Pharmacokinetics of Human Granulocyte-Macrophage Colony-Stimulating Factor Using A Sensitive Immunoassay

By Jonathan Cebon, Peter Dempsey, Richard Fox, George Kannourakis, Eric Bonnem, Antony W. Burgess, and George Morstyn

A sensitive and reliable sandwich enzyme-linked immunoassorbent assay (ELISA) has been developed for recombinant human granulocyte-macrophage colony-stimulating factor (hGM-CSF). The assay is quantitative between 100 pg/mL and 2.5 ng/mL for bacterially synthesized hGM-CSF in human serum and is more sensitive and specific than the semisolid agar bioassay. As part of a phase I study, the pharmacokinetics of intravenous (IV) bolus injection and subcutaneous (SC) administration of hGM-CSF were studied. Following a single IV dose, an initial high blood level of hGM-CSF occurred, followed by a rapid decrease occurring in two apparent phases with a half-life ($t_{1/2}$) of less than five minutes and a $t_{1/2}$ of 150 minutes. After an SC injection, detectable serum levels occurred within 15 to 30 minutes, and serum levels were sustained for a variable time depending on the dose. At the highest SC dose (10 μg/kg), a serum level of >1 ng/mL (65 pmol/L) was maintained for >12 hours after a single injection. This corresponds to the concentration of hGM-CSF supporting near-maximum proliferation in vitro.

The assay is labor-intensive. To overcome the difficulties of the bioassay, we developed a sandwich enzyme-linked-immunoassorbent assay (ELISA) for hGM-CSF. The sensitivity and specificity of this assay has been evaluated and it has been used to determine the pharmacokinetics of bacterially synthesized hGM-CSF being used in a phase I study.

**MATERIALS AND METHODS**

**Patient characteristics.** Patients participated in a phase I study of hGM-CSF. The study involved administration of IV or SC hGM-CSF or daily for ten days to patients with advanced malignancy or neutropenia to assess whether hGM-CSF has anticancer activity and to identify its effects on the hematopoietic system and any adverse reactions. Informed consent was obtained from all patients. The patients did not receive chemotherapy during this study, although previous chemotherapy was permitted provided the last dose was administered at least 6 weeks before hGM-CSF. The characteristics of the patients in whom pharmacokinetics was studied are shown in Table 1. All had a bilirubin level <20 mmol/L and a creatinine level <0.15 mmol/L. The protocol met the ethical guidelines of the National Health and Medical Research Council of Australia.

**Pharmacokinetic study.** Patients received hGM-CSF daily for ten days either by IV bolus or SC injection. Doses tested were 0.3 and 1 μg/kg IV or 0.3, 1, 0, 3.0, and 10 μg/kg SC. Serum for the pharmacokinetic study was collected in sterile tubes at intervals for up to 24 hours after one dose in each patient. Sera

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collected for immunoassay and bioassay were stored at -20°C until assayed.

**Human GM-CSF.** Bacterially synthesized recombinant human GM-CSF (hGM-CSF) was supplied by Drs. T. Nagabhusan and P. Trotta (Schering-Plough, Kenilworth, NJ). Protein concentration was determined by the methods of Bradford and Lowry et al. For laboratory studies, the hGM-CSF was provided as a sterile solution in a phosphate buffer (20 mmol/L pH 7.2). Hydrolytic amino acid analysis was performed for precise quantitation of the hGM-CSF standard used in the immunoassay. For clinical use, hGM-CSF with a specific activity of 10^9 U/mg in a lyophilized powder was supplied. For pharmacokinetic studies, sterile sera stored at 20°C were thawed until use.

**Production of rabbit antiserum to hGM-CSF.** New Zealand white rabbits were immunized with 100 µg hGM-CSF in complete Freund’s adjuvant (CFA, Parkville, Australia) followed by boosts at monthly intervals in incomplete Freund’s adjuvant. Antibody titer was assessed by indirect ELISA using a streptavidin-biotin horseradish peroxidase system (Amersham, Melbourne, Australia). Three rabbit antisera were developed, and one (R4 1.12) was selected because of high titer.

**Sandwich ELISA for detecting hGM-CSF in serum.** hGM-CSF in serum from patients was measured by a sandwich assay. Microtiter plates were coated with the purified monoclonal antibody LMM 111 (200 ng/40 µL) in phosphate-buffered saline (PBS) (16 hours at 20°C) and then blocked with PBS containing 1% wt/vol bovine serum albumin (BSA) (Sigma, St Louis) (20°C for six hours). The plates were rinsed with water, blotted dry, and stored at -20°C until use. After the plates were thawed, 150 µL serum was incubated in each well (4°C for 16 hours). The plates were then washed with wash buffer; PBS containing Tween 20 0.1% vol/vol (Ajax Chemicals, Auburn, Australia). Rabbit anti-iGM-CSF serum (R4 1.12) was diluted 1:1,500 in ELISA diluting buffer (EDB;PBS containing 0.1% Tween 20 vol/vol and 0.1% BSA wt/vol) and incubated in the wells for three hours at 20°C. Bound rabbit antibody was detected using biotinylated anti-rabbit Ig from donkey (Amersham) at 1:500 vol/vol in EDB. The plates were washed again, and streptavidin-biotinylated horseradish peroxidase complex (Amersham) diluted 1:1,000 in EDB was incubated in the wells for 15 minutes. After further washing, 150 µL 2,2’-Azinobis(3-ethylbenzthiazoline sulfonic acid (ABTS) solution) (Sigma) (500 µg/mL in citric acid 100 mmol/L pH 4.2 containing H2O2 (30%) 3 µL/mL vol/vol) was added to each well and read at 30 to 60 minutes by a Titertek MCC/340 absorbance plate reader (Flow Laboratories, Melbourne, Australia) at 450 nm using a reference filter at 405 nm. Background readings were determined from six wells in which PBS containing BSA (0.1% wt/vol) was incubated in the place of serum. The average background reading was calculated and subtracted from all results. The limit of the assay’s sensitivity (0.02 ng/mL) was determined at 3 SD above the mean background level.

**Table 1. Patient Characteristics, Dose of hGM-CSF Administered, and WBC Response**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Dose of hGM-CSF (µg/kg)</th>
<th>Route</th>
<th>Sex</th>
<th>Age</th>
<th>Total WBCs</th>
<th>Neutrophils</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
<th>Pretreatment</th>
<th>Posttreatment*</th>
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<tr>
<td>1</td>
<td>0.3</td>
<td>SC</td>
<td>M</td>
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<td>1.2</td>
<td>0.9</td>
<td>1.5</td>
<td>4.8</td>
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<td>4</td>
</tr>
<tr>
<td>2</td>
<td>0.3</td>
<td>IV</td>
<td>M</td>
<td>52</td>
<td>0.9</td>
<td>0.8</td>
<td>1.1</td>
<td>1.8</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
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<td>72</td>
<td>2.1</td>
<td>2.3</td>
<td>1.3</td>
<td>1.5</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>SC</td>
<td>M</td>
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<td>F</td>
<td>64</td>
<td>1.7</td>
<td>3.6</td>
<td>1.5</td>
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</tr>
<tr>
<td>6</td>
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<td>3.1</td>
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<td>0.8</td>
<td>0</td>
<td>4</td>
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<td>1.7</td>
<td>4.3</td>
<td>2</td>
<td>20</td>
</tr>
</tbody>
</table>

*After ten days of hGM-CSF.
RESULTS

Immunoassay for hGM-CSF. To obtain an immunoassay of optimum sensitivity, a sandwich assay was developed with the murine monoclonal antibody on the solid phase and rabbit antiserum used for the second layer. The schema for this assay is shown in Fig 1. Figure 2 shows a standard curve for the assay. hGM-CSF was added to serum at concentrations from 6 pg/mL to 100 ng/mL, and the solution was incubated on plates prepared with LMM 111 (as described in the Materials and Methods section). hGM-CSF could be detected at >20 pg/mL. At less than 100 pg/mL, the curve was nonlinear and only semiquantitative. Between 100 pg/mL and 2.5 ng/mL, hGM-CSF estimations were quantitative. Samples required dilution for detection of higher concentrations. Comparable sensitivity was achieved when the assay was performed in serum at different dilutions (10% to 90%), culture medium (RPMI 1640 or Dulbecco's modified Eagles medium, DMEM) containing 10% fetal calf serum (FCS) or PBS and BSA 0.1% (data not shown). The substitution of control antibodies CIBr7, an irrelevant IgG1K antibody,37 and preimmune rabbit serum gave a negative result (Table 2).

The specificity of the assay was further demonstrated by adding potential interfering substances into the serum being assayed: granulocyte CSF (G-CSF) 2 μg/mL (Amgen, Thousand Oaks, CA), erythropoietin 0.08 μg/mL (Amgen) IL-2 0.07 μg/mL (Cetus, Emeryville, CA), purified α-interferon (α-IFN) 2,000 U/mL (Wellcome Laboratories, Dartford, UK), recombinant α-IFN 0.5 μg/mL (Schering-Plough). These levels exceed those encountered in vivo and are supramaximal for the in vitro biologic assays. Lipopolysaccharide 100 μg/mL (Bacto lipopolysaccharide Escheri-
Sera from seven normal volunteers were examined with and without exogenously added hGM-CSF to determine how much variation occurred in detection of hGM-CSF in different sera. Figure 4 shows that minor variations existed in the background determinations from each serum. Similarly, minor variation in the quantitation of hGM-CSF at 1 ng/mL was observed between different sera or RPMI containing FCS 10% (bar 1). The mean was 1.03 ng/mL, and the SD was 0.22 ng/mL for the seven human sera.

**Comparison of immunoassay with bioassay.** The standard curves for the hGM-CSF marrow bioassay and ELISA were compared (Fig 5). The specific activity of the batch of hGM-CSF measured in the bone marrow assay was $4 \times 10^0$ U.

**Table 2. Sandwich Elisa Assay for hGM-CSF in Human Serum**

<table>
<thead>
<tr>
<th>Reagents in Immunoassay</th>
<th>Detection of hGM-CSF (Relative Absorbance)</th>
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</thead>
<tbody>
<tr>
<td>First Antibody</td>
<td>Rabbit Donkey Anti-hGM-CSF Anti-Rabbit SBPC</td>
</tr>
<tr>
<td>CIBr7</td>
<td>+</td>
</tr>
<tr>
<td>CIBr7</td>
<td>+</td>
</tr>
<tr>
<td>LMM 111</td>
<td>+</td>
</tr>
<tr>
<td>LMM 111</td>
<td>+</td>
</tr>
<tr>
<td>LMM 111</td>
<td>+</td>
</tr>
<tr>
<td>LMM 111</td>
<td>+</td>
</tr>
<tr>
<td>No hGM-CSF</td>
<td></td>
</tr>
<tr>
<td>hGM-CSF (10 ng/mL)</td>
<td></td>
</tr>
</tbody>
</table>

P, preimmune serum from the same rabbit; SBPC, streptavidin-biotinylated horseradish peroxidase complex; CI Br7, IgG,K—control monoclonal antibody.

Rabbit anti-hGM-CSF, hGM-CSF antiserum from rabbit (R4 1.12). Donkey anti-rabbit, biotinylated donkey immunoglobulin against rabbit immunoglobulin.

*Mean ± SD.

**Fig 3.** Effect of adding potential interfering substances to sera with or without hGM-CSF was assessed by ELISA. The assay was performed in 90% human serum containing either no hGM-CSF or hGM-CSF at 1 ng/mL. G-CSF 2 μg/mL, erythropoietin (EPO) 0.08 μg/mL, IL-2 0.06 μg/mL, α-IFN 3,000 U/mL, γ-IFN 0.05 μg/mL, and lipopolysaccharide (LPS) 100 μg/mL. Each was titrated, but only the highest concentration is illustrated. Results are expressed as average results of triplicates ± SD.

**Fig 4.** Effect of different human sera on the ELISA for hGM-CSF. Absorbance readings were taken from assays performed with culture medium containing 10% FCS (1) and seven different human sera (2 through 8). Background absorbance readings and readings from sera with added hGM-CSF (1 ng/mL) are shown. A slight variation existed between these sera. Mean hGM-CSF determination ± SD of the seven human sera was 1.03 ± 0.22 ng/mL.

**Fig 5.** Comparison of standard curves for hGM-CSF in serum. (A) Sandwich immunoassay. (B) Agar colony-forming assay. Concentration of hGM-CSF refers to that in the sample solution assayed and was therefore ten times higher than the actual concentration in the bioassay culture dish.
U/mg. hGM-CSF at a concentration <1 ng/mL in the undiluted stimulus solution (0.1 ng/mL in the culture) gave no significant colony formation, whereas <0.05 ng/mL could be detected by immunoassay. The immunoassay was therefore ~20 times more sensitive than the bioassay. Bioassay samples required a dilution of stimulus into agar and culture medium of 1:10 before they could be assayed, which accounts largely for the greater sensitivity of the immunoassay.

Pharmacokinetics of hGM-CSF by immunoassay. Blood was collected following IV bolus administration of hGM-CSF from patients receiving 0.3 and 1.0 µg/kg hGM-CSF. Serum was analyzed from a pretreatment specimen and up to 24 hours after hGM-CSF administration. Serum samples were assayed on at least two occasions. Results were expressed as the mean of triplicate determinations ± SD. At the lowest IV dose (0.3 µg/kg), the highest serum level (14 ± 3 ng/mL) was measured at the first time point. The hGM-CSF was cleared rapidly from the blood within 120 minutes with two apparent phases (Fig 6A). When 0.3 µg/kg was administered SC, the maximum concentration of hGM-CSF in the blood (30 ng/mL) occurred 60 minutes after injection. At this dose of hGM-CSFs, no significant effect on the circulating WBC count occurred during the ten-day treatment period (Table 1).

A 1-µg/kg dose of hGM-CSF administered IV resulted in peak serum level of 54 ± 26 ng/mL, with rapid redistribution and elimination phases. Blood levels dropped to <5 ng/mL after 60 minutes and to 1 ng/mL at 120 minutes. The same dose administered SC in two patients resulted in peak levels of 0.75 ± 0.03 and 0.71 ± 0.04 ng/mL ~120 minutes after the injection. At this dose level, an initial increase in WBC count occurred after two days of treatment and a twofold increase in WBC count occurred by day 10 in both patients (Table 1).

Administration of hGM-CSF at the higher doses, 3 and 10 µg/kg SC caused a threefold increase in WBC count by day 10. At the 3-µg/kg level, circulating hGM-CSF was first detected after 15 to 30 minutes with a plateau level of 1 ng/mL, which persisted for nine hours in one patient (Fig 6C), and a higher peak of 2 ng/mL but more rapid elimination, remaining >1 ng/mL for only two hours in the second patient (not shown). A peak of 5 ng/mL followed by a plateau phase with a hGM-CSF level of ~3 ng/mL occurred in the patient receiving 10 µg/kg SC (Figure 6D). In this patient, the hGM-CSF level remained >1 ng/mL for at least 12 hours.

Pharmacokinetics by bioassay. At the IV dose level of 1 µg/kg, serum hGM-CSF pharmacokinetics were also determined by bioassay. Each specimen was tested at several dilutions and compared with a standard curve. This enabled
GM-CSF PHARMACOKINETICS BY IMMUNOASSAY

Fig 7. Correlation of hGM-CSF concentrations in serum measured by bioassay and immunoassay. Sera from patient 5 treated with 1 µg/kg IV were collected at 0.5, 15, 30, 45, 60, and 120 minutes; they were assayed by both methods, and the results were compared by linear regression analysis (r = .97).

calculation of the number of units of activity per milliliter for each sample. The hGM-CSF levels determined by bioassay and immunoassay were compared. Units of activity in the bioassay were plotted against the concentration of immunoreactive hGM-CSF determined by immunoassay for each of the first seven time points (0, 5, 15, 30, 45, 60, and 120 minutes). Because of background colony formation, determinations of hGM-CSF activity beyond the 120-minute time point was not possible. A linear regression analysis was performed, and the correlation gave an r-value of .97 (Fig 7). It was not possible to quantitate the concentration of hGM-CSF by bioassay because of intrinsic colony-stimulating activity in human serum. This was apparent because colonies stimulated by samples from the patients were larger and more numerous than maximally stimulated control colonies grown without human serum.

DISCUSSION

We have described a rapid and reliable immunoassay for recombinant human GM-CSF. The assay is more sensitive than the conventional bioassay method, quantitating hGM-CSF at concentrations as low as 100 pg/mL (6.5 pmol/L). Bacterially synthesized hGM-CSF measured by bioassay can be detected down to concentrations as low as 20 pmol/L.12 This sandwich immunoassay is not subject to major interference by other bioactive molecules or serum components. With the immunoassay, we were able to determine the pharmacokinetics of hGM-CSF given IV or SC. The highest level of hGM-CSF after an IV dose occurred immediately, and two apparent phases of elimination of hGM-CSF were demonstrated. After an SC dose, the peak level of hGM-CSF in the blood occurred at 120 to 240 minutes, and the serum concentration decreased after a variable period of time depending on the dose.

The bioassay for measuring colony-stimulating activity in complex sources such as unfractionated serum or culture supernatants may not clearly distinguish between different colony-stimulating and inhibitory activities. Complex interactions with other factors or serum components may enhance colony formation or suppress clonogenic myeloid proliferation. GM-CSF can induce production of factors that could interact in the bioassay. These difficulties are amplified when unpurified bone marrow cells are used for the bioassay. Another cause of reduced sensitivity in the bioassay, when one measures factors such as GM-CSF present at low concentrations, occurs with the 1:10 dilution of an unknown sample into agar and medium. This dilution can make the difference between near-maximum colony stimulation and background levels of colony formation (Fig 5B) and may require the GM-CSF to be extracted from the sample before assay.

The ELISA for hGM-CSF overcomes many of the difficulties associated with the hGM-CSF bioassay since other factors do not interfere and sample dilution is not necessary unless the concentration of hGM-CSF exceeds the limits of the assay. In the study of these patients, a dilution of 1:10 was required to determine peak levels in patients receiving the IV doses of hGM-CSF. The assay’s specificity is determined by the monoclonal antibody LMM 111, which immunoabsorbs hGM-CSF efficiently from whole serum. The sensitivity is increased by polyvalent rabbit serum, polyvalent detection reagents, and enzyme-mediated reaction. Antibodies raised against human GM-CSF have been reported for use in radioimmunoassays (RIAs). Two RIAs have been described. One RIA for hGM-CSF using a rabbit antiserum to yeast produced hGM-CSF sensitive in the range of 0.5 to 6 ng/mL. A second RIA detects mammalian cell-derived hGM-CSF and is apparently sensitive to 2.9 ng/mL.

The sandwich assay described here appears to be more sensitive, obviates the use of 131I, uses stable reagents that can be stored for prolonged periods, and enables direct measurement of hGM-CSF in serum. A potential disadvantage of immunoassays is that the biologic activity of the antigen is not determined. Degradation of the hGM-CSF molecule may cause reduced biologic activity without affecting its antigenicity. The correlation between the hGM-CSF determinations by bioassay and immunoassay (Fig 7) suggests that the immunoreactive hGM-CSF is biologically active for at least 120 minutes following injection. Correlation would be lost if degradation of hGM-CSF resulted in a nonfunctional but antigenic molecule.

hGM-CSF could not be quantitated by bioassay although pharmacokinetics could be determined qualitatively. The large and more numerous colonies in human serum-containing cultures as compared with control indicate that the serum contained additional colony-stimulating activity. Standard curve assays using the patient’s pretreatment serum may overcome this problem, although this would not allow for moment-to-moment variation in intrinsic colony-stimulating activity.

In this phase I study, bolus IV and SC routes were compared. As expected, a higher peak level was achieved after each IV administration than after each SC administration of the same dose. The anticipated levels of hGM-CSF in serum immediately after an IV injection of 0.3 and 1 µg/kg are 8.4 and 28 ng/mL, respectively (assuming a plasma volume of 2.5 L for a 70-kg adult). The measured peak levels
of 14 ± 3 and 54 ± 26 ng/mL correspond to those predicted. The decrease in hGM-CSF levels occurred with a 1/36 or less than five minutes and a slower 1/28 of 150 minutes, assuming two phases of elimination.

In contrast, after an SC dose, a rise in hGM-CSF blood level occurred within one hour, peaked by two to four hours and fell after two to ten hours. The time during which hGM-CSF remained detectable was dose dependent. At the dose level of 10 µg/kg >1 ng/mL was detectable for >12 hours following a single SC injection. This is significant because in the in vitro assays 1 ng/mL of the clinical preparation gives 100 U of activity, which is close to maximum activity in these assays (Schering-Plough, product information). hGM-CSF was detectable in serum following a SC dose in all patients receiving >1 µg/kg, and all of these patients had an increase in WBC count.

The patient receiving 1 µg/kg IV had a detectable hGM-CSF level for less than four hours. All patients being treated with this IV dose had an increase in WBC count. Detectable serum hGM-CSF levels sustained for as little as four hours each day appear to be capable of increasing WBC count. Intermittent SC hGM-CSF was effective in elevating WBC levels in these patients and is more convenient to administer than continuous IV or SC infusions. Further studies are required to define the optimum route of administration and dose to determine whether blood levels are affected by previous administration of hGM-CSF. We have not determined whether administration of recombinant hGM-CSF results in anti-hGM-CSF antibodies which might affect the assay.

This assay allowed analysis of the pharmacokinetics of exogenous nonglycosylated hGM-CSF. Naturally occurring hGM-CSF is a glycosylated protein. Because of the lack of specificity of the bioassays for hGM-CSF, precise information is not available about its levels in serum, bone marrow, or other biologic fluids. Preliminary data (not shown) indicate that the native glycoprotein is detected by this immunoassay with sensitivity comparable to that of nonglycosylated hGM-CSF. An assay sensitive in the low picomolar range may still not be sufficiently sensitive to define conditions of hGM-CSF deficiency; however, this assay offers the potential to clarify unresolved questions about the in vivo role of hGM-CSF in normal and disease states.

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