagas' disease patient group ($P < 0.05$) when compared with patients with a mild form of the disease or uninfected controls (Fig. 2). Conversely, MCP-1 concentration (Fig. 2) was found not to be altered in patients with chronic Chagas' disease.

DISCUSSION

The expression of $\alpha 4$ -bearing integrins and SLe^x in lymphocytes of patients with Chagas' disease were evaluated in order to better understand the adhesion pathways involving VCAM-1 and P-selectin. The present study shows the first evidence that CD49d-bearing integrins and SLe^x are differentially expressed in PBMC of patients with different clinical forms of chronic *T. cruzi* infection.

Diminished CD49d expression in a non-T-cell population

Table 2. Percentage of CD49d⁺ peripheral blood cells in patients with Chagas' disease and uninfected individuals

Group	CD3 ⁺ (mean % \pm SD)	CD3 ⁺ CD49d ⁺ (mean % \pm SD)	CD3 ⁻ CD49d ⁺ (mean % \pm SD)	CD49d ⁺ (mean % \pm SD)
Uninfected	66 \pm 8 (n = 6)	33 \pm 10	22 \pm 8	54 \pm 10 (n = 10)
Mild	66 \pm 6 (n = 9)	24 \pm 11	11 \pm 6*	41 \pm 17* (n = 16)
Severe	62 \pm 8 (n = 9)	29 \pm 11	23 \pm 14	61 \pm 15 (n = 11)

* Significant differences ($P < 0.05$) compared with severe patients.

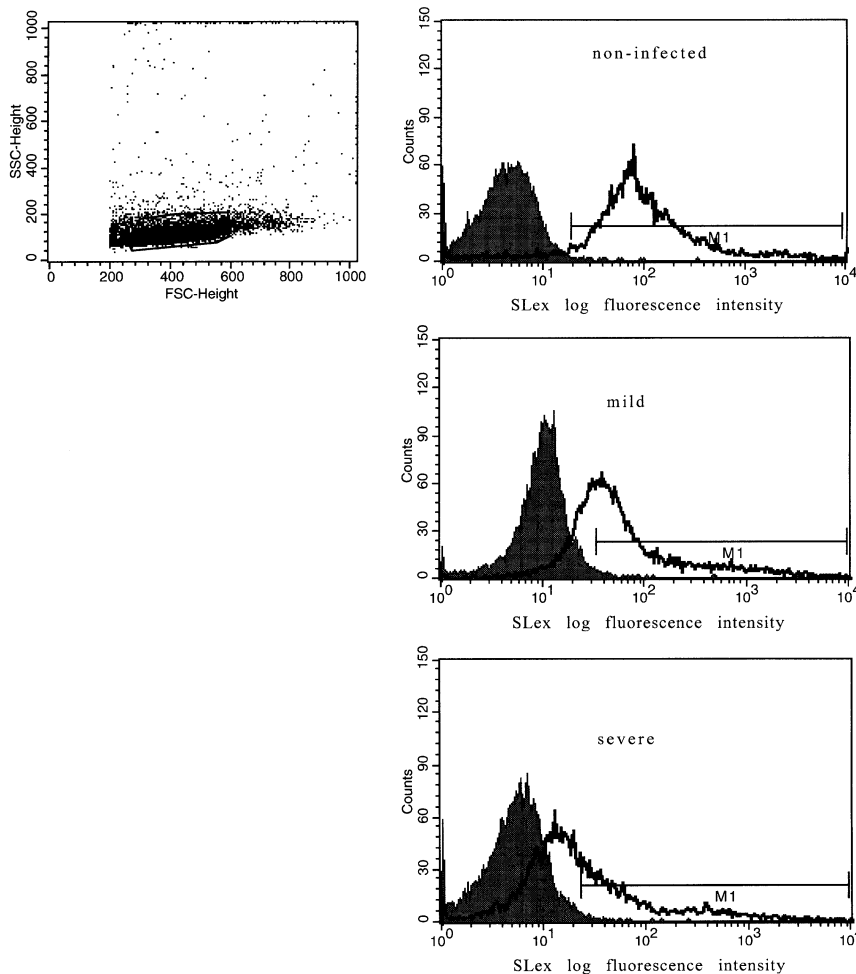


Fig. 1. Expression of SLe^x in peripheral blood lymphocytes from patients with chronic Chagas' disease and uninfected individuals. The top panel shows the selection of the gate for lymphocyte population defined by forward (FSC) and side-scatter parameters (SSC). Cells were stained and analyzed as described under Materials and Methods. Light histograms represent mean SLe^x cell expression in 2 patients with different clinical status of Chagas' disease and an uninfected control whereas dark histograms show staining with a unrelated immunoglobulin (Ig)M antibody. M1 shows the specific fluorescence.

observed in patients with mild chronic Chagas' disease is probably owing to B-cell activation, because a variable decreased in the expression of $\alpha 4\beta 1$ has been shown after activation of human peripheral blood B cells [23]. Increased levels of activated T and B cells have been reported in the circulation of chronic Chagas' disease patients irrespective of the clinical status of the disease [24]. We now demonstrate a higher percentage of activated B lymphocytes in patients with a mild form of the disease compared with those with severe chagasic cardiopathy suggesting a somehow qualitative difference in the immune response between both patient groups.

B-cell activation through the VLA-4/VCAM-1 adhesion pathway might be involved in the immune control of *T. cruzi* infection as the interaction of $\alpha 4\beta 1$ with VCAM-1 reinforces the initial selectin-mediated cell binding to endothelium which is a crucial step in immune and inflammatory responses. In agreement *T. cruzi* infection of endothelial cells was associated with the expression of the endothelial cell adhesion molecules VCAM-1, ELAM-1 and ICAM-1 [25] and a weak expression of VLA-4 has been shown in lymphocytes in inflammatory heart lesions of patients with Chagas' disease [26] or mice chronically infected with *T. cruzi* [27].

Evidence supporting a primary role for the presence of

T. cruzi in the aetiology of Chagas' disease keeps accumulating. As suggested, Chagas' disease is more likely to be the result of persistent parasitization owing to an ineffective immune response [28]. Despite the fact that the immune response is not completely efficient, the majority of *T. cruzi*-infected subjects do not develop severe disease. However, a disruption in the host immune control could trigger an increase in parasite load which in term may upregulate adhesion molecules expression by parasite-induced cytokines and other inflammatory mediators and a detrimental inflammatory process may occur. The notion that the expression of VCAM-1 and P-selectin are elevated in Chagas' disease because of *T. cruzi* infection is supported by the observation that circulating levels of these molecules decreased during specific chemotherapy with benznidazole [29].

The association of increased s-P-selectin levels [18] with a diminished expression of its counter-receptor in the severe form of the disease reinforces a role of P-selectin/SLe^x adhesion pathway in the development of tissue damage. This interpretation is consistent with the reported SLe^x downregulation after the binding to its ligand [30]. However, the association of increased levels of s-P-Selectin with a decreased expression of its counter-ligand in severe Chagas' disease might also reflect impaired in P-Selectin/SLe^x pathway.

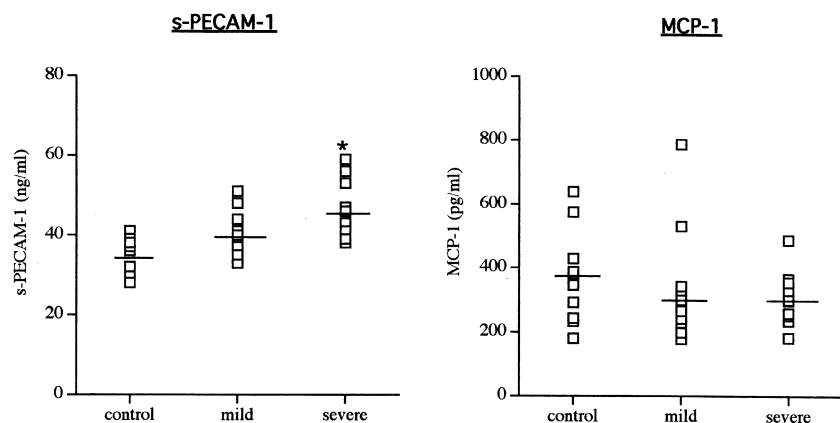


Fig. 2. Levels of circulating cell adhesion molecules and MCP-1 in the plasma of patients with Chagas' disease at different levels of disease severity. The figure shows dot plots for s-PECAM-1 and MCP-1; each square represents one patient. The mean value is shown for each distribution as a horizontal line. Statistical analysis was performed by using an analysis of variance followed by Bonferroni (Dunn) *T*-test. **P* < 0.05 compared with patients with mild Chagas' disease and uninfected individuals.

Interleukin (IL)-4 and tumour necrosis factor (TNF)- α have been shown to provide a mechanism to signal prolonged expression of P-selectin on the endothelial cell surface during chronic inflammation [31]. Likewise, IL-4 and TNF- α are produced during chronic *T. cruzi* infection of mice [32–34] and P-selectin levels were not elevated during the acute phase of Chagas' disease [18].

Functions attributed to PECAM-1 include the initiation of interendothelial cell contacts; the facilitation of leukocyte migration across endothelial cell sheets, the activation of β 1 and β 2-type integrins on leukocytes and the promotion of inflammatory cytokine secretion by monocytes [19, 20]. The presence of a circulating form is suggested to modulate the transendothelial migration of leukocytes [35]. In the present study, levels of s-PECAM-1 were associated with the severity of chronic Chagas' disease showing that the adhesion cascade is not only altered during early steps [18], but also in late events during the transendothelial migration phase of leukocyte recruitment to sites of ongoing inflammation. Moreover, heart and muscle inflammatory lesions from mice during the acute phase of *T. cruzi* infection showed increased expression of PECAM-1 in endothelial cells (S.A. Laucella *et al.* unpublished observations).

CD8⁺ T cells have been shown to be the predominant cell population in inflammatory foci of parasitized tissues [36–38]. Although MCP-1 is a potent T-cell specific chemoattractant [39], a potential role for this chemokine in targeted migratory events during leukocyte recruitment to sites of inflammation in chronic Chagas' disease is not apparent.

Different combinations of adhesion and signalling molecules may be controlling the classes of leukocytes that emigrate into sites of inflammation, and the kinetics of their accumulation during different stages of the disease. Increasing evidence suggests that blockade of the ligand-binding sites between leukocytes and endothelial cells or inhibition of cell adhesion molecules expression diminish haematopoietic cell extravasation and progressive inflammatory events [41–42]. A combination of the specific therapy for *T. cruzi* infection with the inhibition of selected adhesion molecules might become an attractive therapeutic modality for further evaluation.

Collectively, these data support the fact that VCAM-1 and

P-selectin ligands are differentially expressed in chronic Chagas' disease. SLe^x/P-selectin rather than VLA-4/VCAM-1 adhesion pathway might be involved in the pathogenesis of the inflammatory response.

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