Autoantibodies against granulocyte–macrophage colony stimulating factor and interleukin-3 are rare in patients with Felty’s syndrome

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Objectives: Antibodies against granulocyte colony stimulating factor are frequently found in patients with Felty’s syndrome (FS). In this study, we examined the prevalence of antibodies against two other granulopoietic cytokines: granulocyte–macrophage colony stimulating factor (GM-CSF) and interleukin-3 (IL3).

Methods: Sera of 32 patients with FS, 20 normocytic patients with rheumatoid arthritis (RA), and 72 healthy individuals were screened for the presence of antibodies against GM-CSF and IL3 by ELISA and bioassays, using the human erythroleukaemia cell line TF-1.

Results: In two of the 32 patients with FS, antibodies to GM-CSF and IL3 were detectable by ELISA. Binding anti-GM-CSF antibodies were also detected in one of the 72 healthy controls, while in another healthy subject and in one of the patients with normocytic RA, anti-IL3 antibodies were present. Serum from one of the two patients with FS who tested positive for anti-IL3 and anti-GM-CSF antibodies by ELISA showed strong neutralising capacity to the biological effect of IL3, but not to GM-CSF in vitro. Patients with FS had significantly higher serum levels of GM-CSF (median; 2.82 pg/mL; interquartile range 2.64–3.19 pg/mL) compared with patients with RA (2.52 pg/mL; 2.28–2.72 pg/mL; p = 0.012) and healthy controls (2.23 pg/mL; 2.04–2.52; p < 0.001). In addition, serum levels of IL3 in patients were significantly higher in FS (10.05 pg/mL; 8.94–11.98) compared with controls (4.79 pg/mL; 3.72–7.22; p < 0.001), but not compared with RA patients (9.52 pg/mL; 8.32–10.42; p = 0.17).

Conclusions: Antibodies to GM-CSF and IL3 are rare in patients with FS and RA and in healthy subjects. In individual patients with FS, the presence of neutralising anti-IL3 antibodies may contribute to the development of cytopenia.
was to examine sera from patients with FS and healthy subjects for the presence of antibodies against GM-CSF and IL3.

PATIENTS AND METHODS

Patients

Thirty two consecutive patients with FS were included in this study. They were matched by age and sex with 20 patients with RA and no history of current or previous neutropenia and with 72 healthy subjects without haematological abnormalities, or autoimmune or malignant diseases. All patients with RA and FS fulfilled the American College of Rheumatology (formerly the American Rheumatism Association) classification criteria for RA, and have been described previously. FS was defined as chronic neutropenia (absolute neutrophil count (ANC) <1500/μL) of at least 3 months' duration. Neutropenia induced by drugs was ruled out by review of the patient's history and, if necessary, by withdrawal of and rechallenge with any inducible agent. None of the patients had received recombinant human GM-CSF for therapeutic purposes before or at the time of serum sampling.

Determination of haematological parameters

A white blood cell count with differential cell count, a platelet count, and haemoglobin concentration were determined by an automated cell counting system. Blood smears were examined microscopically for the presence of malignant cells and large granular lymphocytes (LGL). Patients with excess LGL were excluded. Bone marrow aspiration cytology and trephine biopsies were available for examination in 23 of the 32 patients with FS and did not provide evidence of haematological malignancies, myelodysplastic syndrome, or other causes of neutropenia unrelated to rheumatic disease.

Determination of GM-CSF and IL3 in serum

GM-CSF and IL3 were determined using highly sensitive quantitative ELISA (Biosource, Camarillo, CA, USA). The sensitivity was <1 pg/mL for the GM-CSF ELISA and <1.25 pg/mL for the IL3 ELISA. The intra-assay coefficients of variation were ≤7.3% for GM-CSF and ≤7.5% for IL3, and the interassay coefficients of variation were 5.4% and 4.8%, respectively.

ELISA for the detection of anti-GM-CSF and anti-IL3 antibodies

Serum samples were screened for the presence of anti-cytokine antibodies according to previously published protocols. Briefly, 96 well microtitre plates were coated with recombinant human (rh)GM-CSF or rhIL3 (both obtained from R&K Systems, Wiesbaden, Germany) at a concentration of 2.5 μg/mL in 100 μL PBS pH 7.0, and incubated overnight at 4°C. Uncoated wells were used as controls to exclude non-specific binding. After 24 hours, plates were washed with PBS containing 0.5% Tween 20 and then blocked with PBS/5% BSA for 30 minutes.

For the detection of IgG antibodies, serum samples were diluted 1:200 in PBS/5% BSA. To exclude non-specific binding in IgM assays due to the presence of rheumatoid factor (RF), all sera for determination of IgM antibodies were preincubated with 1:50 RF adsorbent (Behring Diagnostica, Marburg, Germany) for 15 minutes. All serum samples were also incubated with an Escherichia coli lysate (1:1; Sigma, Munich, Germany) to remove antibodies against E. coli that might cross-react with any residual E. coli antigen in the rhGM-CSF or rhIL3 preparations.

The coated microtitre plates were incubated with 100 μL of serum per well in duplicate for 2 hours at room temperature. After washing, peroxidase conjugated anti-human IgM or IgG (1:2000; Sigma) was added, and samples were incubated for 2 hours at room temperature. After washing four times, a substrate solution containing tetramethylbenzidine (Sigma-FAST OPD; Sigma) was added to each well and incubation continued for 30 minutes. The enzyme reaction was stopped by adding 0.5% sulphuric acid. The plates were read at 450 nm using a microtitre plate reader (Molecular Diagnostics, Chicago, IL, USA). Samples with an absorbance of 3 standard deviations above the mean of normal control absorbance values (n = 72) were deemed positive.

Bioassay for detection of neutralising anti-cytokine antibodies

The neutralising capacity of antibodies against GM-CSF or IL3 was tested using the human erythroleukaemic cell line TF-1 as previously described. These cells proliferate only when either GM-CSF or IL3 is present in the culture medium. Briefly, TF-1 cells (DSMZ, Braunschweig, Germany) from exponentially growing cultures were placed in 96 well microtitre plates and cultured in RPMI-1640 medium containing 5% fetal calf serum and increasing doses of either GM-CSF (0.1–10 000 ng/mL) or IL3 (0.1–10 000 ng/mL) at 37°C and 10³ cells/well (100 μL/well). We found strong proliferation of TF-1 cells in response to 1–10 000 ng/mL GM-CSF (data not shown) or 10–10 000 ng/mL IL3 (fig 1, panel A).

To test for the prevalence of neutralising anti-cytokine antibodies, cells were washed twice and incubated with patient serum (dilution 1:50) at 37°C for 48 hours in RPMI-1640 medium containing 5% FCS, GM-CSF (1000 ng/mL), or IL3 (1000 ng/mL) in 100 μL volume. Control experiments without GM-CSF, IL3, or serum were also performed. Proliferation was measured using a bromodeoxyuridine cell proliferation ELISA according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany). Results are expressed as the mean absorbance in the BrdU ELISA from three experiments for each serum and cytokine, respectively.

Statistical analysis

The Kolmogorov-Smirnov Z test was used to analyse the distribution of the samples. Normally distributed data are expressed as mean (SEM); non-parametrically distributed data are expressed as median (25th and 75th percentiles). For normally distributed data, statistical significance was tested using Student’s t test. The Mann-Whitney U test was used to compare non-parametrically distributed values between two groups. The χ² test with Yates’ correction was applied if appropriate. Regression analysis was used to calculate Spearman’s correlation coefficients. Statistical analysis was performed using SPSS for Windows (version 11.0; SPSS, Chicago, IL, USA).

RESULTS

Prevalence and characterisation of antibodies against GM-CSF and IL3

Binding antibodies against GM-CSF were found in sera of two of the patients with FS, none of the patients with RA, and one of the healthy controls (table 1). In one of the FS patients (patient 26) who tested positive for anti-GM-CSF antibodies, both IgG and IgM antibodies against GM-CSF were detected. The other FS patient (patient 3) and the subject in the control group tested positive in the anti-IgM ELISA only. All patient sera that tested positive for anti-GM-CSF antibodies by ELISA and most of the sera from the other FS and RA patients and the controls were evaluated for their ability to inhibit the biological activity of human GM-CSF in vitro. For this purpose, sera were tested for possible interference with the proliferation enhancing effect of GM-CSF on human TF-1 erythroleukaemic cells. The TF-1
any of the other subjects (table 1). Neutralising capacity of the anti-IL3 antibodies was assessed using TF-1 cells, which showed IL3 dependent proliferation at IL3 concentrations ranging from 10 to 10 000 ng/mL (fig 1). Addition of serum of patient 26, who had tested positive for anti-IL3 IgG and IgM by ELISA, to the culture medium resulted in a >50% inhibition of the proliferation enhancing effect of IL3 (fig 1). Proliferation, assessed by the optical density (absorbance) measured by a proliferation ELISA after incubation with serum from patient 26, was 0.32 compared with a median value of 0.74 (interquartile range (IQR) 0.62–0.92) in the anti-IL3 antibody negative controls (fig 1), which we interpreted as biologically relevant neutralising capacity. This patient also had by far the highest optical density readings in the anti-IL3 antibody ELISA (IgG 0.75, IgM 0.64) compared with controls (mean (SD) IgG 0.22 (0.06), IgM 0.25 (0.09); n = 72), suggesting a high concentration of neutralising anti-IL3 antibodies. In contrast, serum from patient 3, serum from one of the healthy controls who tested positive for anti-IL3 IgM by ELISA, and all tested anti-IL3 negative sera showed no significant neutralisation of IL3 bioactivity in vitro (fig 1).

In order to analyse the clinical significance of the anti-GM-CSF antibodies found in the two patients with FS, these two anti-GM-CSF antibody positive FS patients were compared with the other 30 anti-GM-CSF antibody negative FS patients (table 2). The patient (no. 26) with neutralising anti-IL3 antibodies and non-neutralising anti-GM-CSF antibodies had trictrypedia with moderate anaemia, thrombocytopenia and agranulocytosis. Absolute neutrophil counts of this patient were about 10-fold lower than the mean of the other 28 anti-IL3 and anti-GM-CSF antibody negative FS patients (table 2). The serum of this patient also contained high levels of anti-G-CSF antibodies. IL3 serum levels in this patient were slightly higher than in the other FS patients. In patient 3, who had non-neutralising antibodies against GM-CSF and IL3, neutropenia was less severe than in patient 26; however, thrombocytopenia and modest anaemia were also present. Anti-G-CSF antibodies were not found in serum of patient 3. Unfortunately, insufficient amounts of serum were available to test for the presence of anti-G-CSF antibodies in the remaining patients. As in the majority of FS patients, examination of bone marrow cytology disclosed inflammatory activity in both patients.

**Serum levels of GM-CSF and IL3**

Patients with FS had significantly higher serum levels of GM-CSF (median 2.82 pg/mL; IQR 2.64–3.19 pg/mL) compared with patients with RA (2.52 pg/mL; IQR 2.28–2.72 pg/mL; p = 0.012) and healthy controls (2.23 pg/mL; IQR 2.04–2.52; p < 0.001). Serum levels of GM-CSF in patients with RA were also significantly higher compared with controls (fig 2). In patients with FS, significantly higher serum levels of IL3 were found (10.05 pg/mL (IQR 8.94–11.98) compared with controls (4.79 pg/mL (3.72–7.22); p < 0.001), but not compared with RA patients (9.52 pg/mL; 8.32–10.42; p = 0.17) (fig 2).

**DISCUSSION**

The data presented here expand the results of previous studies showing that not only is the prevalence of binding anti-GM-CSF antibodies low in healthy individuals but that antibodies to IL3 are also very rarely present in sera of healthy subjects and patients with rheumatoid arthritis. As in our previous study, we used stringent criteria defining positivity for anti-cytokine antibodies in ELISAs and prepared all sera to avoid non-specific binding due to the presence of rheumatoid factor and to avoid false positive results due to cross-reactivity with anti-*E. coli* antibodies.
Anti-cytokine antibodies in FS 865

various forms of haematological malignancies treated with GM-CSF antibodies have been found in sera of patients with neutropenia, as observed in other types of neutropenia. CSF are probably a physiological and specific reaction to chronic serum levels of G-CSF in FS compared with IL3 and GM-poietic effect on various cell types. Therefore, the much higher GM-CSF and IL3 are characterised by a broader haemato-G-CSF exclusively regulates the production of neutrophils, levels of G-CSF. In the present study, serum levels of GM-bodies in patients with FS is associated with high serum G-CSF, but not to GM-CSF or IL3 in most patients with FS. IL3, may predispose to the development of autoantibodies to Thus, chronically high levels of G-CSF, but not GM-CSF and IL3 in only two of 32 patients with FS. With these precautions, we found antibodies to GM-CSF and IL3 in only two of 32 patients with FS.

We earlier demonstrated the presence of neutralising and non-neutralising antibodies to another granulopoietic cytokine, G-CSF, in a much larger proportion (73%) of patients with FS. It is unknown why the induction of antibodies against granulopoietic growth factors in FS appears to be restricted to G-CSF in most patients. However, results of the present study and previous observations suggest that chronically or repeatedly elevated levels of a cytokine is one of the major contributing factors leading to the induction of autoantibodies to the respective cytokine. The induction of neutralising anti-erythropoietin antibodies has been reported in patients treated with recombinant erythropoietin for anaemia caused by chronic renal failure. Anti-G-CSF and GM-CSF antibodies have been found in sera of patients with various forms of haematological malignancies treated with rhG-CSF or rhGM-CSF for chemotherapy induced neutropenia. Anti-G-CSF antibodies in patients with FS is associated with high serum levels of G-CSF. In the present study, serum levels of GM-CSF and IL3 were also elevated compared with controls; however, although significant, the observed differences between FS patients and controls were much smaller for CSF and IL3 were also elevated compared with controls; however, although significant, the observed differences between FS patients and controls were much smaller for GM-CSF and IL3 than those observed for G-CSF. While G-CSF exclusively regulates the production of neutrophils, GM-CSF and IL3 are characterised by a broader haemato-poietic effect on various cell types. Therefore, the much higher serum levels of G-CSF in FS compared with IL3 and GM-CSF are probably a physiological and specific reaction to chronic neutropenia, as observed in other types of neutrophenia. Thus, chronically high levels of G-CSF, but not GM-CSF and IL3, may predispose to the development of autoantibodies to G-CSF, but not to GM-CSF or IL3 in most patients with FS.

In agreement with this hypothesis, the sera of two patients with FS in the present study who had higher than expected serum levels of IL3 and GM-CSF (associated with and possibly due to pancytopenia) contained autoantibodies to both GM-CSF and IL3. Serum from one of those patients showed strong neutralising activity to IL3 in a bioassay, suggesting that anti-IL3 antibodies might contribute to the development of cytopenia in this particular patient. To our knowledge, our study is the first report on the prevalence of anti-IL3 antibodies in human disease. However, the development of neutralising anti-IL3 antibodies was shown in rhesus monkeys given intravenous recombinant rhesus monkey IL3 continuously for only 1–2 weeks, showing that elevated levels of homologous IL3 in the circulation can be immunogenic. Although there is no direct evidence for the hypothesis that continuously elevated levels of endogenous cytokines can induce the development of autoantibodies in the same way as recombinant cytokines, the development of antibodies against endogenous cytokines has been observed in the presence of elevated cytokine levels in human disease. Antibodies against endogenous IL6 were found in patients with systemic sclerosis, and serum levels of IL6 were higher in anti-IL6 positive patients compared with anti-IL6 negative patients and healthy subjects. During progressive viral infection, in which interferon-γ levels are usually elevated, increasing levels of anti-interferon-γ antibodies were also found.

In summary, our results support previous observations that not a single mechanism, but rather a great number of different cellular and humoral immune mechanisms contribute

<table>
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<tr>
<th>Table 2</th>
<th>Characteristics of two patients with Felty’s syndrome and autoantibodies to GM-CSF and IL3 compared with 30 FS patients without anti-GM-CSF or anti-IL3 antibodies</th>
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<tbody>
<tr>
<td>Anti-GM-CSF negative FS patients (n = 30)</td>
<td>Patient 26: anti-GM-CSF+, anti-IL3+</td>
</tr>
<tr>
<td>Age (years)</td>
<td>62.7 (2.0)</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>15/15</td>
</tr>
<tr>
<td>ANC (n/L)</td>
<td>2.17 (0.41)</td>
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<tr>
<td>ALC (n/L)</td>
<td>1.00 (0.10)</td>
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<tr>
<td>Platelets (n/L)</td>
<td>12.2 (0.3)</td>
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<tr>
<td>Haemoglobin (g/dL)</td>
<td>11.51 (0.65)</td>
</tr>
<tr>
<td>GM-CSF (pg/mL)</td>
<td>2.82 (2.65–3.17)</td>
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<tr>
<td>IL3 (pg/mL)</td>
<td>9.85 (8.94–11.91)</td>
</tr>
<tr>
<td>Bone marrow</td>
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<tr>
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<td>Lymphocytosis</td>
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<td>RF positive</td>
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<td>Splenomegaly</td>
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Serum from patient 26 contained neutralising antibodies to IL3 and non-neutralising antibodies against GM-CSF. Serum from patient 3 contained non-neutralising antibodies against IL3 and GM-CSF. Values for parametrically distributed values (ANC, ALC, haemoglobin, platelets) of the anti-GM-CSF/IL3 antibody negative patients with FS are displayed as means (SEM). Non-parametrically distributed values are displayed as medians (IQR).
to the development of neutropenia in FS, of which one or only a limited number are simultaneously operative in an individual patient.

ACKNOWLEDGEMENTS

The authors wish to thank Ms N Pfänder for technical assistance and Dr E Csernok for measurement of anti-G-CSF antibodies.

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