Induction of Anti-Recombinant Human Granulocyte-Macrophage Colony-Stimulating Factor (Escherichia coli-Derived) Antibodies and Clinical Effects in Nonimmunocompromised Patients

By Peter Ragnhammar, Heinz-Jürgen Friesen, Jan-Erik Frödin, Ann-Kari Lefvert, Moustapha Hassan, Anders Österborg, and Håkan Mellstedt

The pharmacokinetics of recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF), induction of anti-GM-CSF antibodies, and clinical effects related to the induction of the antibodies were analyzed in patients with metastatic colorectal carcinoma (CRC) who were not on chemotherapy (n = 20, nonimmunocompromised patients). rhGM-CSF (250 μg/m²/d; Escherichia coli-derived) was administered subcutaneously for 10 days every month for 4 months. Eight patients with multiple myeloma (MM) on intensive chemotherapy followed by rhGM-CSF treatment were also included (immunocompromised patients). After a single injection of GM-CSF at the first cycle in CRC patients, the maximum calculated concentration (Cmax) was 5.24 ± 0.58 ng/mL; the half-life (T1/2) was 2.91 ± 0.8 hours; and the area under the concentration curve (AUC) was 30.86 ± 6.03 hours × ng/mL (mean ± SE). No anti–GM-CSF antibodies were detected. During the subsequent cycles, 95% of the CRC patients developed anti–GM-CSF IgG antibodies, which significantly altered the pharmacokinetics of rhGM-CSF at the third and fourth cycles with decreased Cmax (2.87 ± 0.57 ng/mL; P < .05), T1/2 (1.57 ± 0.2 hours; P < .05), and AUC (14.90 ± 4.10 hours × ng/mL; P < .005). The presence of anti–GM-CSF antibodies significantly reduced the GM-CSF–induced enhancement of granulocytes, and there was a clear tendency for a decreased increment of monocytes. Antibodies diminished systemic side effects of rhGM-CSF. Only 1 of 8 MM patients showed a very low anti–GM-CSF antibody titer after GM-CSF therapy, as shown by enzyme-linked immunosorbent assay and Western blot. Therefore, in nonimmunocompromised patients, exogenous nonglycosylated GM-CSF induced an anti–GM-CSF IgG antibody response in practically all patients, which seemed to be of clinical significance. In immunocompromised patients, virtually no significant antibody response was shown. © 1994 by The American Society of Hematology.

Colony-stimulating factors (CSFs) have pleiotropic effects, inducing maturation and functional activation of blood cells. Several hematopoietic growth factors (ie, interleukin-1, IL-3, IL-6, IL-11, erythropoetin, stem cell factor, macrophage-CSF, granulocyte-CSF, and granulocyte-macrophage–CSF [GM-CSF]) have been identified with the major clinical applications to support myelopoiesis, differentiation of major histocompatibility complex class I1 antigen expression on monocytes,2 activation of blood cells. Several hematopoietic growth factors (ie, interleukin-1, IL-3, IL-6, IL-11, erythropoetin, stem cell factor, macrophage-CSF, granulocyte-CSF, and granulocyte-macrophage–CSF [GM-CSF]) have been identified with the major clinical applications to support myelopoiesis, differentiation of major histocompatibility complex class I1 antigen expression on monocytes,2 activation of blood cells.

GM-CSF might also be a useful cytokine for biotherapy of malignant diseases. GM-CSF induces (1) differentiation of monocytes to large macrophage-like cells,3,2 augmentation of major histocompatibility complex class II antigen expression on monocytes,2,3 (3) enhancement in vitro of macrophage and granulocyte spontaneous cytotoxicity and antibody-dependent cellular cytosis,8 and (4) increased expression of adhesion molecules on granulocytes and monocytes.2,6 The immunogenicity of tumor cells may be enhanced by GM-CSF through facilitating "tumor antigen" presentation.8 GM-CSF has also been shown to cooperate with other cytokines in the expansion of specific T cells.9 Transfection of the GM-CSF gene to tumor cells with the aim to increase the immunogenicity of the cells and to locally stimulate effector cells might also be an interesting approach.10 GM-CSF alone might induce tumor regression in humans, probably by activating specific and unspecific killer cells.11,12 Therapeutic unconjugated monoclonal antibodies (MoAbs) may eradicate tumor cells in vivo by activating various immune functions.13 Some of these mechanisms such as antibody-dependent cellular cytosis and induction and amplification of an idiotypic network response may be augmented by GM-CSF.14-16 Based on our therapeutic experience with the anticolon carcinoma MoAb 17-1A (mouse IgG2A) alone,15 on in vitro results combining GM-CSF and MoAb 17-1A for killing of human carcinoma cell lines16 and on the above-mentioned effects of GM-CSF, a therapeutic trial in patients with metastatic colorectal carcinoma (CRC) was initiated combining GM-CSF and MoAb 17-1A. Long-lasting complete remissions were noted.17 The effects on the cytotoxic capability of peripheral blood mononuclear cells in vivo and on cell subsets involved in the cytotoxic reactions have recently been described.18

Detailed information on various aspects of exogenously administered GM-CSF should be of value to optimize the use of this cytokine as an antineoplastic and hematopoietic stimulatory agent. In this report, we describe the pharmacokinetics of GM-CSF and the induction of anti–GM-CSF antibodies as well as some clinical effects connected to the induction of these antibodies. The antibody response was also related to whether the patients were nonimmunocompromised (no concomitant chemotherapy) or immunocompromised (concomitant intensive chemotherapy).
MATERIALS AND METHODS

Patients

CRC. Twenty patients (12 males and 8 females) entered a phase II trial (Table 1). The median age was 65 years (range, 14 to 78 years). All patients had metastatic CRC and a Karnofsky index of ≥80%. One patient had received chemotherapy and irradiation 5 months earlier, and another had chemotherapy 2 months previously. All others were, except for primary surgery, untreated.

Multiple myeloma (MM). Eight patients (2 men and 6 women) with MM clinical stage IIA or IIB according to Durie and Salmon29 were included (Table 2). The diagnostic criteria for MM have been described previously.30 The median age was 69 years (range, 50 to 74 years). All patients were previously untreated and had a Karnofsky index of ≥80%.

Treatment Schedule

CRC patients. Recombinant human GM-CSF (rhGM-CSF) produced in Escherichia coli (specific activity, 5 x 10^7 IU/mg protein; Behringwerke AG, Marburg, Germany) was administered at a dose of 250 µg/m²/SC daily for 10 days. Each cycle was repeated every fourth week. Four cycles were administered.15 If the disease progressed during the study period, the patients were withdrawn from the study and were offered chemotherapy.

MM patients. The MM patients entered a phase I multicenter trial studying escalating doses of cyclophosphamide in combination with interferon-α (IFN-α) and betamethasone. The dose of cyclophosphamide (Cyclofosfamid; Orion, Espoo, Finland) ranged from

Table 1. Characteristics of Patients With CRC

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (Yr/Sex)</th>
<th>Clinical Site</th>
<th>Previous Therapy</th>
<th>PS-T (Mo)</th>
<th>Site of Metastases</th>
<th>No. of GM-CSF Treatment Cycles</th>
<th>Total Dose of GM-CSF (µg)</th>
<th>Maximum Anti-GM-CSF (IgG) Titer (OD)</th>
<th>Day of Maximum Antibody Titer From Start of Therapy</th>
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<tr>
<td>1</td>
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<td>10,500</td>
<td>0.500</td>
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<td>D</td>
<td>S</td>
<td>17</td>
<td>Lung</td>
<td>3</td>
<td>13,500</td>
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<td>4</td>
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<td>Rectum (M)</td>
<td>D</td>
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<td>2</td>
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<td>19,000</td>
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<tr>
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<td>4</td>
<td>19,600</td>
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<td>Liver, Lymph nodes</td>
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<td>16,200</td>
<td>0.430</td>
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Abbreviations: M, moderately differentiated; P, poorly differentiated; MACC, modified Astler Coller class; S, surgery; Chemo, chemotherapy; Irrad, irradiation; PS-T, time from primary surgery to start of therapy.
500 mg/m² to 950 mg/m² IV. CY was administered on days 1 and 3 of a 4-day treatment cycle. IFN-α (WellferonR; Wellcome Ltd, Beckenham, London, UK) was administered daily SC on days 1 through 4. Betamethasone (30 mg daily; BetapredR; Glaxo, Middlesex, UK) was administered orally on days 1 through 4. The cycle was repeated every fourth week. All MM patients received 5 µg/kg/d of GM-CSF (mologramostim; E coli-derived; LeucomaxR; Schering-Plough Corp, Kenilworth, NJ) SC from day 5 of a cycle until the day when the granulocyte count exceeded 1.5 × 10⁹/l, and the nadir platelet count was passed. The individual number of treatment cycles as well as the total dose of rhGM-CSF are shown in Table 2. In all MM patients, GM-CSF was administered for 7 to 12 consecutive days, with a median time of 9 days.

Serum samples for anti–GM-CSF antibody analyses were drawn at least 10 days after a GM-CSF injection.

**Blood Cell Counts**

The total number of white blood cells (WBCs) were counted using a Zeiss standard RA microscope in ordinary light (magnification × 1,000). Two hundred cells were counted. The percentage of WBC subsets was determined by a differential count on smears of peripheral blood stained with May–Grunwald and Giemsa. The total number of blood cell populations was calculated from these figures.

**Serum Sampling**

Venous blood was collected in sterile tubes. The serum was separated and stored at −70°C until assayed.

**GM-CSF Assay**

The pharmacokinetics of GM-CSF were determined on the first day of a cycle. GM-CSF was assayed in enzyme-linked immunosorbent assay (ELISA), using flat-bottomed microtiter ELISA plates (96 wells; Costar, Cambridge, MA), coated with a mouse monoclonal antihuman GM-CSF antibody (IgG; Genzyme, Cambridge, MA, USA) overnight at 4°C. After blocking with 8% bovine serum albumin (Semper, Stockholm, Sweden) diluted in phosphate buffer (pH 9.7) for 2 hours at 37°C, the serum samples and the GM-CSF standard (in human normal AB serum [1:6]; Behringwerke AG) diluted in phosphate buffer (pH 7.2) were added in duplicates and incubated overnight at 4°C. The serum samples as well as the GM-CSF standard were passed through a Protein A Sephadex column (CL-4B; Pharmacia, Uppsala, Sweden) overnight at 4°C. After blocking with 8% bovine serum albumin (Semper, Stockholm, Sweden) diluted in phosphate buffer (pH 9.7) for 2 hours at 37°C, the serum samples as well as the GM-CSF standard were coated with 125 µL per well of rhGM-CSFBehringwerke preparation (1 µg/mL) in diethanolamine buffer, pH 9.8, the absorbance was read at 405 nm using an automatic ELISA reader (Multi-Scan plus; Laboratory Systems, Helsinki, Finland).

The samples (diluted 1:50 in OWBE) were preincubated without or with E coli lysate (2%) for 1 hour at room temperature. The filter was then incubated with the samples at room temperature overnight under continuous agitation. After 5 washes in phosphate-buffered saline containing 0.5% Tween 20, the filter was incubated for 1 hour at room temperature with a goat antirabbit IgG antiserum (Behringwerke AG) and incubated at room temperature for 1 hour. (This procedure was introduced to remove E coli-directed antibodies.) A total of 100 µL of the serum samples was then added to microtiter wells in duplicate and incubated at room temperature for 2 hours. The wells were washed in phosphate-buffered saline containing 0.5% Tween 20. The plates were then incubated with 0.1 mL per well of horseradish peroxidase conjugated antihuman IgM (1:40; product no. OSDJ; Behringwerke AG) or antihuman IgG (1:40; product no. OSDH; Behringwerke AG), respectively, for 2 hours at room temperature. After washing 4 times, 0.1 mL of the substrate tetramethylbenzidine (product no. OUVI0; Behring Diagnostica) was added to each well, and the plate was incubated for 30 minutes at room temperature. The enzyme reaction was stopped by adding 0.5 N sulfuric acid. The plate was read at 450 nm using a Behring ELISA Processor BEP II (Behring Diagnostica).

Serum samples with an optical density (OD) value above the range of 300 normal blood donors were considered positive, were further quantitated by ELISA, and were applied to Western blot analysis as a confirmatory assay. The OD values were related to a synthetic standard composed of rabbit antihuman–GM-CSF Fab′ fragments chemically linked to human IgG or IgM molecules, respectively (patent no. EPA 291086 [Boehringer Mannheim] and DEOS 3112334 [Behringwerke AG]). The synthetic standard has the property of binding to GM-CSF by the Fab′ fragment of rabbit Ig and to antihuman Ig antibodies through the human Ig part of the molecule.

**Western Blotting**

rhGM-CSF was separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 16%) using the Laemmli buffer system. The protein was electroblotted into nitrocellulose filters (Hybond E-Transfer; Amersham) according to the method of Towbin et al using 25 mmol/L Tris to 192 mmol/L glycine/20% methanol buffer (pH 8.3; vol/vol). A voltage gradient of 6 V/cm was applied for 1 hour. Each blot was checked by AuroDye protein staining (Amersham). After blotting, the filter was immersed in blocking solution (TRIS/HCl buffer, pH 8.1), containing lactofeinn, gelatine, bovine serum, and 0.5% Tween 20 (product no. OWBE) and agitated for 1 hour at room temperature.

The samples (diluted 1:50 in OWBE) were preincubated without or with E coli lysate (2%) for 1 hour at room temperature. The filter was then incubated with the samples at room temperature overnight under continuous agitation. After 5 washes in phosphate-buffered saline containing 0.5% Tween 20, the filter was incubated for 1 hour at room temperature with a goat antihuman IgG labeled with colloidal gold (Amersham). The signal was amplified, according to the manufacturer’s instructions, using a silver enhancement kit (IntenseSE; Amersham). In each test, a positive serum was included showing the same staining pattern as the synthetic standard.

**Serum Immune Complexes**

Immune complexes in serum were analyzed using the C1q binding technique as described in detail previously.

**Pharmacokinetics**

The pharmacokinetics parameters for subcutaneous administration were evaluated and fitted by a one-compartment model with the
first order of absorption. Parameter estimation using nonlinear least squares analysis was performed using PCNONLIN (Statistical Consultants Lexington, KY).

**Statistical Analyses**

The differences between means were analyzed using Student’s t-test or Wilcoxon signed rank test for paired and unpaired observations. The linear regression model was used to estimate correlation between independent observations.

**RESULTS**

**Pharmacokinetics of GM-CSF**

GM-CSF was not detectable in serum before therapy. After an SC injection of 250 μg/m² of GM-CSF at cycle I, the maximum calculated concentration (C_{max}) was 5.24 ± 0.56 ng/mL and was reached at 3.25 ± 0.25 hours after the injection. The C_{max} at cycles III and IV in the same patients was 2.87 ± 0.57 ng/mL after 2.67 ± 0.33 hours. The difference in serum C_{max} was statistically significant (P < .05). The corresponding serum half-lives (T_{1/2}) were 2.91 ± 0.8 hours and 1.59 ± 0.2 hours, respectively (P < .05).

There was also a statistically significant difference in the area under the concentration curve (AUC) comparing cycle I (30.86 ± 6.03 hours × ng/mL) with cycles III and IV (14.90 ± 4.10 hours × ng/mL; P < .005; see Fig 1). The decrease in C_{max}, T_{1/2}, and AUC by increasing number of treatment cycles was related to the induction of GM-CSF antibodies (see below).

**Induction of Anti–GM-CSF Antibodies**

A high proportion of normal individuals has antibodies against E coli, usually of high affinity. Such antibodies may interfere in the assay system because the rhGM-CSF preparation for coating of the plates might contain minute amounts (2.8 ppm) of E coli proteins. To avoid false-positive results, the serum samples were preincubated with an E coli lysate to adsorb anti-E coli antibodies. Analyses of serum samples not preincubated with the E coli lysate gave higher OD values (data not shown). Therefore, our assay system has an improved specificity for anti–GM-CSF antibodies.

An OD value of greater than 0.3 was used as cutoff for anti–GM-CSF IgG antibodies, and an OD value of greater than 0.15 was used for IgM antibodies. In the control population of 300 healthy blood donors, greater than 95% of the individuals had OD values below these levels. Of the 300 donors, 5 had antibodies against GM-CSF detected by ELISA, which was confirmed by a weak but specific staining in Western blot (data not shown).

No CRC patients had antibodies against GM-CSF before therapy. Anti–GM-CSF IgM antibodies were not detected with the present sampling design. During GM-CSF therapy in the CRC patients, increasing titers of specific anti–GM-CSF IgG antibodies were noted (Figs 2 and 3 and Table 1). The GM-CSF antibody titers had disappeared at about 30 weeks after the last injection (Fig 2). At the end of cycles II and IV, 50% and 90%, respectively, of the tested patients had IgG antibodies (Fig 3). In total, 19 of the 20 CRC patients (95%) developed anti–GM-CSF IgG antibodies (Table 1). Three groups of CRC patients could be distinguished with regard to the level of antibody titers induced: no antibody titer (n = 1; CRC patient no. 17); low antibody titers (n = 5; CRC patients no. 2, 6, 13, 15, and 20); and high antibody titers (n = 14; CRC patients no. 1, 3, 4, 5, 7, 8, 9, 10, 11, 12, 14, 16, 18, and 19).

The presence of anti–GM-CSF IgG antibodies was confirmed by Western blot analyses in all patients, as shown in Fig 4. In addition to the GM-CSF bands (14.5 kD), several weak bands with a molecular weight (MW) corresponding to the MW of E coli proteins (ca 20 kD) could be observed after protein staining (Fig 4, lane A). Immunoblotting of sera preincubated with E coli lysate using an antihuman IgG antiserum showed no bands at the place of E coli proteins but showed only antibodies reacting with GM-CSF (Fig 4, lane B). Sera not preincubated with E coli lysate showed the
presence of antibodies against *E coli* as well as GM-CSF (Fig 4, lane C). Blotting of the synthetic antibody standards (IgG and IgM) showed a similar pattern to that of the induced antibodies (Fig 4, lanes D and E). In 2% of the 300 normal donors, anti-*E coli* IgG antibodies could be detected (Fig 4, lane F) that disappeared after preincubation with *E coli* lysate (Fig 4, lane G).

From an immunologic point of view, the CRC patients might be regarded as having a noncompromised immune system because they had not previously received chemotherapy (with the exception for 2 patients who had been administered cytostatics 2 to 5 months earlier). All CRC patients had a good performance status. For comparison, a group of MM patients who had been treated with an intensive chemotherapy regimen followed by GM-CSF administration to support bone marrow regeneration (immunocompromised patients) was included. The MM patients received rhGM-CSF in a similar scheduling as the CRC patients (see Materials and Methods). The number of cycles administered before testing for anti–GM-CSF antibodies in MM patients varied between 2 and 6 (median no., 4). Only 1 MM patient (MM patient no. 8) had detectable anti–GM-CSF antibodies after 4 GM-CSF treatment cycles. The antibody titer was very low. The OD value (0.312; see Table 2 and Fig 5) was just above the cutoff level (0.300), and a weak band was noted on Western blot (data not shown).

In the remaining 7 patients, no anti–GM-CSF antibodies were detected by ELISA and Western blot (Fig 5). The difference in number of patients with regard to anti–GM-CSF antibody induction between the two groups was statistically, highly significant ($\chi^2 = 5.23; P < .001$).

The two patient groups both received *E coli*-derived GM-CSF. However, CRC patients were administered a recombinant protein produced by Behringwerke, and MM patients received rhGM-CSF from Schering-Plough. As can be observed in Fig 5, CRC patients treated with rhGM-CSF/Behringwerke developed antibodies reacting with rhGM-CSF/Behringwerke as well as with rhGM-CSF/Schering-Plough.

### Clinical Effects of Anti–GM-CSF Antibodies

**Pharmacokinetics.** The induction of anti–GM-CSF antibodies was associated with a significant reduction of $C_{\text{max}}$, $T_{1/2}$, and AUC of exogenously administered GM-CSF (see above and Fig 1).

![Fig 3. Frequency of patients with anti–GM-CSF IgG antibodies at day 1 and day 10 of each treatment cycle (I through IV). Patients with no detectable anti–GM-CSF antibodies (■) and with anti–GM-CSF antibodies (■). Figures with brackets indicate the number of patients receiving the respective GM-CSF cycle.](image)

![Fig 4. Western blotting of anti–GM-CSF antibodies (CRC patient no. 9). Mobilities of the MW standards are indicated at the left margin: (1) bovine trypsin inhibitor, 6.2 kD; (2) lysozyme, 14.3 kD; (3) β-lactoglobulin, 18.4 kD; (4) carbonic anhydrase, 28.5 kD; and (5) ovalbumin, 43.8 kD. (Horizontal arrow indicates the localization of the *E coli* bands.) Lane A shows AuroDye staining of blots. Note the GM-CSF main band with an MW of 14.5 kD and the main *E coli* bands at ca. 20 kD. Lane B shows immunoblotting of a patient serum after preincubation with *E coli* lysate. Note only visible bands at the place of GM-CSF. Lane C is the same as for lane B but without preincubation with *E coli* lysate. Note also the presence of anti-*E coli* antibodies. Lanes D and E show immunoblotting after adding the anti–GM-CSF IgM (D) and anti–GM-CSF IgG (E) synthetic standards. Lane F shows immunoblotting of a normal blood donor serum without preincubation with *E coli* lysate. Note the detection of anti-*E coli* antibodies. Lane G is the same as for lane F but with preincubation with *E coli* lysate. Note the disappearance of anti-*E coli* antibodies.](image)
Fig 5. Anti–GM-CSF IgG antibody titers (ELISA) in 10 CRC patients (see Table 1) and 8 MM patients (see Table 2) tested against plates coated with GM-CSF/Behringwerke (■) or GM-CSF/Schering-Plough (□).

WBCs. At treatment cycle IV, in CRC patients, the maximum total numbers of WBCs, neutrophils, and eosinophils were significantly lower than the peak values at the previous cycles (P < .05). There was also a clear tendency by each treatment cycle for gradually decreased monocyte counts at day 10 (P = .06). No tendency for a decreased increment of lymphocytes was noted (Table 3). Patients with low anti–GM-CSF antibody titers showed significantly higher values of WBCs, eosinophils, and neutrophils at day 10 of cycle IV as compared with patients with high anti–GM-CSF antibody titers (Table 4). The individual WBC counts at day 10 of each cycle for those 13 patients completing 4 treatment courses are shown in Fig 6. The total number of WBCs was unchanged during the 4 treatment cycles in the patient (CRC17) not developing anti–GM-CSF antibodies, whereas there was a modest decrease in the low-titer patients and a marked decrease at treatment cycles III and/or IV in the high-titer group.

Moreover, in the high-titer group, there was a statistically significant correlation between the anti–GM-CSF antibody titer and total number of WBCs (r = −0.438; P < .005), neutrophils (r = −0.467; P < .001), and eosinophils (r = −0.334; P < .05) when comparing day 10 of all treatment cycles. By increasing anti–GM-CSF titers, the cell counts decreased. In patients with low antibody titers, no such correlation could be noted.

After each treatment cycle, WBC counts returned to baseline level within 5 days. There was no significant influence on the total number of WBCs during long-term follow-up (Fig 7).

Side effects. The main side effects in all CRC patients at cycle I that were considered to depend on the GM-CSF treatment were myalgia (50%), local irritation (45%), fever (greater than 38.5°C; 35%), bone pains/sternalgia (35%), rash/pruritus (35%), conjunctivitis (30%), headache (25%), nausea (15%), and chills (15%). Myalgia was easily controlled by dextropropoxiphene. However, in 1 patient (CRC patient no. 1), the GM-CSF dose had to be reduced because of severe myalgia. Myalgia appeared at days 2 to 3 and disappeared at days 8 to 9 of the cycle. Fever (greater than

<table>
<thead>
<tr>
<th>Blood Cells</th>
<th>Cycle I (n = 20)</th>
<th>Cycle II (n = 20)</th>
<th>Cycle III (n = 18)</th>
<th>Cycle IV (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 10</td>
<td>Day 1</td>
<td>Day 10</td>
</tr>
<tr>
<td>WBC</td>
<td>6.66 ± 0.35</td>
<td>26.24 ± 1.78*</td>
<td>6.35 ± 0.39</td>
<td>30.41 ± 1.97*</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>4.42 ± 0.35</td>
<td>17.12 ± 1.58†</td>
<td>4.21 ± 0.37</td>
<td>19.24 ± 1.31†</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.27 ± 0.05</td>
<td>4.47 ± 0.57†</td>
<td>0.21 ± 0.03</td>
<td>6.38 ± 0.79†</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.02 ± 0.01</td>
<td>0.06 ± 0.02</td>
<td>0.02 ± 0.01</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.42 ± 0.05</td>
<td>1.16 ± 0.23</td>
<td>0.37 ± 0.05</td>
<td>0.96 ± 0.17†</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1.53 ± 0.10</td>
<td>2.72 ± 0.24*</td>
<td>1.54 ± 0.12</td>
<td>3.27 ± 0.32†</td>
</tr>
</tbody>
</table>

* P < .05 and †P < .01 comparing day 10 of each cycle.

† P < .05 and †P < .01 comparing day 10 of cycle I and day 10 of cycle IV.
Table 4. Total No. of Blood Cells (Mean ± SE; \times 10^9 L) in CRC Patients With Low (n = 4) and High (n = 8) Anti–GM-CSF Antibody Titers, Respectively, at Days 1 and 10 of Treatment Cycle IV

<table>
<thead>
<tr>
<th>Blood Subsets</th>
<th>Low Anti–GM-CSF Antibody Titer</th>
<th>High Anti–GM-CSF Antibody Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 10</td>
</tr>
<tr>
<td>WBC</td>
<td>6.63 ± 0.84</td>
<td>7.72 ± 0.71</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>4.34 ± 1.07</td>
<td>5.40 ± 0.64</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.19 ± 0.09</td>
<td>0.37 ± 0.16</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.52 ± 0.14</td>
<td>0.40 ± 0.04</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1.92 ± 0.36</td>
<td>1.41 ± 0.20</td>
</tr>
</tbody>
</table>

Only patients with low (CRC patients no. 2, 13, 15, 20) and high (CRC patients no. 5, 8, 10, 11, 12, 14, 18, 19) anti–GM-CSF titers completing 4 GM-CSF treatment courses are depicted in this table.

* P < .05 comparing the low- and high-titer groups at the same day of the treatment cycle.

38.5°C) was noted 2 to 3 hours after the injection and lasted for 2 to 6 hours. There was a clear tendency towards reduced frequency of systemic adverse reactions such as myalgia, fever, rash/pruritus, conjunctivitis, headache, and nausea by treatment cycle IV. The side effects during each cycle in those 13 CRC patients completing 4 GM-CSF treatment courses are shown in Table 5. Furthermore, in the high anti–GM-CSF antibody titer group, a total of 4 systemic adverse events were recorded among 9 patients at treatment cycle IV, whereas 7 were recorded among the 4 low antibody titer patients.

There were no clinical signs of immune complex-related adverse reactions. The kidneys are supposed to be the most sensitive organ to immune complexes. No patient had proteinuria or red blood cells in the urine, which are sensitive indicators of renal immune complexes depositions. Serum immune complexes were analyzed at each treatment cycle. No circulating immune complexes could be detected.

**DISCUSSION**

Nonimmunocompromised patients with metastatic CRC received 10 days of GM-CSF treatment repeated every month for 4 months with the aim being to activate cellular cytotoxic functions as well as antigen presenting cells and to amplify an idiotypic immune network response. The pharmacokinetics (C_max, T_1/2, and AUC) of GM-CSF during cycle I was similar to that described by others. However, after repeated cycles, pharmacokinetics analyses of GM-CSF showed significantly reduced C_max, T_1/2, and AUC that correlated with the appearance of anti–GM-CSF antibodies.

To ascertain that our assay system detected true anti–GM-CSF antibodies, particular attention was paid to avoiding measuring anti-E coli antibodies, because minute amounts of E coli proteins might be present in the drug preparations administered to the patients as well as in the rhGM-CSF preparations used to capture anti–GM-CSF antibodies. Moreover, anti-E coli antibodies are common among normal individuals and increase with an individual’s age. By preincubation of the sera with an E coli lysate false-positive results could be avoided. These precautions should be taken in all immune assays using recombinant proteins produced in E coli. Furthermore, human antimouse antibodies (HAMAs) were induced in all CRC patients, but HAMAs did not seem to interact with anti–GM-CSF antibodies in the assay. The incubation medium contained whole bovine serum that effectively suppressed nonspecific binding to the solid phase. Nonbound HAMAs were washed away before adding the conjugate. Moreover, there was no correlation between the HAMA response and the anti–GM-CSF response. The induction of HAMAs was more rapid than that of anti–GM-CSF antibodies. Most of the CRC patients had developed HAMAs at start of cycle II, whereas no patient had anti–GM-CSF antibodies at this time-point. At 30 weeks after completion of therapy, no patient had detectable anti–GM-CSF titer, whereas all patients still had HAMAs (submitted for publication).

The induction of anti–GM-CSF antibodies was probably a primary immune response even though no IgM antibodies were detected. The kinetics of the IgG antibodies were clearly that of a primary immunization. Reports on induction of anti–GM-CSF antibodies are scanty. Of 16 patients with advanced malignancies who were heavily pretreated or on intensive chemotherapy, 4 developed anti–GM-CSF IgG antibodies against rhGM-CSF. Of 16 patients with myelodysplastic syndrome receiving GM-CSF after previous treatment with chemotherapy, 1 developed a low titer of anti–GM-CSF antibodies. In spite of an abundance of studies using rhGM-CSF to support myeloregeneration after myelo-suppressive therapy, to our knowledge, these are the only studies that show anti–GM-CSF antibody induction. These two reports are in agreement with the results in the MM patients of the present study, where 1 of 8 patients on intensive chemotherapy had a low anti–GM-CSF antibody titer after 4 cycles of GM-CSF administration.

The high frequency of anti–GM-CSF antibodies in the CRC patients may have several explanations. All patients had a good performance status, and only 2 of them had previously received chemotherapy/irradiation. Therefore, the CRC patients should be regarded as nonimmunocompromised as compared with the MM patients who, like the patients of the other studies, were most likely immunocompromised as a consequence of intensive chemotherapy.
Therefore, our results might support the assumption that chemotherapy prevented the development of antibodies to rhGM-CSF. Moreover, we administered GM-CSF SC, which is an excellent route for antigen presentation as compared with IV administration. Furthermore, GM-CSF was administered in repeated cycles that boosted the patients.

Interestingly, antibodies developed against GM-CSF/Behringwerke also reacted with GM-CSF/Schering-Plough. (Both GM-CSFs were produced in *E. coli.*) The standards as well as most of the positive serum samples showed higher signals on GM-CSF/Schering-Plough–coated plates as compared with GM-CSF/Behringwerke-coated plates (Fig 5). This might partly be because of the fact that, in this set-up of analyses, freshly coated GM-CSF/Schering-Plough plates were used, whereas the GM-CSF/Behringwerke plates were precoated and stored with a drying agent in sealed bags at +4°C until use. However, there was no constant factor from sample to sample between plates coated with the different GM-CSFs. This is not surprising. The two GM-CSFs were prepared by different production procedures. The epitope pattern of the two different GM-CSFs was expected to differ, and the antibody response may vary from patient to patient with regard to epitopes recognized.

The presence of GM-CSF antibodies seemed to have biologic effects. The altered pharmacokinetics of GM-CSF with decreased $C_{\text{max}}$ and AUC as well as shorter $T_{1/2}$ resulted probably in reduced amounts of bioavailable rhGM-CSF, which was also suggested by Gribben et al. In that study, the increase in neutrophils was not that expected in 1 of 4 patients developing anti–GM-CSF antibodies. In our patients, there was a clear relationship between the anti–GM-CSF antibody titers and the increase in WBCs, neutrophils, and eosinophils (ie, the higher the antibody titer, the lower the cell increase). With repeated cycles, there was a clear tendency towards a lower increment in total number of monocytes, although the difference did not reach statistical significance. Lymphocyte counts seemed to be unaffected.

The results are in agreement with the notion that GM-CSF mainly affects maturation/proliferation of hematopoetic cells.
of the myeloid and monocytic lineages. An alternative explanation to the lower leucocyte counts at subsequent cycles may be a decreased capacity of bone marrow to be stimulated by GM-CSF. Moreover, at cycle IV, systemic side effects of GM-CSF (myalgia, fever, rash/pruritus, conjunctivitis, headache, and nausea) were markedly reduced as compared with the previous cycles, which may be related to the induction of anti–GM-CSF antibodies. Also in this respect, there seemed to be a difference between those patients developing high anti–GM-CSF titers and those with low titers. This finding is similar to that of another study showing that the appearance of antibodies to rIFN-α2, reduced the side effects of IFN-α2.20

The induction of anti-rhGM-CSF antibodies did not seem to have a negative impact on the levels of WBCs during long-term follow-up. This might be because of the fact that antibodies induced against exogenous E. coli-derived GM-CSF recognized epitopes on the native protein backbone, normally protected by O-linked glycosylation but exposed on E. coli-produced GM-CSF.28 Therefore, endogenous GM-CSF may not be recognized by these antibodies because this molecule might not express the relevant immunotopes. Anti–GM-CSF antibodies also gradually disappeared after discontinuation of GM-CSF, probably as a result of no further antigenic stimulation. Therefore, the effects on endogenous GM-CSF might not be hampered by antibodies against exogenous E. coli-derived GM-CSF.

In summary, this study showed that repeated SC administration of E. coli-derived GM-CSF in nonimmunocompromised patients induced a primary humoral immune response against exogenous GM-CSF in practically all patients. The pharmacokinetics and the variations in WBC counts during therapy indicated that the biologic effects of the exogenous GM-CSF were reduced by the induced antibodies. However, antibody induction in immunocompromised patients was rare and of low titers. This has to be taken into consideration when designing therapeutic protocols using recombinant cytokines (probably not only GM-CSF) in patients prone to mount an immune response.

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REFERENCES


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Induction of anti-recombinant human granulocyte-macrophage colony- stimulating factor (Escherichia coli-derived) antibodies and clinical effects in nonimmunocompromised patients

P Ragnhammar, HJ Friesen, JE Frodin, AK Lefvert, M Hassan, A Osterborg and H Mellstedt