MACROPHAGE COLONY-stimulating factor (M-CSF) is one of a number of growth factors now recognized as governing hematopoiesis. In vitro, M-CSF stimulates the proliferation of monocyte/macrophage precursors, and the differentiation of these precursor cells into mature macrophages. It is synergistic with other hematopoietic growth factors in inducing the proliferation of monocyte precursors. In vivo, M-CSF has been implicated in the regulation of macrophage production, although a sustained effect of M-CSF given exogenously has been difficult to demonstrate. M-CSF acts on monocytes and mature macrophages to increase oxidative metabolism and antitoxin. Monoclonal antibodies against tumor cells directed and enhance macrophage cytotoxicity in both murine and human systems. M-CSF enhances antitumor antibody-dependent cell-mediated cytotoxicity (ADCC) by murine macrophages. We have recently reported that recombinant human M-CSF (rhM-CSF) increases the number of circulating monocytes in nonhuman primates, and that these monocytes mediate increased antitumor ADCC after a brief period of in vitro differentiation. This study has implications for the design of possible future clinical trials combining antitumor monoclonal antibodies and rhM-CSF.

In light of these findings, we were encouraged to explore the effects of exogenously administered rhM-CSF on nonhuman primates. Using cynomolgus monkeys, we examined the effect of rhM-CSF on peripheral white blood cell count, monocyte number, immunophenotype of circulating mononuclear cells, and in vitro antitumor ADCC by peripheral blood monocytes.

MATERIALS AND METHODS

rhM-CSF. Recombinant human M-CSF is a 90 Kd homodimer consisting of two disulfide-bonded monomers of 233 amino acids each, with two N-glycosylation sites and numerous O-glycosylation sites. Our preparation was greater than 99% pure by polyacrylamide gel electrophoresis, and had a specific activity of 50 units/mg as measured by an in vitro murine bone marrow stimulation assay. The purified protein was free of detectable pyrogen and endotoxin. It was suspended in Tris-buffered saline with 10 mmol/L citrate and glycine for parenteral administration.

Animals. Male cynomolgus monkeys weighing between 3 and 7 kg were used in all experiments. All research was carried out according to guidelines established and certified by local and federal authorities governing the use and treatment of laboratory animals. The monkeys were fed Purina Laboratory Chow and given water ad libitum. For continuous intravenous infusions, a catheter was surgically implanted into the iliac vein of the primate under general anesthesia using sterile technique. A Broviac indwelling access catheter was exteriorized and connected to an ambulatory infusion
pump (Parker Micro-Pump, Model 2004, Parker, Irvine, CA). The entire pump apparatus was then held in a jacket worn by the animal. This allowed a continuous intravenous delivery of rhM-CSF at selected doses over specified time intervals. One animal in the study received twice-daily rhGM-CSF given subcutaneously. Since the data from this animal were not discernibly different from those animals receiving continuous intravenous infusion, they are included in our analysis. Animals were lightly anesthetized with ketamine before blood drawing procedures. Complete blood cell counts and cytotoxic differentials were done using a Technicon H1 analyzer (Technicon Corp, Terrytown, NY). Differential were reviewed by a commercial veterinary pathology laboratory and by technicians at Genetics Institute. Selected slides were also reviewed by Dr Arthur Skarin, Dana Farber Cancer Institute, Harvard Medical School, Boston, MA. Serum biochemical analyses, including hepatic and renal function studies, were done using an automated multiparameter analyzer (SMa-20).

Monoclonal antibodies. The murine monoclonal antibody 3F8 (IgG2a, anti-Ga), developed in our laboratory, has been previously described.24 Antibody R24 (IgG2a, anti-Ga) was the generous gift of Dr Alan Houghton, Memorial Sloan-Kettering, New York, NY.

Separation of mononuclear cells. Heparinized whole blood and serum were collected under sterile conditions. Blood was sent on ice to our animal facility from the laboratory, where immunophenotyping and cytotoxicity assays were done. Because blood from cynomolgus monkeys is not amenable to separation by conventional techniques, the following procedure was used to purify mononuclear cells from whole blood: Heparinized blood was diluted 1:1 in sterile RPMI 1640 medium with 3% gelatin (Sigma Chemical Co, St Louis, MO), and centrifuged at room temperature for 10 minutes at 100 g. The leukocyte-rich plasma was collected, and the red blood cell pellet was resuspended in RPMI plus 0.1% methylcellulose (Sigma) and allowed to gravity sediment in a 37°C water bath. When a sharply defined red blood cell pellet had formed (usually 20 to 30 minutes), the supernatant was collected and pooled with the results of the first sedimentation. Residual red blood cells were removed by hypotonic lysis, and the white blood cells were layered on a single step gradient of 52% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) in isotonic medium (52% percoll, 5.8% 10x Hanks' balanced salt solution, and 42.2% RPMI). After centrifugation at 1,000 g for 15 minutes, the interface layer, which contained the mononuclear cell fraction, was collected and washed. This protocol effectively separated mononuclear cells (lymphocytes and monocytes) from granulocytes and erythrocytes, as confirmed by Wright's stain morphology and flow cytometry.

The mononuclear cell fraction was suspended in RPMI plus 10% bovine serum (BS) and allowed to adhere for 1 to 2 hours at 37°C in 96-well flat-bottomed tissue culture plates (Falcon, Oxnard, CA), at a concentration of 1.6 x 10⁶ cells per well. (With human blood, this concentration of cells had been found to yield 3 x 10⁴ adherent monocytes per well after washing.) The plates were washed vigorously in RPMI plus 0.1% human serum albumin using a multichannel pipette. The cells adhered well, despite their overnight shipment on ice. Before plating, the percentage of monocytes in the mononuclear cell fraction was estimated by counting glass-adherent cells under phase contrast microscopy. Control monkeys typically had 15% to 30% glass-adherent cells in the mononuclear fraction. If the percentage of these cells exceeded 30%, the cell concentration was adjusted to give an approximate seeding density of 5 to 6 x 10⁴ monocytes per well (one half to two thirds of which could be expected to adhere, on the basis of our experience with human monocytes). Adherent cells were cultured for 3 to 11 days at 37°C, 5% CO₂, in 10% pooled monkey serum supplemented with 100 U/mL rhM-CSF, and then assayed for antitumor cytotoxicity.

Enzyme-linked immunosorbent assay for cytotoxicity. Cytotoxicity was measured as previously described.24 Target cells (SKMel-1, human melanoma), obtained from the American Type Culture Collection (Rockville, MD), were added directly to the 96-well plates containing cultured monkey monocytes at 1 x 10⁴ targets per well (giving an approximate effector:target (E:T) ratio of 4:1). Antitumor antibody (3F8, 2 µg/mL) or medium was added to each well, and the plates were incubated for 72 hours. Control wells receiving antibody but no monocytes typically had 5% to 20% fewer target cells after 72 hours, when compared with targets incubated in medium alone. To adjust for this mild antiproliferative effect of antibody, all cytotoxicity data were calculated based on parallel controls (target cells cultured under identical conditions of antibody, medium, and rhM-CSF). After incubation, the plates were centrifuged to recover any detached cells, the supernatant was aspirated, and the plates were air-dried and fixed with glutaraldehyde. After thorough washing in phosphate-buffered saline (PBS), the plates were incubated with 10% BS in PBS to block nonspecific binding of antibody, then washed and incubated with a second antitumor monoclonal antibody (R24). After washing, peroxidase-conjugated goat anti-mouse (Fisher-Biotech, Pittsburgh, PA) was added, and the hydrolysis of o-phenylene diamine by bound peroxidase was measured as optical density (OD) at 490 nm. The percentage of surviving target cells was calculated using the formula: % Surviving = [OD (targets + effectors) - OD (effectors alone)]/OD (targets alone). This was converted to percent cytotoxicity using the relationship: % Cytotoxicity = 100% - % surviving targets. Determinations were performed in duplicate, and the mean is reported.

Flow cytometry. Mononuclear cells were purified as described above, and stained with monoclonal antibodies against CD16, CD14, CD11b, LFA3, and HLA-DR. A secondary antibody (goat anti-mouse fluorescein isothiocyanate [FITC]) was added, and the cells were analyzed using a FACScan cytofluorometer (Becton-Dickinson, Mountain View, CA). For two-color immunofluorescence, cells were first stained with anti-CD11b, then with biotin-conjugated goat anti-mouse antibody, followed by avidin-phycocerythrin and an anti-CD16 FITC conjugate. Anti-CD16 (Leu-11) was obtained from Becton-Dickinson. Anti-CD14 (MY4) and anti-CD11b (904) were the generous gift of Dr James Griffin, Dana Farber Cancer Institute, Harvard Medical School. Anti-LFA3 was the generous gift of Dr Steve Burakoff, Dana Farber Cancer Institute, Harvard Medical School. Anti-HLA-DR (L243) and anti-CD11b (OKM-1) were obtained from the American Type Culture Collection and used as culture supernatant.

RESULTS

We treated four cynomolgus monkeys with rhM-CSF at doses previously seen to have biologic effect (50 to 100 µg/kg/d). Each monkey received three courses of 14 days' duration, with a 2 week rest period between courses. Three animals received continuous intravenous infusions, and one received twice-daily subcutaneous injections. Since the hemato logic effects observed were similar in all animals, the data were pooled for analysis.

Parenteral administration of rhM-CSF produced a rise in the total circulating leukocyte count and a pronounced increase in the number of circulating monocytes. During treatment with rhM-CSF, a population of large, granular, extensively vacuolated cells emerged that morphologically resembled mature macrophages. Figure 1 shows an example of these "macrophage-like cells" (MLC). Figure 2 shows serial white blood cell counts for a typical animal receiving
rhM-CSF. There was a prompt rise in the absolute number of monocytes and MLC (Fig 2A) with a concomitant increase in the total leukocyte count (Fig 2B). The elevation in monocyte number was not sustained, declining after 7 to 10 days despite continued administration of rhM-CSF. The peak monocytosis typically occurred on days 5 through 7. Table 1 summarizes several of the hematologic parameters, averaged for all the animals in the study.

A mild dose-dependent decrease in platelet count was observed in each animal (Fig 2C), but was not pronounced or clinically significant at the doses used in this study. No biochemical evidence of renal, hepatic, or other organ system toxicity was seen in our study.

Figure 3 shows an immunophenotypic analysis of the cell population induced by rhM-CSF. Animals treated with rhM-CSF had a marked increase in the number of circulating cells staining positively with the marker CD14. These cells fell within the cytofluorometric gates established for monocytes based on forward light scatter and side scatter (Fig 3A and B). To further confirm that the cell population induced by rhM-CSF was of monocyte lineage, we stained the cells with several other monoclonal antibodies that, in our experience and that of others, had been shown to be reactive with monocytes from cynomolgus monkeys. Figure 3C shows that these cells were 93% CD14-positive, 98% HLA-DR-positive, 98% LFA3-positive, and 96% CD11b-positive. (Because some differences in staining pattern were observed between the two anti-CD11b antibodies OKM-I and 904, we present the data for both.) All of these markers were also found on normal monocytes from control animals. We did not detect any alteration in the level of expression of these antigens (as measured by mean fluorescence intensity) during rhM-CSF treatment.

We found that 77% of the monocytes induced by rhM-CSF expressed CD16 (the low-affinity Fc receptor, FcRIII). Figure 4 shows that rhM-CSF–treated monkeys had an expanded population of cells co-expressing CD16 and CD11b.
M-CSF IN NONHUMAN PRIMATES

Fig 3. Immunophenotype of circulating cells induced by rhM-CSF. Peripheral blood mononuclear cells from cynomolgus monkeys undergoing treatment with rhM-CSF were obtained during the peak monocytosis and stained as described in Materials and Methods. Stained cells were analyzed using a FACSscan flow cytometer. Forward light scatter (FSC) and side scatter (SSC) gates for monocytes were established based on the monocyte marker CD14 (MY4). (A) The gate shown included greater than 95% of CD14 positive cells in normal control animals, and was used to select a cell population for further analysis: (B) the number of cells falling within this gate was increased in rhM-CSF-treated animals, compared with control animals; and (C) the reactivity of this expanded population (gated as shown in B) with a panel of monocyte markers, compared with an irrelevant control. The data are from a representative animal treated with rhM-CSF at a dose of 100 µg/kg/d.

All of these cells were monocytes by forward and side scatter criteria, and also co-expressed CD14 (data not shown).

To examine the cytotoxic potential of the circulating monocytes and MLC, we used an antitumor ADCC assay developed in our laboratory that uses immunologic detection of surviving target cells as a measure of cytotoxicity. Fresh monocytes do not demonstrate active antitumor ADCC using this assay, but they progressively acquire efficient cytotoxicity during in vitro culture. To obviate differences in culture conditions between control and rhM-CSF-treated monkeys (who might have some amount of exogenous rhM-CSF in their serum), we used a culture medium supplemented with 10% pooled monkey serum and optimal amounts of rhM-CSF (100 µM/mL). Monocytes from both treated and untreated animals survived well in this medium, and underwent the morphologic changes associated with in vitro differentiation of monocytes.

Figure 5 shows the development of antitumor cytotoxicity over the first 4 days of culture. In this experiment, human melanoma target cells and antitumor monoclonal antibody were added to replicate monocyte cultures after 1 to 4 days of pre-incubation. The cultures were assayed 24 hours later for the presence of residual tumor cells by enzyme-linked-immunosorbent assay (ELISA), as described in Materials and Methods. In the presence of rhM-CSF, monocytes from both treated and control monkeys developed ADCC over time, but cytotoxicity emerged substantially earlier in the treated group. A pre-incubation period of 72 hours was chosen for subsequent assays because it permitted the development of measurable ADCC by the control monocytes but did not allow the treatment group to reach a plateau at 100%.

Figure 6 shows the average antitumor cytotoxicity by rhM-CSF–treated monkey monocytes from eight experiments. Monkeys were treated with 50 to 100 µg/kg/d of rhM-CSF intravenously or subcutaneously for 5 to 7 days before blood sampling. Monkeys not receiving rhM-CSF served as controls. ADCC was significantly higher in the treatment group (82% versus 19%, P < .05). Antibody-independent killing was not significantly different in the treatment and control groups (16% versus 29%, NS).

DISCUSSION

This study demonstrates that rhM-CSF given parenterally induces a peripheral monocytosis in cynomolgus monkeys, with the emergence of a substantial population of large, vacuolated, granular cells that morphologically appear to be in the monocyte/macrophage lineage. These cells express a variety of monocyte markers. It is not clear from morphology alone whether they represent immature monocytes (promonocytes) or mature monocytes undergoing activation and differentiation. Since there are few well-studied monocyte differentiation markers available for monkeys, it is difficult to draw conclusions on this point from immunophenotyping data. It is clear, however, that the population of cells which emerges during treatment with rhM-CSF is of the monocyte/macrophage lineage.

CD16 (FcRIII) is not normally found on circulating human monocytes, and may represent an activation or differentiation marker in humans. CD16 was present on 77% of monocytes induced by rhM-CSF in the treated monkeys. However, there was also a population of CD16-positive monocytes detectable in untreated control animals. Thus the presence of CD16 on rhM-CSF–induced monocytes...
Fig 4. Expanded population of CD16 (FcRIII)-positive monocytes in animals treated with rhM-CSF. Peripheral blood mononuclear cells from the animal shown in Fig 3 (B) and from a control animal (A) were double stained for CD16 and CD11b. Contour plots of the whole mononuclear cell fraction (lymphocytes and monocytes) are shown. The control animal showed a small population of cells expressing high levels of both CD16 and CD11b (CD16-positive monocytes), while the rhM-CSF-treated animal had a greatly expanded population of these double-positive cells. All of the cells in the region outlined were monocytes by FSC and SSC (see Fig 3). The double-negative cells were greater than 98% small lymphocytes by light scatter. Similar experiments were performed using the monocyte marker CD14 in place of CD11b, with identical results (data not shown).

may represent an inter-species difference, rather than an effect of therapy.

The monocytes induced by rhM-CSF developed antitumor ADCC more rapidly in culture than control monocytes.

While in vitro monocyte differentiation has certain parallels with in vivo differentiation into mature macrophages, it is not clear to what degree these two phenomena are comparable. It is possible that circulating monocytes from treated animals were “pre-activated,” and thus differentiated more rapidly in vitro. However, it is also possible that differentiation pro-

Fig 5. Development of antitumor ADCC during in vitro culture of monkey monocytes. Peripheral blood monocytes from treated and control monkeys were purified by adherence to plastic as described in Materials and Methods, and then cultured for 1 to 4 days in 10% pooled monkey serum in RPMI medium, with or without 100 µM/mL rhM-CSF. Human melanoma cells and antitumor antibody (3F8) were added on days 1, 2, and 4, and surviving target cells were measured by ELISA after 24 hour co-incubation. In the presence of rhM-CSF, monocytes from both the treated animal and control animal developed ADCC over time, although detectable ADCC emerged earlier in the treated monkey. Without suplemental rhM-CSF in the culture medium, no significant ADCC emerged in either animal. The E:T ratio in all assays was 4:1.

Fig 6. Enhanced antitumor ADCC mediated by monocytes from rhM-CSF-treated monkeys. Peripheral blood monocytes from rhM-CSF-treated animals and control animals were purified as described in Materials and Methods, and then precultured for 3 days in medium containing 10% monkey serum and 100 µM/mL rhM-CSF. Target cells (human melanoma) with or without antitumor antibody (3F8) were added on day 3, and co-incubated for 72 hours. Cytotoxicity was determined by ELISA. For incubations with antibody, there was significantly higher ADCC in the animals receiving rhM-CSF (P < .05, average of eight experiments) than in control. For antibody-independent killing, there was no significant difference between the two groups (average of seven experiments). The E:T ratio was 4:1 in all experiments.
ceeding at the same rate for both groups, but that the degree of cytotoxicity achieved was greater in the treatment group. Thus, while we conclude that some form of monocyte activation for ADCC appears to take place during treatment with rhM-CSF, its exact nature remains to be investigated.

We did not find a significant increase in antibody-independent killing during treatment with rhM-CSF. This may be attributable in part to the fact that our assay was developed primarily to detect macrophage ADCC (which is very efficient) and may not be sensitive enough to reliably detect lower levels of killing.

The monocytosis induced by rhM-CSF was not sustained. We observed a tendency for the counts to fall after 7 to 10 days of continuous treatment. Retreatment with rhM-CSF after a 1 to 2 week rest period again resulted in a rise in the monocye count. We do not believe that this pattern is attributable to the emergence of anti-rhM-CSF antibodies, both because repeated courses of the growth factor continued to produce good responses, and because in other studies with nonhuman primates, we have not seen development of anti-rhM-CSF antibodies after 14 days of treatment with 1 mg/kg/d of rhM-CSF.

The principal adverse effect associated with rhM-CSF administration was a mild dose-dependent decrease in the platelet count. At the doses used in the study, this effect was slight and did not result in clinical symptoms. The decrease was promptly reversible after discontinuation of therapy. No other significant adverse effects were seen in this study.

This is consistent with our experience of rhM-CSF in other studies. In monkeys undergoing necropsy as part of a separate, formal toxicology study, no organ toxicities were seen at a dose of 100 μg/kg/d of rhM-CSF. At a 10-fold higher dose (1 mg/kg/d), symptomatic thrombocytopenia developed in some animals. Necropsy of monkeys in that study receiving 1 mg/kg/d showed increased organ weights of liver and spleen, with foci of extramedullary hematopoiesis in both organs. The livers showed perivascular mononuclear cell infiltrates, without biochemical evidence of hepatic dysfunction; no other organ abnormalities were seen. The bone marrows were hypercellular with increased numbers of cells in the monocyte lineage.

The etiology of the mild thrombocytopenia seen in our study is not clear. We did not measure platelet production, anti-platelet antibodies, or platelet survival in this study. In the toxicology study mentioned above, we saw normal to increased numbers of megakaryocytes in the bone marrow of monkeys receiving 1 mg/kg/d of rhM-CSF. Other experiments done in rabbits have demonstrated in vivo phagocytosis of platelets by macrophages in animals receiving rhM-CSF. These findings suggest peripheral destruction of platelets by activated monocyte/macrophages, but further investigation of this phenomenon is needed.

In conclusion, parenteral rhM-CSF appears to be well-tolerated, and results in a marked monocytosis, with evidence of enhanced monocyte antitumor cytotoxicity in the presence of tumor-specific antibody. This has implications for the design of combined growth-factor/antibody trials using rhM-CSF and antitumor monoclonal antibodies. The increase in total white blood cell count during rhM-CSF administration suggests that rhM-CSF might also prove useful in chemotherapy-induced neutropenia. Human studies in both these areas are planned.

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Effects of parenteral recombinant human macrophage colony-stimulating factor on monocyte number, phenotype, and antitumor cytotoxicity in nonhuman primates

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