We evaluated the expression of chemokine receptors (CCR1, CCR2, CCR5, and CXCR4) on the surface of peripheral blood mononuclear cells obtained from patients with chronic chagasic cardiomyopathy (CCC) and noninfected individuals. Only CCR5 and CXCR4 expression was different on the surface of the subsets (CD4, CD8, and CD14) evaluated. Patients with mild CCC had elevated leukocyte expression of CCR5, compared with noninfected individuals or those with severe disease. CXCR4 expression was lower on leukocytes from patients with severe CCC. The differential expression of both receptors on leukocytes of patients with CCC was consistent and clearly correlated with the degree of heart function such that the lower the heart function, the lower the expression of either CCR5 or CXCR4. These results highlight the possible participation of the chemokine system in early forms of chagasic cardiomyopathy and the relevance of heart failure–induced remodeling in modifying immune parameters in infected individuals.
but also because of the development of heart failure, which itself can lead to major changes in chemokine/chemokine receptor expression [16–19]. In the present study, we evaluated the expression of chemokine receptors (CCR1, CCR2, CCR5, and CXCR4) on the surface of peripheral blood mononuclear cells (PBMCs) obtained from noninfected individuals and patients with CCC. The chemokine receptors we investigated were those whose ligands have been shown to be produced in vitro or in experimental models of T. cruzi infection [10–15, 21]. Moreover, the choice of chemokine receptors was also limited by the availability of commercially obtainable antibodies at the time the study was initiated.

SUBJECTS, MATERIALS, AND METHODS

Study population. The studies described here received ethical clearance from the ethical review board of Universidade Federal de Minas Gerais. The diagnosis of Chagas disease was based on the presence of at least 2 positive serological examinations using distinct techniques (ELISA, indirect hemagglutination, or indirect immunofluorescence) in an individual with a relevant epidemiological history. Noninfected individuals had negative results in all 3 serological examinations. All individuals were recruited and clinically evaluated at the Center for Reference and Treatment of Infectious Diseases at the Hospital das Clinicas, Universidade Federal de Minas Gerais. All individuals underwent a complete clinical examination and the following laboratory workup: full blood count, free T4, thyroid-stimulating hormone, glucose, potassium, creatinine, blood urea nitrogen, electrocardiogram (EKG), chest X-ray, a 24-h Holter examination, echodopplercardiography, and a treadmill exercise test. Patients with hypertension, diabetes, thyroid or renal disturbances, or other systemic diseases, as well as those using steroidal drugs were excluded from the study, because these conditions could prevent the adequate interpretation of cardiac disease severity on immune parameters. Informed consent was obtained from all patients and uninfected individuals. Human experimental guidelines of the Brazilian Ministry of Health were followed in the conduct of the experiments described here.

The left-ventricle ejection fraction (LVEF) was obtained through Simpson’s method using the software provided with the equipment by an observer blinded to the clinical status [22]. Twenty-four-hour Holter monitoring was performed using a portable 3-channel cassette tape recorder (Dynamis; Cardios). Patients were encouraged to continue with their normal everyday activities during the recordings, with the avoidance of physical exercise or drugs that could interfere with autonomic function. Tapes were analyzed when at least 18 h of good-quality tracings were available. The recordings were analyzed on a Burdick/DMI Hospital Holter System (Spacelabs Burdick) by a semiautomatic technique. Minimal means and maximal heart rate, the number of ectopic ventricular and supraventricular beats, the occurrence of pauses, and heart blocks were recorded. A maximal stress testing was performed according to the standard Bruce protocol [23]. Patients were encouraged to exercise until they reached their limit of maximal effort. Heart rate and blood pressure were measured at rest, during each stage of exercise, at peak exercise, and during recovery. Exercise capacity, in estimated maximal oxygen consumption, was estimated on the basis of a previously published normogram [23].

Chagasic patients were categorized into 2 groups, according to the degree of heart dysfunction: mild CCC and severe CCC. Patients with mild CCC (n = 20) were those with normal EKG and chest X-ray results or with only minor alterations in their echocardiogram (e.g., regional contraction defects). These were patients whose classification corresponded to the indeterminate form, or CCC1, respectively, according to previously published clinical criteria from our group [1]. Patients with severe CCC (n = 23) were those with severe conduction defects (i.e., left–bundle branch block, left–anterior branch hemiblock with right–bundle branch block, or total atrioventricular block) or ventricular enlargement, as observed on the echodopplercardiogram. These were patients whose classification corresponded to classes CCC4 or CCC5, respectively, according to previously published clinical criteria from our group [1]. The control group consisted of 12 noninfected individual without any clinical or laboratory evidence of heart disease (table 1).

Detection of chemokine receptor on the surface of human PBMCs. Blood was collected in heparinized vaccutainer flasks (Becton Dickinson) and added to Ficoll-Hypaque (Sigma). Isolated PBMCs were harvested and washed in RPMI

### Table 1. Characteristics of noninfected individuals or patients with mild or severe chronic chagasic cardiomyopathy (CCC).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Noninfected (n = 12)</th>
<th>Mild CCC (n = 20)</th>
<th>Severe CCC (n = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD, years</td>
<td>39.1 ± 3.6</td>
<td>46.0 ± 2.1</td>
<td>42.0 ± 2.6</td>
</tr>
<tr>
<td>Male, %</td>
<td>67</td>
<td>56</td>
<td>55</td>
</tr>
<tr>
<td>NYHA, %</td>
<td>I 100</td>
<td>100</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>LVEF, mean ± SD, %</td>
<td>71.7 ± 0.8</td>
<td>64.6 ± 1.0</td>
<td>47.3 ± 2.7*</td>
</tr>
<tr>
<td>LVETD, mean ± SD, mm</td>
<td>48 ± 1</td>
<td>50 ± 1</td>
<td>59 ± 2*</td>
</tr>
<tr>
<td>log VPB, mean ± SD</td>
<td>ND</td>
<td>87 ± 55</td>
<td>3132 ± 1140*</td>
</tr>
</tbody>
</table>

**NOTE.** The left-ventricle ejection fraction (LVEF) and left-ventricle end diastolic diameter (LVETD) were assessed by echodopplercardiography. The no. of ventricle premature beats in 24 h (VPB) was assessed by 24-h Holter monitoring. ND, not determined; NYHA, New York Heart Association. * P < 0.05, vs. noninfected individuals or patients with mild CCC.
nutritive medium, and $25 \times 10^3$ cells were added to phycoerythrin-conjugated anti–human chemokine receptor monoclonal antibodies (CXCR4, CCR1, CCR2, or CCR5) or to fluorescein isothiocyanate–conjugated surface markers (CD3, CD4, CD8, and CD14), both of which had been diluted 1:5 in PBS that contained 0.1% bovine serum albumin. After incubation at 4°C for 20 min, cells were harvested, washed, and fixed in a solution that contained 10 g/L paraformaldehyde, 10.2 g/L sodium cacodylate, and 6.65 g/L NaCl. Flow cytometric analysis was performed using a FACScan flow cytometer (Becton Dickinson). Lymphocytes and monocytes were differentiated by characteristic side and forward light-scatter properties and confirmed by CD3 and CD14 staining. The threshold level was based on the maximum staining of a matched isotypic antibody with irrelevant specificity used in the same concentration. Isotype-control antibody bound to <1% of cells, and results are reported as the proportion of cells with fluorescence signals above the cutoff, as defined by the isotype controls.

**Statistical analysis.** For comparisons between different individuals, 1-way analysis of variance and the Bonferroni multiple-comparison procedure were used. For the association between chemokine expression and clinical features, data were analyzed by linear regression. Probability values were considered to be significant at $P<.05$. Data are expressed as mean ± SE. The analysis was done using the computer program PRISM (GraphPad).

**RESULTS**

**Clinical characteristics of patients with CCC.** Noninfected individuals and patients with mild or severe CCC had similar age and sex distribution (table 1). All noninfected individuals and patients with mild CCC were New York Heart Association class I, whereas 50% of patients with severe CCC were class II or greater. The latter findings were reflected in significant lower ejection fraction, greater left ventricle diastolic diameter, and greater number of ventricular premature beats in a 24-h period in patients with severe CCC (table 1).

**Expression of chemokine receptors on PBMCs.** The expression of chemokine receptors—CCR1, CCR2, CCR5, and CXCR4—was evaluated on different populations of leukocytes—CD4+, CD8+, and CD14+. As seen in table 2, there were no differences in the expression of CCR1 or CCR2 on the surface of CD4+, CD4+, or CD8+ T cells obtained from noninfected persons or patients with CCC. However, there were significant differences in the expression of CCR5 and CXCR4 (figure 1). The expression of CCR5 on CD4+ and CD8+ from patients with mild CCC was significantly greater than on cells from noninfected individuals or those with severe CCC (figure 1A and 1C). The expression of CXCR5 on CD14+ cells was greater on PBMCs from patients with mild CCC than those with severe CCC and was not different from that of noninfected patients (figure 1E). There were no differences in the expression of CXCR4 between noninfected individuals and those with severe CCC (figure 1).

Similarly to CCR5 expression, the expression of CXCR4 on the surface of CD4+ cells was higher in patients with mild CCC than in noninfected individuals (figure 1B). However, the expression of CXCR4 was lower on the surface of CD8+ and CD14+ cells patients with more severe disease than those with mild CCC or noninfected persons (figure 1D and 1F). Indeed, the expression of CXCR4 on CD14+ cells of patients with the severe form of CCC was approximately one-half that of noninfected individuals (figure 1F).

**Correlation between the expression of CCR5 and CXCR4 on PBMCs and parameters of heart dysfunction.** The evaluation of the expression of either CCR5 or CXCR4 on different leukocyte subsets demonstrated that their expression was lower in patients with more severe disease. Indeed, CCR5 expression was higher in patients with mild CCC, and CXCR4 expression was lower in those with severe CCC (figure 1). Next, we examined whether the changes in chemokine receptor expression observed in the various groups was associated with the degree of heart dysfunction of the patients. To this end, we evaluated
whether there was a correlation between the expression of chemokines on the various leukocyte subsets of patients with CCC and the degree of heart dysfunction, as assessed by the LVEF, the left-ventricle diastolic diameter (LVDD), and the number of ventricular premature beats in 24 h. These data are summarized in table 3. Overall, there was a clear direct correlation between the expression of CCR5 or CXCR4 on the various leukocyte subsets and the LVEF. Conversely, there was an inverse correlation between the expression of CCR5 or CXCR4 on the various leukocyte subsets and the LVDD (table 3). Thus, with the decrease in heart function, there was a decrease in CCR5 and CXCR4 receptor expression on PBMCs. Figure 2 illustrates 3 examples of these correlations. In contrast, the number of ventricular premature beats on the 24-h Holter examination did not correlate with the expression of chemokine receptors on PBMCs of patients with CCC (table 3).
Table 3. Correlation between the expression of chemokine receptors by peripheral blood mononuclear cells and indices of ventricular function in patients with Chagas disease.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>LVEF, %</th>
<th>LVEDD, mm</th>
<th>log VPB</th>
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<tbody>
<tr>
<td>CD4+</td>
<td>0.409 (.01)</td>
<td>0.346 (.03)</td>
<td>0.253 (.137)</td>
</tr>
<tr>
<td>CCR5+</td>
<td>0.363 (.02)</td>
<td>0.304 (.03)</td>
<td>0.192 (.137)</td>
</tr>
<tr>
<td>CXCR4+</td>
<td>0.436 (.02)</td>
<td>0.304 (.03)</td>
<td>0.253 (.137)</td>
</tr>
<tr>
<td>CD8+</td>
<td>0.438 (.006)</td>
<td>0.410 (.01)</td>
<td>0.178 (.293)</td>
</tr>
<tr>
<td>CCR5+</td>
<td>0.458 (.04)</td>
<td>0.399 (.01)</td>
<td>0.126 (.437)</td>
</tr>
<tr>
<td>CXCR4+</td>
<td>0.458 (.04)</td>
<td>0.454 (.004)</td>
<td>0.277 (.082)</td>
</tr>
</tbody>
</table>

NOTE. Data are Pearson’s coefficients (P). The left-ventricle ejection fraction (LVEF) and the left-ventricle end diastolic diameter (LVEDD) were assessed by echo-doppler-cardiography. The no. of ventricle premature beats in 24 h (VPB) was assessed by 24-h Holter monitoring. There were 20 and 23 patients with the mild or severe forms, respectively, of chronic chagasic cardiomyopathy. The different leukocyte populations—CD4+, CD8+, and CD14+ cells—were labeled with fluorescein isothiocyanate, and the chemokine receptors—CCR5 and CXCR4—were labeled with phycoerythrin; the % of double-positive cells was evaluated.

DISCUSSION

Chronic myocarditis is the main pathological finding associated with Chagas disease morbidity. There are several hypotheses that have been raised to explain the severe heart lesions found in some patients, including autoimmune mechanisms, microvascular dysfunction, and parasite persistence [5, 6, 24]. Regardless of the underlying reason to explain severe chronic myocarditis, it is thought that inflammatory injury to heart cells determines the degree of heart lesion and, consequently, the prognosis of patients [1]. Chemokines are group of mediators of the inflammatory process that are known to be important inducers of leukocyte recruitment and activation by acting on their G-protein–coupled receptors [8]. There has been some evidence in experimental models of T. cruzi infection that chemokines may be relevant mediators of leukocyte influx and disease pathogenesis [7, 11, 21, 25].

In an attempt to evaluate some of the components of the chemokine system in human Chagas disease, we studied the expression of the chemokine receptors CCR1, CCR2, CCR5, and CXCR4 on the surface of CD4+, CD8+, and CD14+ cells, which are major constituents of CCC [3, 4, 26, 27]. Only the expression of CCR5 and CXCR4 was different on the surface of the various subsets evaluated. Of interest, leukocytes from patients with mild CCC had elevated expression of CCR5, compared with noninfected individuals or those with severe disease. The expression of CXCR4 was lower on leukocytes from patients with severe CCC, compared with noninfected or those with mild disease. The differential expression of both receptors on leukocytes of patients with mild and severe CCC was evaluated.
sistent and was clearly correlated with the degree of heart function such that the lower the heart function, the lower the expression of either receptor. These results contrast with the results of other studies that have demonstrated enhanced overall chemokine receptor mRNA (as assessed by using RNase protection assay) in heart sections and PBMCs of patients with end-stage heart failure of nonchagasic origin [16, 17]. The reasons for these differences could lie in the different techniques used for assessing chemokine receptor expression. Although Damas et al. [16] used mRNA-based strategies, our studies evaluated the expression of chemokine receptor protein using flow cytometry. Even though protein evaluation is a better estimate of function than the expression of mRNA, studies that assess the migration of leukocytes from these patients must be performed in the future, to firmly establish a role for any given chemokine receptor.

CCR5 is the receptor for CCL3, CCL4, and CCL5 and has been previously associated with the determination of susceptibility to T. cruzi infection in the development of chagasic heart disease [15]. A polymorphism in the CCR5 promoter region (CCR5 59029 A→G promoter-point mutation) that is associated with lower expression of CCR5 on leukocytes was more frequent in patients with less severe than those with more severe Chagas disease [15]. The suggestion of the latter authors was that decreased CCR5 expression on Th1 cells might protect against the development of chagasic cardiomyopathy. CCR5 and its ligands are expressed during experimental murine T. cruzi infection [11, 12, 21], and preliminary results from experiments in our laboratory have suggested that CCR5 is essential for the host to mount a protective immune response against the parasite. In the present study, and in contrast to the suggestion of Calzada et al. [17], patients with mild cardiomyopathy had greater surface expression of CCR5 on circulating CD4+, CD8+, and CD14+ cells than uninfected individuals. In contrast, patients with more severe disease had similar expression of CCR5 to noninfected individuals and lower expression than patients with mild disease. Thus, the expression of higher levels of CCR5 on the surface of circulating leukocytes is induced in chronic Chagas disease and associates with the development of mild disease. There are least 2 possibilities that might explain the lower expression of CCR5 on the surface of leukocytes from patients with severe disease, compared with those who have mild disease. Because CCR5 appears to be relevant for the control of acute disease, it is possible that patients with lower CCR5 expression are those who do not control infection adequately and are thus prone to develop a more severe chronic infection. However, there is no proof that more severe acute disease is followed by a greater risk of developing severe chronic disease. Of note, a few patients with mild disease had low CCR5 expression. It will be of interest to follow these patients to evaluate whether they are more likely to develop a more severe disease. Alternatively, the lower CCR5 expression in patients with more severe disease could be due to the presence of heart dysfunction in this latter group of patients. In this way, it is possible that the increase in expression of CCR5 observed in mild CCC is lost when patients develop heart dysfunction. The negative correlation between the expression of CCR5 and the degree of heart dysfunction tends to support the latter possibility.

In contrast to CCR5, there was no difference in the expression of CXCR4 between noninfected individuals and patients with mild CCC. This observation would tend to suggest that Chagas disease itself does not induce a major change in CXCR4 expression. Similarly, there was no change of CCR1 or CCR2 expression on leukocytes. However, the expression of CXCR4, especially on CD14+ cells, was lower in patients with severe disease than those with mild disease or noninfected persons. Akin to the discussion above, it is possible that the lower expression of CXCR4 could identify a group of patients who are prone to develop severe CCC when challenged with T. cruzi infection or that heart failure could be associated with the lower CXCR4 expression on leukocytes of patients with severe CCC. Again, the good negative correlation between CXCR4 expression and the degree of heart dysfunction would suggest that the latter possibility is more likely.

In conclusion, our results suggest that there is an augmented expression of chemokine receptors, especially CCR5, in patients with mild CCC and that the diminished CCR5 and CXCR4 expression observed in patients with severe CCC appears to be mostly due to the onset of heart failure in this latter group of patients. Our results highlight the possible participation of the chemokine system in the early stages of chagasic cardiomyopathy and the relevance of heart failure–induced remodeling in modifying immune parameters in infected individuals.

Acknowledgment

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