Reduced Expression of Th1-Associated Chemokine Receptors on Peripheral Blood Lymphocytes at Diagnosis of Type 1 Diabetes

T. Lohmann,1 S. Laue,1 U. Nietzschmann,2 T.M. Kapellen,2 I. Lehmann,3 S. Schroeder,3 R. Paschke,1 and W. Kiess1

We investigated the expression of Th1- and Th2-associated chemokine receptors on peripheral blood lymphocytes at diagnosis and in the first phase of type 1 diabetes. Peripheral blood mononuclear cells (PBMCs) of 25 patients with newly diagnosed type 1 diabetes, 10 patients with longstanding type 1 diabetes, and 35 healthy control subjects were examined for expression of the chemokine receptors CXCR4 (naive T-cells), CCR5 and CXCR3 (Th1 associated), and CCR3 and CCR4 (Th2 associated) on CD3+ lymphocytes. Furthermore, we analyzed chemokine serum levels (monocyte chemoattractant protein [MCP]-1, macrophage inflammatory protein [MIP]-1α, MIP-1β, and RANTES (regulated on activation, normal T-cell expressed and secreted)) and phytos hemagglutinin (PHA)-stimulated cytokine secretion of Th1- ([γ-interferon [IFN-γ] and tumor necrosis factor-α [TNF-α]]) and Th2 ([interleukin [IL]-4 and -10]-associated cytokines by PBMC. The patients with newly diagnosed type 1 diabetes were followed for these parameters at 6–12 months after diagnosis. The PBMCs of patients with newly diagnosed but not with longstanding type 1 diabetes showed reduced expression of the Th1-associated chemokine receptors CCR5 (P < 0.001 vs. control subjects) and CXCR3 (P < 0.002 vs. control subjects). This reduction correlated with reduced IFN-γ and TNF-α production of PBMCs after PHA stimulation and reversed 6–12 months after diagnosis to normal levels. CCR4 cells were reduced in both newly diagnosed and longstanding type 1 diabetic patients, which correlated to reduced PHA-stimulated IL-4 production. MIP-1α and MIP-1β levels were considerably elevated in a subgroup of patients with newly diagnosed diabetes. We assume that Th1-associated peripheral T-cells are reduced in a narrow time window at the time of diagnosis of diabetes, possibly due to extravasation in the inflamed pancreas. Thus, chemokine receptor expression of peripheral blood lymphocytes may be a useful surrogate marker for the immune activity of type 1 diabetes (e.g., in intervention trials). Diabetes 51:2474–2480, 2002

Type 1 diabetes is presumed to be a T-cell-mediated autoimmune disease (1,2). T-cells invading pancreatic islets are observed at the time of diagnosis of type 1 diabetes (2). However, timing for this invasion (long time or only shortly before diagnosis) is unknown (1,3,4). Knowledge about this timing is of importance for the planning of intervention trials aiming to prevent type 1 diabetes (5). Recently, a new class of small molecules guiding cellular migration has been described, the so-called chemokines (6,7,8). Chemokines have been divided into four subfamilies according to the position of cystein residues: the CXC family with an amino acid between two cysteins, the CC family with none, the C family with only one cysteine, and the CX3C family with three amino acids between two cysteins (7,8). Chemokines act on their effectors via specific receptors that belong to a seven-transmembrane domain G protein-coupled family. These receptors are expressed on leukocytes, among them T-cells.

Chemokine receptors are critically involved in the extravasation of cells into inflamed tissue (e.g., the insulitis in type 1 diabetes) (9,10). Moreover, the repertoire of chemokine receptors is associated with the functional aspects of cells. Lanzavecchia et al. (11) have reported that Th1 cells are associated with CCR5 and CXCR3 and Th2 cells with CCR3 and CCR4 chemokine receptors. This finding is intriguing for the pathogenesis of type 1 diabetes since this disease is likely to be triggered by Th1 lymphocytes, whereas Th2 lymphocytes may be protective (12). Indeed, in another T-cell-mediated autoimmune disease, multiple sclerosis, Th1-associated chemokine receptors on peripheral blood lymphocytes were found to be a marker for immune activity of the disease (13). Whether similar mechanisms also apply for type 1 diabetes is presently not known. The aim of this study, therefore, was to determine the expression of Th1- and Th2-associated chemokine receptors on peripheral blood lymphocytes of patients with type 1 diabetes at the time of diagnosis and thereafter. A further aim was to find potential surrogate markers.
for the immune activity in type 1 diabetes as previously described for multiple sclerosis.

**RESEARCH DESIGN AND METHODS**

**Patients/Methods.** Peripheral blood mononuclear cells from newly diagnosed diabetic children, healthy control subjects, and patients with longstanding diabetes. A total of 25 consecutive patients with newly diagnosed type 1 diabetes attending the Children’s Hospital at the University of Leipzig between August 1999 and June 2000 were included in this prospective study. Blood samples were drawn at admission before starting insulin treatment for the isolation of freshly obtained peripheral blood mononuclear cells (PBMCs). All blood samples were transported to our laboratory within 2 h. PBMCs were isolated by Ficoll-Paque density gradient centrifugation (Biochrom, Berlin, Germany) and immediately used for fluorescence-activated cell sorter (FACS) staining or peripheral blood mononuclear cell (PHA)-stimulated cultures.

The mean age of the patients was 9.9 ± 4.1 years (range 2.6–16.6 years). The mean HbA1c of the patients was 10.5 ± 2.1% (normal range of our laboratory: 4.8–6.2%). Of the 25 patients, 11 had ketoacidosis at the time of diagnosis. None of the patients had clinical or laboratory signs of severe infections. All patients were HLA typed and found positive for at least one of the following islet cell-specific autoantibodies: ICA, GADA, or I2A-Ab. A further patient group consisted of 10 longstanding type 1 diabetic patients with a diabetes duration of 6 months to 9 years (mean 3.2 ± 1.2 years). These patients were metabolically relatively well controlled (HbA1c < 7.5%) and had a mean age of 27.3 ± 5.1 years (range 19–34 years). A total of 35 healthy probands without diabetes and without islet cell–specific antibodies served as control subjects. A total of 25 control subjects were age and sex matched to the newly diagnosed diabetic children (“control group 1,” mean age 10.1 ± 3.4 years, range 2.7–17.8 years) and the other 10 control subjects were of comparable age to the longstanding type 1 diabetic patients (“control group 2,” mean age 27.5 ± 5.9 years, range 21–38 years). All control subjects were also HLA typed. Ten of the 25 children control subjects and all 10 adult control subjects had at least one of the diabetes-associated HLA risk alleles DR4/DQB1*0302 or DR3/DQB1*0201. After finishing the experiments described above, a new series of 10 newly diagnosed type 1 diabetic patients (five of them also at a follow up 6–12 months after diagnosis), 10 longstanding type 1 diabetic patients, and 10 healthy control subjects were tested for expression of the Th2-associated chemokine receptor CCR4 and double expression of the Th1-associated chemokine receptors CCR5 and CXCR3. All probands gave informed consent before their inclusion in the study. This study was approved by the Ethical Board of the Medical Faculty of the University of Leipzig (registration no. 052/99), and all investigations were performed according to the principles of the appropriate version of the Declaration of Helsinki.

**FACS staining for chemokine receptors.** The PBMCs of the patients and control subjects were characterized for the expression of chemokine receptors by three-colored direct immunofluorescence and flow cytometry using a FACSscan (Becton Dickinson). Cells were washed with PBS (Gibco, Karlsruhe, Germany) and reconstituted in PBS with 1% FCS and 0.1% sodium azide (staining buffer). The following antibodies were added with 20 µl of 106 cells in 100 µl staining buffer according to the recommendations of the manufacturer: 1) monoclonal antibody (mAb) to CD3 conjugated with Cy-chrome (Pharmingen/Becton-Dickinson, Heidelberg, Germany); 2) fluorescein isothiocyanate (FITC)-conjugated mAb to CD4 or CD8 (Pharmingen); 3) phycoerythrin (PE)-conjugated mAb to CXCR4, CCR5, or CXCR3 (Pharmingen and CCR2, CCR5, and CXCR4 (R&D Systems, Wiesbaden, Germany). After 30 min at 4°C, cells were washed twice with staining buffer, fixed with PBS/0.1% formaldehyde, and analyzed on the FACSscan using CellQuest software.

**PHA-stimulated cytokine production.** The PHA-stimulated cytokine production of peripheral blood cells in patients and control subjects was tested by a whole-blood assay described previously (14,16). Briefly, blood was drawn in heparinized tubes (Vacutainer) and mixed by repeated inversion. Then, 150-µl aliquots were diluted 1:10 in RPMI (Gibco) with 10% FCS, pipetted in 24-well tissue culture plates, and stimulated with 10 µg/ml PHA (Sigma Taufkirchen, Germany). Cells were incubated at 37°C in a 5% CO2 atmosphere for 48 h, after which the plasma supernatants were aspirated, pooled, and stored at −20°C until assayed for cytokines. Cytokines were measured by commercially available assays for γ-interferon (IFN-γ), tumor necrosis factor (TNF-α), interleukin-10 (IL-10), and IL-10 (OPTEIA kits; Pharmingen/Becton Dickinson, Heidelberg, Germany) according to the instructions of the manufacturer. After blocking of nonspecific binding sites with PBS/10% FCS, samples were pipetted in prepared wells, covered with plate sealer, and incubated for 2 h at room temperature. Then, the content of wells was aspirated and the wells were washed five times with wash buffer (PBS/0.05% Tween [20]) before the plate was blotted on absorbent paper. Thereafter, 100 µl of working detector solution was added to each well and incubated for 1 h at room temperature. The wells were washed again seven times before 100 µl of substrate solution was added to each well and incubated for 30 min at room temperature. Then, 50 µl of stop solution was added to each well and within 30 min the absorbance at 450 nm was read. All samples were analyzed in duplicate and the mean of duplicates is presented. Some probes had to be diluted before assay. The sensitivity of the assays is provided by the manufacturer with 4 pg/ml for all four cytokines (IFN-γ, TNF-α, IL-4, and IL-10).

**Measurement of chemokines in the serum.** The chemokines monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, and RANTES (regulated on activation, normal T-cell expressed and secreted) were measured in serum probes initially drawn at the time of diagnosis and stored at −20°C until assayed. Commercially available enzyme-linked immunosorbent assays (ELISAs) from R&D systems (Quantikine) were used according to the instructions of the manufacturer. Briefly, 10 µl serum was diluted 50-fold with 400 µl Calibrator Diluent RD5H (1X) at room temperature and added with 100 µl per well to prepared ELISA plates. After incubation for 2 h and washing, 200 µl chemokine conjugate was added to each well and incubated for a further hour. Then, wells were washed again and 200 µl Substrate Solution was added per well and incubated for another 20 min before adding 50 µl of Stop Solution. The optical density of each well was determined within 30 min using a microplate reader set at 540 nm. The standards for the calculation of results (standard curve) were delivered by the manufacturer. All samples were measured in duplicate and results are presented as mean of the duplicates. The sensitivity of the assays was provided by R&D Systems with 5 pg/ml (MCP-1), 10 pg/ml (MIP-1α), and 8 pg/ml (RANTES).

**Suppression of CCR5 expression by MIP-1α.** CD3+ cells of three healthy control subjects were incubated with increasing amounts of MIP-1α (10, 100, 1,000 ng/ml; R&D Systems, Wiesbaden, Germany) for 24–72 h. The highest concentration of MIP-1α measured in diabetic patients was <1 ng/ml so that the selected concentrations were at least one log above this level. Before and after incubation, CCR5/CD3 cells were analyzed by FACS as described above. Data are presented for 24-h incubation and were similar for 48- or 72-h incubation.

**Statistics.** The comparison between patient groups and between patients and control subjects was done by two-tailed Mann-Whitney U tests. The unpaired U test was used for comparisons of groups and the paired t test for the follow-up analysis. Bonferroni’s correction for multiple analyses was done as indicated. Data were analyzed with SPSS for Windows (Version 9.0.1). P < 0.05 was regarded as statistically significant.

**RESULTS**

**Chemokine receptor expression on peripheral blood lymphocytes.** We found a significant reduction of the Th1-associated chemokine receptors CCR5 and CXCR3 on CD3 lymphocytes in newly diagnosed diabetic children compared with age- and sex-matched control subjects (for CCR5 P < 0.001, for CXCR3 P < 0.002; Fig. 1 and Table 1). This reduction of CCR5 and CXCR3 receptors was also observed for CD4 but not for CD8 cells (P < 0.0001 for CCR5/CD4+ cells, P < 0.002 for CXCR3/CD4+ cells; Table 1). These differences remain highly significant using Bonferroni’s correction for multiple analyses. We have also analyzed in a separate series the number of CD3 cells with double expression of CCR5 and CXCR3 and found this percentage to be comparable (20.62 ± 8.63% in 10 control subjects and 33.02 ± 8.07% in newly diagnosed type 1 diabetic patients). Therefore, the reduction of CCR5 and CXCR3 T-cells in newly diagnosed diabetic patients takes place in both single-positive (either CCR5 or CXCR3) and double-positive (CCR3 + CXCR5) CD3 cells.

Interestingly, there was no difference comparing longstanding (1–5 years) diabetic adult patients with age-matched control subjects for CCR5/CD3+ cells (P = 0.25; Fig. 1 and Table 1) or CXCR3/CD3+ cells (P = 0.11; Fig. 1 and Table 1). The reduction of CCR5/CD3+ and CXCR3/CD3+ cells in newly diagnosed pediatric diabetic patients was already reversed in most of the patients 6 months
after diagnosis (Table 1). For all 25 patients, we have confirmed this rise in the follow-up investigation after 12 months (data not shown). Therefore, the striking reduction of Th1-associated chemokine receptors on peripheral blood lymphocytes seems to be present only in a narrow time window of some weeks around the diagnosis of type 1 diabetes. This finding was further confirmed in two patients followed from a prediabetic state to diagnosis and 6–12 months thereafter (Table 3) showing the fall in CCR5- or CXCR3-positive CD3 cells just at diagnosis.

There was no difference for the Th2-associated chemokine receptor CCR3 between the groups (results not shown). However, for the other Th2-associated chemokine receptor CXCR4, we found in a separate series of 10 patients and 10 control subjects a reduction of CCR4 expression on CD3 cells (17.15 ± 4.39% CD3/CXCR4 cells in 10 control subjects compared with 12.46 ± 3.44% in 10 newly diagnosed type 1 diabetic patients, $P < 0.01$). This reduction could be reproduced in longstanding diabetic patients (13.47 ± 5.28% CD3/CXCR4 cells) and in the follow up investigation of five of the newly diagnosed patients after 6–12 months (12.31 ± 5.28% CD3/CXCR4 cells).

We found no significant difference for the chemokine receptor CXCR4 expression (associated to naive T-cells) on CD3 cells in the peripheral blood of newly diagnosed diabetic patients and their control subjects ($P = 0.69$; Fig. 1, Table 1) or of longstanding diabetic patients and their control subjects ($P = 0.09$). Similarly, we found no significant difference of CXCR4 expression between diabetic patients and matched control subjects when analyzing CD4 or CD8 cells ($P > 0.5$, Table 1). The expression of CXCR4 on CD3 cells did not change at follow up at 6 and 12 months (Table 1).

**PHA-stimulated cytokine production of peripheral blood lymphocytes.** Because the observed reduction of Th1-associated chemokine receptors seems to contradict published data on cytokine production of peripheral blood lymphocytes in diabetic patients, we sought to correlate our data on chemokine receptor expression to PHA-stimulated secretion of Th1 (IFN-γ, TNF-α) and Th2-associated (IL-4, IL-10) cytokines. Again, we found a reduction of Th1-associated cytokines in newly diagnosed diabetic patients compared with matched control subjects ($P < 0.001$ for IFN-γ; $P < 0.001$ for TNF-α; Fig. 2, Table 2). Also these differences remain highly significant using Bonferroni’s correction. In contrast, there was no difference in PHA-stimulated IFN-γ or TNF-α levels between longstanding diabetic patients and adult control subjects ($P = 0.25$ for IFN-γ, $P = 0.53$ for TNF-α; Fig. 2, Table 2). Similar to our finding of Th1-associated chemokine receptors, the reduced Th1-associated cytokine stimulation in newly diagnosed diabetic patients was reversed at the follow up investigation at 6 months ($P < 0.01$ for IFN-γ, $P < 0.03$ for TNF-α; Table 1) and at 1 year (data not shown). We found no significant difference for PHA-stimulated secretion of IL-10 between pediatric control subjects and newly diagnosed diabetic patients, whereas adult control subjects had lower IL-10 levels compared with longstanding diabetic patients ($P < 0.01$; Fig. 2, Table 2). However, in agreement with published results, we observed a signifi-

### TABLE 1

Expression of chemokine receptors on CD3, CD4, and CD8 cells

<table>
<thead>
<tr>
<th></th>
<th>Control group 1</th>
<th>Newly diagnosed diabetic patients/baseline</th>
<th>Follow-up</th>
<th>Control group 2</th>
<th>Longstanding diabetic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR5/CD3</td>
<td>8.98 ± 2.78</td>
<td>5.31 ± 3.94*</td>
<td>8.13 ± 4.87†</td>
<td>10.2 ± 1.86</td>
<td>12.7 ± 6.18</td>
</tr>
<tr>
<td>CCR5/CD4</td>
<td>2.48 ± 1.14</td>
<td>1.23 ± 1.09*</td>
<td>1.66 ± 0.93‡</td>
<td>2.53 ± 0.81</td>
<td>2.95 ± 2.46</td>
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<tr>
<td>CCR5/CD8</td>
<td>1.86 ± 0.86</td>
<td>1.44 ± 1.42</td>
<td>2.05 ± 1.29</td>
<td>3.31 ± 1.41</td>
<td>2.23 ± 1.85</td>
</tr>
<tr>
<td>CXCR3/CD4</td>
<td>7.07 ± 2.59</td>
<td>4.3 ± 2.04*</td>
<td>6.29 ± 2.43†</td>
<td>8.18 ± 2.08</td>
<td>8.27 ± 4.37</td>
</tr>
<tr>
<td>CXCR3/CD8</td>
<td>7.89 ± 3.53</td>
<td>7.17 ± 4.51</td>
<td>8.74 ± 4.82</td>
<td>8.35 ± 3.82</td>
<td>7.82 ± 4.98</td>
</tr>
<tr>
<td>CXCR4/CD3</td>
<td>33.61 ± 9.23</td>
<td>32.8 ± 12.2</td>
<td>32.72 ± 11.05</td>
<td>39.7 ± 8.36</td>
<td>33.2 ± 7.3</td>
</tr>
<tr>
<td>CXCR4/CD4</td>
<td>20.03 ± 7.02</td>
<td>18.74 ± 9.65</td>
<td>18.81 ± 6.01</td>
<td>23.53 ± 8.05</td>
<td>17.48 ± 5.71</td>
</tr>
<tr>
<td>CXCR4/CD8</td>
<td>7.96 ± 3.27</td>
<td>7.66 ± 4.05</td>
<td>8.77 ± 4.31</td>
<td>9.84 ± 3.51</td>
<td>8.21 ± 4.61</td>
</tr>
</tbody>
</table>

Data are means ± SD. Expression of chemokine receptors CCR5, CXCR3, and CXCR4 on CD3, CD4, and CD8 cells are shown for newly diagnosed and longstanding diabetic patients and age-matched control subjects. Control group 1 is matched to newly diagnosed, control group 2, and longstanding diabetic patients. *$P < 0.001$, ‡$P < 0.01$ for diabetic patients versus control subjects. †$P < 0.01$, §$P < 0.001$ for comparison between baseline and follow-up in newly diagnosed patients.
Significant reduction of PHA-stimulated IL-4 secretion in both newly diagnosed and longstanding diabetic patients compared with matched control subjects ($P < 0.005$ newly diagnosed diabetic patients compared with pediatric control subjects, $P < 0.015$ longstanding diabetic patients compared with adult control subjects; Fig. 2, Table 2). Both differences remain significant with Bonferroni’s correction. Neither PHA-stimulated IL-4 nor IL-10 levels changed significantly at follow up (Table 2). Therefore, we conclude that the reduction of stimulated Th1-associated cytokine secretion is also observed only during a short time around the diagnosis of type 1 diabetes whereas the reduction of IL-4 secretion may be a consistent feature of diabetic patients.

**Chemokine levels in the serum.** We have measured the chemokines MCP-1, MIP-1α, MIP-1β, and RANTES in the serum of newly diagnosed diabetic patients and control subjects. There was a significant difference for MIP-1α levels between patients and pediatric control subjects ($P < 0.001$; Fig. 3) compared as groups. However, there were clearly patients with elevated MIP-1α serum levels: 14 of 25 patients had MIP-1α levels above mean $+ 3$ SD of control subjects and 10 of 25 patients had excessively high levels $>100$ pg/ml MIP-1α (46.9 pg/ml is described as the highest serum level in control subjects by the manufacturer). In contrast, there was no patient with longstanding type 1 diabetes with a MIP-1α serum level $>21$ pg/ml. A similar distribution has been found for MIP-1β: 7 of 21 newly diagnosed patients had elevated MIP-1β levels above the mean $+ 3$ SD of age-matched control subjects (230 pg/ml) that has not been found in any longstanding diabetic patient.

We found no difference for MCP-1 ($P = 0.07$; Fig. 3) or RANTES serum levels ($P = 0.71$; Fig. 3). Although there seems to be a tendency to higher MCP-1 serum levels in patients compared with control subjects, we did not find MCP-1 serum level in patients above mean $+ 3$ SD of control subjects. At follow-up, there was no change in MCP-1 or RANTES serum levels. The fall in MIP-1α levels after 6 months (Table 2) was not significant. There were no differences in MIP-1α, MCP-1, or RANTES serum levels between adult control subjects and longstanding diabetic patients (Fig. 3 and Table 2).

**Suppression of CCR5 level on CD3 cells by MIP-1α.** In a first series of experiments, no change of CCR5 levels on CD3 cells was seen with MIP-1α from 0.1 to 10 ng/ml or with IFN-γ from 1 to 100 ng/ml, with TNF-α from 0.1 to 10 ng/ml, or with a mixture of IFN-γ and TNF-α with these concentrations (data not shown). The concentrations of cytokines were selected to be at least one log above the highest concentrations of these cytokines measured after PHA stimulation in patients or control subjects. In three

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**TABLE 2**

<table>
<thead>
<tr>
<th></th>
<th>Control group 1</th>
<th>Newly diagnosed diabetic patients/baseline</th>
<th>Follow-up</th>
<th>Control group 2</th>
<th>Longstanding diabetic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>2,319.2 ± 1871.1</td>
<td>357.9 ± 260.3*</td>
<td>1,734.8 ± 1094.1†</td>
<td>1,900 ± 800</td>
<td>4,400 ± 4,600</td>
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<tr>
<td>IL-10</td>
<td>587.9 ± 262.3</td>
<td>616.5 ± 380.1</td>
<td>993.3 ± 432.1</td>
<td>693.4 ± 269.1</td>
<td>1,290.2 ± 1,053.5‡</td>
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<tr>
<td>TNF-α</td>
<td>298 ± 139.6</td>
<td>124.9 ± 109*</td>
<td>199 ± 97.1</td>
<td></td>
<td>208.3 ± 135.2</td>
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<tr>
<td>IL-4</td>
<td>39.8 ± 38.4</td>
<td>14.7 ± 9.6†</td>
<td>23.2 ± 18.7</td>
<td>41.4 ± 32.4</td>
<td>15.9 ± 6.9¶</td>
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<tr>
<td>MCP-1</td>
<td>328.8 ± 148.7</td>
<td>287 ± 86.7</td>
<td>326.8 ± 117.6</td>
<td>267.6 ± 50.5</td>
<td>299.3 ± 70.6</td>
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<tr>
<td>MIP-1α</td>
<td>21.2 ± 9.0</td>
<td>145.4 ± 292.6*</td>
<td>62.7 ± 98.8</td>
<td>16.9 ± 1.0</td>
<td>18.27 ± 0.86</td>
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<tr>
<td>RANTES</td>
<td>55.5 ± 25.8</td>
<td>64.1 ± 53.8</td>
<td>50.1 ± 37.7</td>
<td>33.4 ± 25.0</td>
<td>35.9 ± 11.3</td>
</tr>
</tbody>
</table>

Data are means ± SD. Cytokine secretion (in pg/ml) of PBMC after stimulation by PHA and chemokine serum levels of MIP-1α (pg/ml), MCP-1 (pg/ml), and RANTES (ng/ml) are shown for the groups as in Table 1. *$P < 0.001$, †$P < 0.05$ for diabetic patients versus control subjects. ¶$P < 0.01$, ⌈$P < 0.001$, ‡$P < 0.05$ for comparison between baseline and follow-up in newly diagnosed patients.
separate experiments, the suppression of CCR5 levels on CD3 cells by incubation with higher amounts of MIP-1α was tested in vitro. The selected concentrations of MIP-1α (10–1,000 ng/ml) were at least one log higher as the highest concentrations measured in the serum of newly diagnosed patients with type 1 diabetes. As shown in Table 4, only concentrations >100 ng/ml suppressed CCR5 levels >50% in all three experiments. The results were similar for 24, 48, and 72 h of incubation with MIP-1α. Therefore, at least two to three log higher concentrations of MIP-1α, as present in the sera of newly diagnosed patients with type 1 diabetes, are necessary to suppress CCR5 expression on CD3 cells in vitro.

**DISCUSSION**

The invasion of pathogenic T-cells into the islets is a critical step in the pathogenesis of type 1 diabetes. However, it is presently unknown whether this happens in a chronic fashion long before diagnosis (1) or shortly before diagnosis by switching a nondestructive to a destructive insulitis, as described in NOD mice (3,4,17). The differentiation between these two models for the natural history of type 1 diabetes is relevant for the timing of intervention trials aiming to prevent type 1 diabetes (5). Chemokines such as MCP-1 or MIP-1α are critically involved in the migration of pathogenic T-cells into the islets in type 1 diabetes (9,10). However, for human type 1 diabetes, there are very few data on chemokines or chemokine receptors during the course of the disease. In another T-cell–mediated autoimmune disease, multiple sclerosis, Th1-associated chemokine receptors (CCR5 and CXCR3) on peripheral blood lymphocytes have been identified as surrogate markers for the immune activity of the disease (13). This finding prompted us to search for similar phenomena in human type 1 diabetes since surrogate markers for the immune activity are urgently needed in type 1 diabetes to guide ongoing intervention trials (19).

Surprisingly, we found a striking reduction of the Th1-associated chemokine receptors CCR5 and CXCR3 on peripheral blood lymphocytes at the time of diagnosis of type 1 diabetes. This finding was reproduced for CD3 and CD4 cells and correlated with a reduced stimulation of the Th1-associated cytokines IFN-γ and TNF-α by PHA. The latter finding seems to contradict published results on PHA-stimulated cytokine production in type 1 diabetes (15). However, our study differs from the study of Kallmann et al. (15) in that we only investigated patients at diagnosis of type 1 diabetes before first insulin therapy (day 0), whereas Kallmann et al. tested patients with type 1 diabetes up to 4 weeks after initiation of insulin therapy. In another study of PHA-stimulated cytokine secretion (20), only patients at the time of diagnosis of type 1 diabetes were compared with healthy control subjects. In agreement with our results, a reduction of IFN-γ stimulation in type 1 diabetes was found. In contrast to our findings regarding Th1-associated chemokine receptors CCR5 and CXCR3, we found no significant differences in the constitutively expressed CXCR4 or the Th2-associated CCR3 receptor levels between patients and control subjects. However, CCR4 (a further Th2-associated chemokine receptor [11]) expression was reduced on CD3 in a separate series of patients and control subjects. Nevertheless, longstanding type 1 diabetic patients also had reduced CCR4 levels on CD3, which suggests a lack of regulatory cells in type 1 diabetes in general, thus correlating well to the findings of Kukreja et al. (21) and our finding of reduced PHA-stimulated IL-4 production in both newly diagnosed and longstanding diabetic patients. However, recent studies have associated CCR4 with either a regulatory (22) or a memory phenotype of both Th1 and Th2 (23), which complicates the interpretation of our findings.

**TABLE 3**

Longitudinal study of CCR5 and CXCR3 expression in two individuals from a prediabetic state

<table>
<thead>
<tr>
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<th>−11 months</th>
<th>Baseline</th>
<th>+6 months</th>
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<tr>
<td></td>
<td>CCR5</td>
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<td></td>
<td>CXCR3</td>
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<table>
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<th>Baseline</th>
<th>+12 months</th>
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<td>CCR5</td>
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<td>6.34</td>
</tr>
<tr>
<td></td>
<td>CXCR3</td>
<td>22.15</td>
<td>12.60</td>
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</table>

Follow-up of CCR5- and CXCR3-positive CD3 cells in two patients from a prediabetic state (11 and 6 months after diagnosis, respectively) to diagnosis (baseline) and 6 or 12 months thereafter.

**TABLE 4**

Reduction of CCR5 expression by high MIP-1α concentrations in vitro

<table>
<thead>
<tr>
<th>MIP-1α concentration in vitro (ng/ml)</th>
<th>0</th>
<th>10</th>
<th>100</th>
<th>1,000</th>
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<tbody>
<tr>
<td>Experiment 1</td>
<td>8.39</td>
<td>7.12</td>
<td>5.38</td>
<td>2.99*</td>
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<tr>
<td>Experiment 2</td>
<td>4.90</td>
<td>4.84</td>
<td>4.22</td>
<td>2.31*</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>9.22</td>
<td>8.02</td>
<td>6.27</td>
<td>3.16*</td>
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</tbody>
</table>

Data are % Reduction of CCR5 expression on CD3 cells after incubation in vitro for 24 h with MIP-1α in increasing concentrations from 0 to 1,000 ng/ml in three separate experiments. *Reduction of CCR5 expression >50%.

FIG. 3. Chemokine serum levels of MCP-1, MIP-1α, and RANTES are shown for newly diagnosed and longstanding diabetic patients and their age-matched control subjects, as in Figs. 1 and 2. Note that levels are expressed in picograms per milliliter in a logarithmic scale. The level of significance for the comparison of MIP-1α levels between patients and control subjects is shown above the graph.
We found a significant difference of the serum chemokine levels of MIP-1α and MIP-1β between patients and control subjects. However, interestingly, there were clearly elevated MIP-1α and MIP-1β levels in some but not all patients with type 1 diabetes. CCR5 cells were found greatly reduced in diabetic patients at the time of diagnosis in our study, but there was no general correlation between MIP-1α or MIP-1β serum levels and CCR5 expression. Our result of MIP-1α and MIP-1β elevation in some but not all type 1 diabetic patients is very similar to recently published findings on serum levels of IP-10 (IFN-γ-inducible protein 10), a Th1-associated CXCR3 ligand, in type 1 diabetes (24). To date, we have not found any characteristic feature of these patients with elevated MIP-1α levels that differentiates them from those patients with normal MIP-1α levels in terms of metabolic data, HLA type, antibody positivity and/or level, or chemokine receptor levels. We believe that elevated MIP-1α levels may be indicative of islet destruction, but we have no direct proof of this. Because there are no surrogate markers for islet destruction, pancreatic biopsies are necessary to prove this hypothesis but are subsequently impossible for us to perform. CCR5- or CXCR3-positive cells seem to not produce higher amounts of MIP-1α compared with their negative counterparts after stimulation with anti-CD3, anti-CD8, and anti-CD28, or PHA (data not shown), although these cells produce more IFN-γ than CCR5- or CXCR3-negative cells after stimulation by PHA, confirming their Th1-associated phenotype (our unpublished results, 13).

MIP-1α binds to CCR1 and CCR5 and is also associated with Th1 responses (11). MIP-1α is most likely involved in the pathogenesis of experimental allergic encephalomyelitis, the animal model of multiple sclerosis (25,26), and has been found in brain lesions of multiple sclerosis patients (13). Also in NOD mice, the animal model for type 1 diabetes, MIP-1α is secreted by antigen-specific Th1 cells invading the pancreatic islets (9) and is correlated with a destructive insulitis in the pancreas (10). The latter study found increased MIP-1α: MIP-1β + MCP levels in the pancreas of NOD mice with destructive insulitis. Most importantly, NOD.MIP-1α−/− mice exhibited reduced destructive insulitis and were protected from diabetes (10). In the study by Bradley et al. (9), other chemokines such as MCP-1 or RANTES (which have both been investigated in our study) were also associated with Th1 pancreatic infiltrates. However, MCP-1 generally seems to correlate more closely with the early nondestructive insulitis than with destructive insulitis at diabetes onset (10,27) and primes Th2 polarization of T-cells (28). The interpretation of RANTES serum levels, as offered in our study, has to be seen with caution since not only PBMCs but also thymocytes can deliver large amounts of this chemokine (29).

We do not think that the reduction of CCR5 or CXCR3 receptors on CD3+ cells in type 1 diabetes is due to metabolic changes at the time of diagnosis or to the HLA type. We have tested a group of newly diagnosed type 2 diabetic patients with similarly elevated blood glucose levels (HbA1c levels >10%) and found no difference in chemokine receptor expression compared with control subjects (data not shown). Moreover, both type 1 diabetic patients with and without ketoacidosis showed reduced expression of CCR5 and CXCR3 receptors on T-cells. There may be a fluctuation of chemokine receptors (by infections, for example). But we can exclude severe infections in our patients by clinical and laboratory findings. The stability of chemokine receptor levels at 6 and 12 months (data not shown) in our individual patients argues against high fluctuations of these levels per se. Finally, the HLA type does not seem to influence chemokine receptor expression on CD3+ cells since control subjects with at least one diabetes-associated HLA haplotype (10 control subjects for children and 10 for adult control subjects had at least one of the diabetes-associated DR4/DQB1*0302 or DR3/DQB1*0201 alleles) were analyzed separately for expression of CCR5 and CXCR3 on T-cells and did not differ from the remaining control subjects.

There are several possible explanations for our finding of reduced Th1-associated chemokine receptors on lymphocytes at the time of diagnosis of type 1 diabetes: 1) Th1 cells may be extravasated in the inflamed pancreatic islets at this time; 2) the receptors are primarily downregulated by chemokines or cytokines (30); 3) the receptors are cut or shedded as it is described for adhesion molecules (e.g., intracellular adhesion molecule [ICAM]-1) (31); and 4) these cells are killed at this time, possibly by apoptosis. We favor the first scenario since a migration of pathogenic T-cells very shortly before or at the time of diabetes onset fits well with data of the NOD mouse animal model (3,4,18). Moreover, an enrichment of exactly the same cells, CCR5+ and CXCR3+ T-cells, in cerebrospinal fluid compared with peripheral blood has been described during autoimmune attacks in multiple sclerosis (32). Similarly, an enrichment of CCR5+ cells has been found in the synovial fluid of patients with different forms of arthritis (33–36). Of course, the other possibilities outlined above have to be formally excluded. At least for possibility 2, downregulation of chemokine receptors by chemokines or cytokines, we have in vitro data showing that at least two to three-log higher concentrations of MIP-1α, as measured in the serum of newly diagnosed patients, were necessary to significantly reduce CCR5 levels on T-cells. Similarly, TNF-α, IFN-γ, and a mixture of both in concentrations up to 0.1 μg/ml were unable to significantly reduce the CCR5 levels on T-cells, at least in our setting. In another study, TNF-α under different circumstances has been reported to reduce CCR5 expression on T-cells (37).

Because a Th1-like dominance of peripheral blood lymphocytes has been described in first-degree relatives of type 1 diabetic patients (38), prospective studies on the expression of chemokine receptors before onset of diabetes are necessary to identify the time point when the reduction of Th1-associated chemokine receptors on peripheral blood cells occurs. Our preliminary data on two patients followed from a prediabetic state indicate that the reduction may happen in a time window 6–12 months before diagnosis. This may be too late to intervene in trials since the final invasion of pathogenic T-cells may have already occurred. A larger study is important to determine the prognostic relevance of these markers as potential surrogate markers for the immune activity in type 1 diabetes. Such markers are needed especially for intervention trials since the natural course of autoimmune diabetes may be rather heterogeneous (39).
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