Altered cytokine expression of peripheral blood lymphocytes in polymyositis and dermatomyositis


Objective: To investigate the intracellular and soluble cytokine levels and T cell subsets in peripheral blood of patients with active and inactive polymyositis and dermatomyositis.

Methods: The frequencies of T and B lymphocytes, T helper (Th), and T cytotoxic (Tc) cells and of interferon γ (IFNγ), interleukin (IL)4, and IL10 expression of CD4+ or CD8+ cells were determined by flow cytometry. The concentrations of soluble cytokines were measured with commercial enzyme linked immunosorbent assays.

Results: In active dermatomyositis there was a decreased percentage of T (CD3+) lymphocytes and Tc (CD8+) lymphocytes, decreased IFNγ expression of CD4+ and CD8+ cells, but an increase in B and IL4 producing CD4+ lymphocyte frequencies. These prominent changes disappeared in the inactive stage of the disease. In polymyositis no significant change in these lymphocyte subsets or in intracellular cytokine expression could be detected in either the active or the inactive form. The frequency of IL4+/IFNγ+ Tc cells was calculated and a significantly increased Th2/Th1 frequency was found in active dermatomyositis, and a decreased frequency in inactive dermatomyositis, compared with the control population.

Conclusions: There appears to be a difference between polymyositis and dermatomyositis in the level of peripheral blood lymphocytes and their intracellular cytokine content. These findings provide further evidence for a difference in the pathogenesis of polymyositis and dermatomyositis.

Polymyositis and dermatomyositis are idiopathic inflammatory myopathies of unknown origin, although immunological mechanisms may be involved in their pathogenesis.1,2 There are data suggesting that these two diseases may be pathogenetically similar,3–5 while other reports provide evidence that their origin essentially differ.

In polymyositis T cell mediated cytotoxicity rather than humoral immunity may be the main effector mechanism, as immunohistochemical studies on muscle biopsies have shown that most of the T cells in the endomysium surrounding and invading single myofibres are of CD8+ type.6,7 Cytotoxic granules of CD8+ T cells release perforin and serine esterases, which contribute to muscle cell death. Oligoclonal expansions of CD8+ T cells have been found in the blood and within muscle biopsies in polymyositis, but not in dermatomyositis.10–12 On the other hand in dermatomyositis humoral mediated damage to muscle blood vessels seems to be more important in the pathogenesis. Inflammatory infiltrates mainly consist of activated B cells and are concentrated in the interstitial and perivascular areas.13,14 Deposition of the membrane attack complex of complement is often seen in the muscle vessels in dermatomyositis, and the vessel injury appears to be complement mediated.15 The pattern of perifascicular atrophy of muscle fibres is characteristic and attributed to ischaemic effects.

Cytokines have important functions in inflammatory processes and are likely to participate in the pathogenesis of idiopathic inflammatory myopathies. The role of cytokines in these diseases is, however, poorly understood. Cytokine studies have shown raised serum interleukin 1 (IL1) receptor antagonist in patients with active polymyositis and dermatomyositis, but low levels of tumour necrosis factor α and IL1β.16 Immunohistochemical investigations of cytokines showed that cytokine expression in muscle tissue is dominated by IL1α, IL1β, and transforming growth factor (TGF)β1–3.17 Local release of T cell derived cytokines is not remarkable in inflammatory myopathies. To detect the cytokine secretion of peripheral blood mononuclear cells the ELIspot assay was used previously in one study, but no published data are available on intracellular cytokine production measured by flow cytometry in polymyositis and dermatomyositis.

To gain better insight into the pathogenesis of these myopathies we investigated cytokine levels in the sera of patients with polymyositis and dermatomyositis. We also used a flow cytometric assay to detect the peripheral blood lymphocyte subsets and the intracellular cytokines produced in these cells. An additional aim was to investigate the proportion of T helper (Th) 1 (CD4+/IFNγ+), Th2 (CD4+/IL4+), T cytotoxic (Tc) 1 (CD8+/IFNγ+), and Tc2 (CD8+/IL4+) lymphocytes in polymyositis and dermatomyositis. Patients were tested in both the active and the inactive stages of the disease.

METHODS
Patients and controls

Individuals enrolled in this study were patients suffering from polymyositis (37 women, 13 men; mean (SD) age 45.9 (13.7) years, range 20 to 69) or dermatomyositis (33 women, 16 men; age 46.9 (13.5) years, range 13 to 73) followed up in our outpatient department. Their diagnosis was defined on the basis of the international criteria of Bohan and Peter18 and the disease activity assessment was based on the clinical symptoms, muscle strength, and laboratory indices (serum creatinine kinase (CK) and lactate dehydrogenase (LDH) activity, table 1). The number of patients with active polymyositis was 13 (eight women, five men; mean (SD) age, 43.1 (14.1) years), while 24 patients were in the inactive stage of the disease (29 women, eight men; age 46.9 (13.6) years) at the initiation of the study. The number of patients with active dermatomyositis was 29 (18 women, 11 men; age...
Within the defined lymphocyte gate, based on their antigen positivity.

Measurement of intracellular cytokine expression of Th and Tc lymphocytes in whole blood

As resting lymphocytes do not contain substantial quantities of cytokines, we stimulated T cells in whole blood by phorbol myristate acetate (PMA, 25 ng/ml, Sigma) and ionomycin (1 μg/ml, Sigma) for four hours at 37°C. Activation was done in the presence of Brefeldin-A (Sigma, 10 μg/ml) which inhibits intracellular transport so that cytokines produced during the activation will be retained inside the cells. After the stimulation of the Th and Tc cells, samples were marked by quantum red labelled anti-CD4 or anti-CD8 (Sigma) mAb for 30 minutes at room temperature in the dark. Then FACS lysing solution (Becton Dickinson, San Jose, California, USA) was used for 10 minutes to lyse the erythrocytes and fix the whole blood leukocytes. After a washing step, the plasma membrane of the cells was permeabilised by the FACS permeabilising solution (Becton Dickinson) for another 10 minutes. The fixed and permeabilised leukocytes were labelled by anti-IFNγ-FITC and anti-IL4-PE (Becton Dickinson) or anti-IL10-PE mAb (CalTag, Burlingame, California, USA) for 30 minutes at room temperature in the dark. The samples were measured by a Coulter EPICS XL flow cytometer. Data from about 5000 Th or Tc lymphocytes were collected in each sample. These cells were gated, based on their side scatter/forward scatter and CD4 or CD8 positivity. The proportion of IFNγ, IL4, and IL10 positive cells was determined among the CD4+ and CD8+ T cells.

Measurement of autoantibodies

Anti-ENA and anti-Jo-1 (Hycor) antibodies were detected by enzyme linked immunosorbent assay kits (ELISA). We detected antinuclear factor (ANF) antibodies in HEP-2 cells by an indirect immunofluorescence technique.

Measurement of serum cytokine concentrations by ELISA

We measured the levels of circulating cytokines (IFNγ, IL4, and IL10) in the serum of patients and controls. Serum samples were collected on every experimental day and were stored at –80°C until testing with commercial cytokine ELISAs (OptEIA™ system, Pharmingen) according to the manufacturer’s instructions.

Statistical analysis

The differences between the healthy controls and the patients with polymyositis or dermatomyositis were calculated by Student’s unpaired t test. Correlations between variables were assessed by Pearson regression using the Statistica for...
Windows software. A significant difference was defined as $p < 0.05$.

RESULTS

Distribution of lymphocyte subpopulations in whole blood of patients suffering from active or inactive dermatomyositis or polymyositis

We found a major difference in lymphocyte phenotypisation only in the case of dermatomyositis. Patients in the active state of this disorder had a significantly decreased percentage of CD3$^+$ cells ($p = 0.01$) and CD8$^+$ cells ($p = 0.01$), and an increased percentage of CD19$^+$ cells ($p = 0.01$) compared with healthy controls and with the inactive disease state (table 2).

Beside the regular lymphocyte subtypes we also determined the percentage of activated T cells in our samples based on the HLA-DR or CD69 expression of CD3 positive T cells. Independent of the activity of the disorders we observed significantly increased frequencies only in the CD3$^+$/HLA-DR$^+$ cells both in dermatomyositis and polymyositis compared with the controls (control $\times$ active dermatomyositis, $p = 0.01$; $\times$ inactive dermatomyositis, $p < 0.01$; $\times$ active polymyositis, $p < 0.01$; $\times$ inactive polymyositis, $p < 0.01$; tables 2 and 3).

Intracellular cytokine expression in peripheral lymphocytes of patients with dermatomyositis

The frequency of IFN$\gamma^+$ Th1 cells was significantly lower ($p < 0.01$) in active dermatomyositis than in controls or in patients with inactive disease (active disease, 10.6 (7.1)%; inactive disease, 22.7 (10.0); control, 22.1 (10.0)). The frequency of IFN$\gamma^+$ Tc1 lymphocytes was also significantly decreased in active dermatomyositis compared with the controls (active disease, 24.4 (16.5)% $\times$ 43.4 (8.5)% in controls, $p < 0.01$) (fig 1A).

The percentage of IL4$^+$ Th2 cells was markedly increased in active dermatomyositis and decreased in inactive dermatomyositis compared with the controls (active disease, 1.01 (0.66)%; inactive disease, 0.33 (0.3)%; controls, 0.62 (0.54)%, and the difference between the values of the active and inactive states was significant ($p = 0.05$) (fig 1B). In CD8$^+$ cells we could not detect any measurable change in intracellular IL4 expression.

The frequencies of IL10-containing CD4$^+$ and CD8$^+$ T cells were higher in both active and inactive dermatomyositis, but the differences were significant only for inactive dermatomyositis (CD4$^+$/IL10$^+$: control, 2.8 (4.1)%; inactive dermatomyositis, 6.5 (5.6)%; $p = 0.04$; CD8$^+$/IL10$^+$: control, 3.7 (4.3)%; inactive disease, 8.8 (6.7)%; $p = 0.01$).

Table 3  T cell subpopulations and activated T cells of peripheral blood in controls and in patients with active and inactive polymyositis

<table>
<thead>
<tr>
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<th>Patients with aPM (n = 13)</th>
<th>Patients with iPM (n = 37)</th>
<th>Controls (n = 32)</th>
<th>aPM $\times$ control</th>
<th>iPM $\times$ control</th>
<th>aPM $\times$ iPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3$^+$ cells (%)</td>
<td>66.2 (9.4)</td>
<td>70.4 (8.1)</td>
<td>70.3 (7.2)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD4$^+$ cells (%)</td>
<td>42.3 (12.4)</td>
<td>46.9 (9.8)</td>
<td>45.2 (7.6)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD8$^+$ cells (%)</td>
<td>21.6 (10.2)</td>
<td>20.6 (6.4)</td>
<td>19.6 (5.9)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD19$^+$ cells (%)</td>
<td>13.8 (7.4)</td>
<td>10.9 (7.4)</td>
<td>11.7 (4.1)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD3$^+$ and HLADR$^+$ cells (%)</td>
<td>13.6 (14.3)</td>
<td>9.4 (7.8)</td>
<td>3.0 (2.3)</td>
<td>$p &lt; 0.01$</td>
<td>$p &lt; 0.01$</td>
<td>NS</td>
</tr>
<tr>
<td>CD3$^+$ and CD69$^+$ cells (%)</td>
<td>1.5 (1.0)</td>
<td>1.2 (0.9)</td>
<td>1.0 (0.8)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean (SD). The samples were measured by flow cytometry and lymphocytes were identified on the basis of their scatter properties.

aPM, active polymyositis; iPM, inactive polymyositis.

Figure 1  Expression of intracellular interferon $\gamma$ (IFN$\gamma$) (panel A) and interleukin 4 (IL4) (panel B) in stimulated peripheral T helper (Th) and T cytotoxic (Tc) cells of patients with dermatomyositis (DM) and polymyositis (PM). The samples were measured by flow cytometry, and lymphocytes were identified on their scatter properties and CD4/CD8 positivities.
The calculated Th2/Th1 frequencies were significantly lower in active dermatomyositis and higher in inactive disease than in the controls (fig 2).

**Intracellular cytokine expression in peripheral lymphocytes of patients with polymyositis**

The frequency of IFNγ and IL4 positive Th and Tc cells did not differ significantly compared with the healthy controls (fig 1A and 1B). Though the percentages of IL10 positive Th and Tc lymphocytes— independently of disease activity—were higher in the patients than in the controls, these differences were not significant (data not shown).

**Levels of soluble cytokines in serum of patients with dermatomyositis and polymyositis**

Besides the intracellular levels of cytokines we determined the cytokine patterns in the sera of patients with polymyositis and dermatomyositis using commercially available ELISA systems. Probably because of the high degree of individual variation and the low cytokine levels, hardly any significant differences could be observed between patients and controls.

In the case of dermatomyositis and polymyositis, independently of disease activity— were higher in the patients than in the controls, these differences were not significant (data not shown).

**DISCUSSION**

This study was undertaken to gain further insight into the pathogenesis of idiopathic inflammatory myopathies. The most important finding in our work was the pronounced difference in the frequency of the peripheral blood lymphocyte subsets and also in the intracellular cytokine content of these cells. We observed a significant increase in the frequency of IL4/IFNγ secreting cells. While in active dermatomyositis an altered Th1 and Th2 lymphocyte frequency was observed, as the percentage of IFNγ containing CD4+ and CD8+ cells was significantly decreased compared with the control population. This prominent change disappeared in the inactive disease state. On the other hand, in active dermatomyositis a marked increase in the frequency of CD4+IL4+ cells was observed, while in inactive dermatomyositis these changes disappeared and we detected a decrease in the IL4+CD4+ cell number compared with the controls. The frequency of IL4 producing cytokytic T lymphocytes was not different between patients and controls.

Studies of lupus-prone mice and humans indicate that immune abnormalities may be more closely associated with changes in the frequency of type 2-type 1 cytokine secreting cells, than with changes in the absolute number of cells producing any single cytokine. For this reason, we calculated the frequency of IL4/IFNγ secreting cells. While in active dermatomyositis a significantly increased Th2/Th1 frequency was detected, in inactive disease a decrease in Th2/Th1 frequency was found compared with the control population. Such alterations were not detected in polymyositis.

We also studied the number of IL10 producing Th and Tc cells. In dermatomyositis and polymyositis we observed an increase in the percentage of IL10 secreting cells, although this was significant only in inactive dermatomyositis. The reason for this discrepancy between the numbers of IL4 and IL10 producing T cells may be that IL10 is not a pure type 2 cytokine. IL10 is produced by both Th1 and Th2 lymphocytes and is an important immunoregulatory cytokine. IL10 has the ability to downregulate several major functions of Th1 cells and macrophages and can also control the Th2 mediated inflammatory processes. We detected higher levels of IL10 secreting T cells in dermatomyositis than in polymyositis, but the difference was not so marked between the two diseases as was observed in the number of IL4+ or IFNγ+ cells.

Lundberg and colleagues carried out indirect immunohistochemistry studies of muscle tissue sections with a panel of 16 different cytokine specific monoclonal antibodies in 15 untreated patients with polymyositis, dermatomyositis, and inclusion body myositis. They concluded that the cytokine expression in muscle tissue of patients with inflammatory myopathy is dominated by IL1α, IL1β, and TGFβ1–3. INFγ staining was detected in six of 15 patients, IL10 was observed in five patients, but IL4 was absent in all patients and the authors assumed that local release of T cell derived cytokines may not be a requirement for tissue injury in the inflammatory myopathies. There did not appear to be a qualitative difference in cytokine expression patterns in polymyositis, dermatomyositis, or inclusion body myositis. These results do not contradict our findings, as in autoimmune diseases the cytokine content of lymphocytes is not necessarily the same in the peripheral
blood and in the affected tissues. It is usually accepted that thyroid autoimmunity is an autoimmune disease mediated by Th1 cells, but recent results show a major difference in the balance of Th1 and Th2 cytokines secreted by cultures of thyroid and peripheral blood lymphocytes.\textsuperscript{24} To study the cytokine content of the peripheral blood mononuclear cells Hagiwara and colleagues used the ELIspot technique and showed that in dermatomyositis patients with active disease had significantly fewer peripheral blood mononuclear cells spontaneously secreting IFN-\gamma than normal controls.\textsuperscript{25} Moreover they showed that subjects with active dermatomyositis had fewer cells secreting IFN-\gamma than patients with inactive disease. Their results are consistent with our findings in the case of dermatomyositis, as they also detected an altered type 1 cytokine pattern. They did not investigate the number of IL4 containing cells but detected the frequency IL6 producing cells. They showed a modest but statistically significant increase in the number of cells secreting IL6 in patients with dermatomyositis. They also found an altered type 2/type 1 cytokine frequency in dermatomyositis, similar to our results, but they counted this frequency from the IL10/IFN\gamma rate. Instead of IL10 we used IL4 in our calculation, as type 1 T cells also produce IL10. In the case of polymyositis our results are inconsistent with the findings of Hagiwara and colleagues, as they found that the number of cells producing type 1 cytokines in serum, and other sources of production.\textsuperscript{25–27} Serum cytokine levels in polymyositis patients, however, cannot be used to determine the number of cells secreting cytokines. This is not surprising, as serum cytokine levels do not correlate with results of the intracellular cytokine staining.\textsuperscript{28–30}}

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