

Effects of Recombinant Human Granulocyte-Macrophage Colony Stimulating Factor on Hematopoiesis in Normal Cynomolgus Monkeys

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(Received 23 July 1992/Accepted 24 November 1992)

ABSTRACT. The *in vivo* efficacy of nonglycosylated recombinant human granulocyte-macrophage colony stimulating factor (rh GM-CSF) expressed in *Escherichia coli* was studied in cynomolgus monkeys (*Macaca fascicularis*). A single intravenous (IV) administration of rh GM-CSF (100 µg/kg) resulted in a two-fold increase in peripheral white blood cell (WBC) count with a predominance of neutrophils after 12 hr. The increased WBC count returned to the initial level within 48 hr after administration. In a study with consecutive IV administrations for 10 days, animals treated with 20 µg/kg/day rh GM-CSF underwent marked leukocytosis (four fold) and thrombocytosis (two fold). The increase in WBC count was due to increased number of neutrophils, eosinophils, lymphocytes, monocytes and basophils. Red blood cell count was unaffected. The leukocytosis resolved within one week after the termination of administration. A lower mean platelet volume compared to the pre-treatment level was observed in rh GM-CSF treated animals receiving 20 µg/kg/day for 20 days. This coincided with the elevation in platelet count.—KEY WORDS: cynomolgus monkey, leukocytosis, mean platelet volume, rh GM-CSF, thrombocytosis.

— J. Vet. Med. Sci. 55(2): 221–225, 1993

Human granulocyte-macrophage colony stimulating factor (GM-CSF) has been purified and molecularly cloned [9, 23]. It has been shown to support the *in vitro* proliferation and differentiation of multi-lineage progenitor cells [2, 14].

Recently it was reported that recombinant human GM-CSF (rh GM-CSF) effectively induced leukocytosis *in vivo* in primates [5, 12] and its application in clinical use in bone marrow transplantation has been further studied [1, 8, 15, 16]. However, there is not enough information about the *in vivo* efficacy of human GM-CSF yet because its biological activity has been studied exclusively *in vitro*. This study was undertaken to investigate the details of the *in vivo* activity of rh GM-CSF.

We analyzed peripheral blood of rh GM-CSF treated primates and compared the results with those of recombinant human granulocyte-colony stimulating factor (rh G-CSF) which stimulates the terminal differentiation of neutrophil precursors [20]. Rh GM-CSF administration resulted in an elevation of peripheral WBC counts and PLT counts. The thrombocytosis observed in this study is interesting, since effects of rh GM-CSF on platelet production remains to be determined.

MATERIALS AND METHODS

Animals: Nine male and two female adult cynomolgus monkeys (*Macaca fascicularis*), approximately 3 to 4 years old, were housed individually in steel cages in an air-conditioned room at 23 to 27°C. The monkeys were fed with certified primate pellets.

Sources of rh GM-CSF and rh G-CSF: *Escherichia coli* (*E. coli*) cells expressing a high level of rh GM-CSF were

constructed according to the method established by Wong *et al.* [23]. The rh GM-CSF accumulated intracellularly was extracted and purified to homogeneity by a series of chromatographic steps [23], including high-performance liquid chromatography (HPLC). It had a specific activity of 2.0×10^8 U/mg protein in a colony forming assay. The purity of this material was more than 98% by HPLC assay.

Rh G-CSF was purified [22] from *E. coli* cells expressing rh G-CSF and had a specific activity of 1.2×10^8 U/mg in the colony forming assay with the purity of more than 95% by SDS gel electrophoresis.

Endotoxin levels of both samples were not detected by the Limulus amebocyte lysate assay (Wako Pure Chemical Industries, Ltd.).

Treatment with rh GM-CSF and rh G-CSF: An experimental outline is shown in Table 1. The dosing solutions were prepared by diluting rh GM-CSF with 0.01 M phosphate-buffered solution (PBS, pH 7.0). Rh G-CSF was diluted with 0.01 M acetic acid buffer solution (pH 4.0). Volume of 0.4 ml/kg was administered to primates intravenously twice daily (except in the single administration study). The control animals received IV administrations of PBS in a similar manner. The doses were determined according to the studies on the *in vivo* effects of GM-CSF or G-CSF in primates [12, 21].

Examinations of hematology and blood biochemistry: Blood samples were successively collected and were evaluated for the following; red blood cell (RBC) count, WBC count, platelet (PLT) count, hematocrit, hemoglobin, mean platelet volume (MPV, Sysmex Micro Cell Counter CC-120 and F-800) and differential WBC counts (May-Giemsa staining).

In addition, each serum obtained from blood samples

Table 1. Experimental outline in cynomolgus monkeys

Study	Test substance	Administration		No. of animals (sex)
		Dose ($\mu\text{g/kg/day}$)	Period (day)	
Single administration study	Vehicle	0	1	1(male)
	GM-CSF	100	1	1(male)
	G-CSF	100	1	1(male)
10-day administration study	Vehicle	0 \times 2	10	1(male)
	GM-CSF	10 \times 2	10	3(2 males, 1 female)
	G-CSF	10 \times 2	10	1(male)
20-day administration study	Vehicle	10 \times 2	10	1(male)
	GM-CSF	10 \times 2	20	2(1 male, 1 female)

was analyzed for the following; glutamine oxalacetic transaminase, glucose, total protein, albumin, blood urea nitrogen, creatinine, total bilirubin, total cholesterol and triglyceride levels (Spectrophotometer UV-210A, Simazu).

RESULTS

A single IV administration of rh GM-CSF: Three male cynomolgus monkeys were used in this single IV administration study. Two monkeys underwent a single IV administration of rh GM-CSF or rh G-CSF at 100 $\mu\text{g/kg}$. The other monkey was served as a control.

The rh GM-CSF-treated animal, as well as the rh G-CSF-treated animal, showed a rapid rise in WBC count (Fig. 1). In the rh GM-CSF-treated animal, the WBC count increased from a baseline of 12,500/ μl to a maximum of 31,300/ μl at 12 hr after administration, and from a baseline of 16,300/ μl to a maximum of 37,500/ μl after 8 hr in the rh G-CSF-treated animal. At 12 hr, 59% of peripheral WBC were neutrophils in the rh GM-CSF-treated animal, 68% in the rh G-CSF-treated animal and 35% in the control. The increased WBC count in the rh GM-CSF-treated animal was predominantly due to the increase of neutrophils and it returned to the pre-treatment value within 48 hr. In the control animal, the diurnal variation of WBC count was observed. Similar results were obtained in an additional complementary study with the same three monkeys (data not shown).

Effects of rh GM-CSF-treated for 10 days: Three animals (2 males, 1 female) were treated with rh GM-CSF (20 $\mu\text{g/kg/day}$) for 10 days and one animal (male) was similarly treated with rh G-CSF (20 $\mu\text{g/kg/day}$). One additional animal (male) served as a control.

In the rh GM-CSF-treated animals, the WBC counts increased gradually during the 10-day treatment from a mean baseline of 10,700/ μl (range 9,700–12,000/ μl) to a mean maximum of 41,900/ μl (24,300–59,300/ μl) at day 10 (Fig. 2-B). The increase in WBC count was predominantly due to increase of neutrophils, eosinophils, and lymphocytes. Within the lineages that were expanded by rh GM-CSF treatment, the neutrophil levels increased from a mean baseline of 3,600/ μl (1,300–6,800/ μl) to a mean

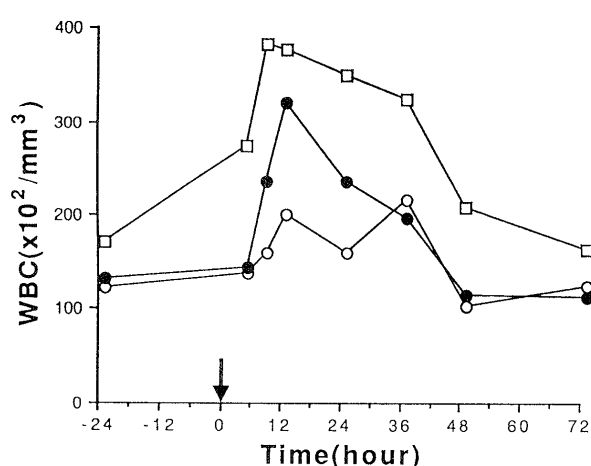


Fig. 1. Time course of peripheral blood WBC of three monkeys. One monkey served as a control (○), one monkey received rh GM-CSF at a dose of 100 $\mu\text{g/kg}$ (●) and the other received an equivalent dose of rh G-CSF (□). An arrow represents the time of injection.

maximum of 22,900/ μl (16,100–27,700/ μl); the eosinophil levels increased from a mean baseline of 100/ μl (0–400/ μl) to a mean maximum of 6,700/ μl (2,200–8,900/ μl); the lymphocyte levels changed from a mean baseline of 7,000/ μl (5,600–8,400/ μl) to a mean maximum of 13,200/ μl (4,100–23,800/ μl , the values of two animals increased and the other slightly decreased); the monocyte levels changed from a mean baseline of 100/ μl (0–400/ μl) to a mean maximum of 700/ μl (0–1,700/ μl); the basophil levels changed from less than 100/ μl to a mean maximum of 400/ μl (0–800/ μl). The increased WBC count decreased to 12,600/ μl (8,300–15,000/ μl) within 48 hr after the cessation of rh GM-CSF treatment.

Compared with the rh G-CSF-treated animal, the rh GM-CSF-treated animals experienced delayed elevations in peripheral WBC count during the treatment period. These monkeys showed a maximum level of WBC 4 days later than the rh G-CSF-treated animal (Fig. 2-B, C). In the rh G-CSF-treated animal, leukocytosis resulted from a substantial increase in the level of neutrophils.

We observed the appearance of unusual eosinophils in

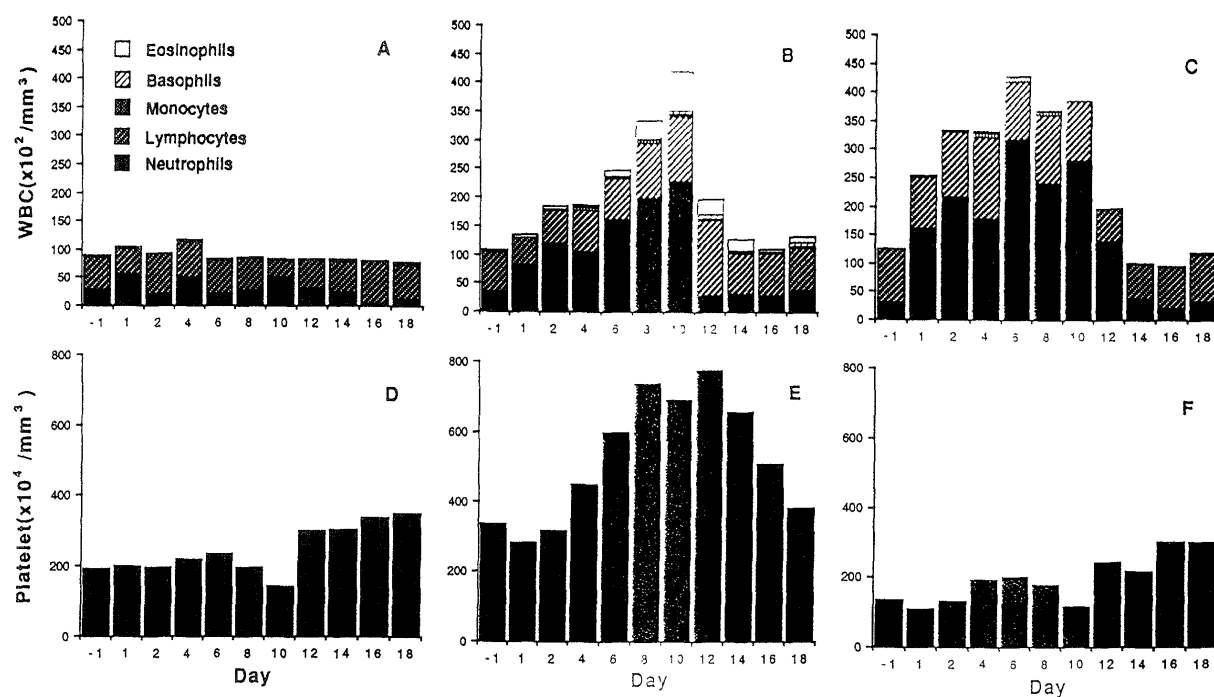


Fig. 2. Differential cell counts (A, B, C) and platelet counts (D, E, F) of a control (A and D), rh GM-CSF ($20 \mu\text{g/kg/day}$) treated monkeys ($n=3$, B and E) for 10 days and an rh G-CSF treated animal ($20 \mu\text{g/kg/day}$) for 10 days (C and F).

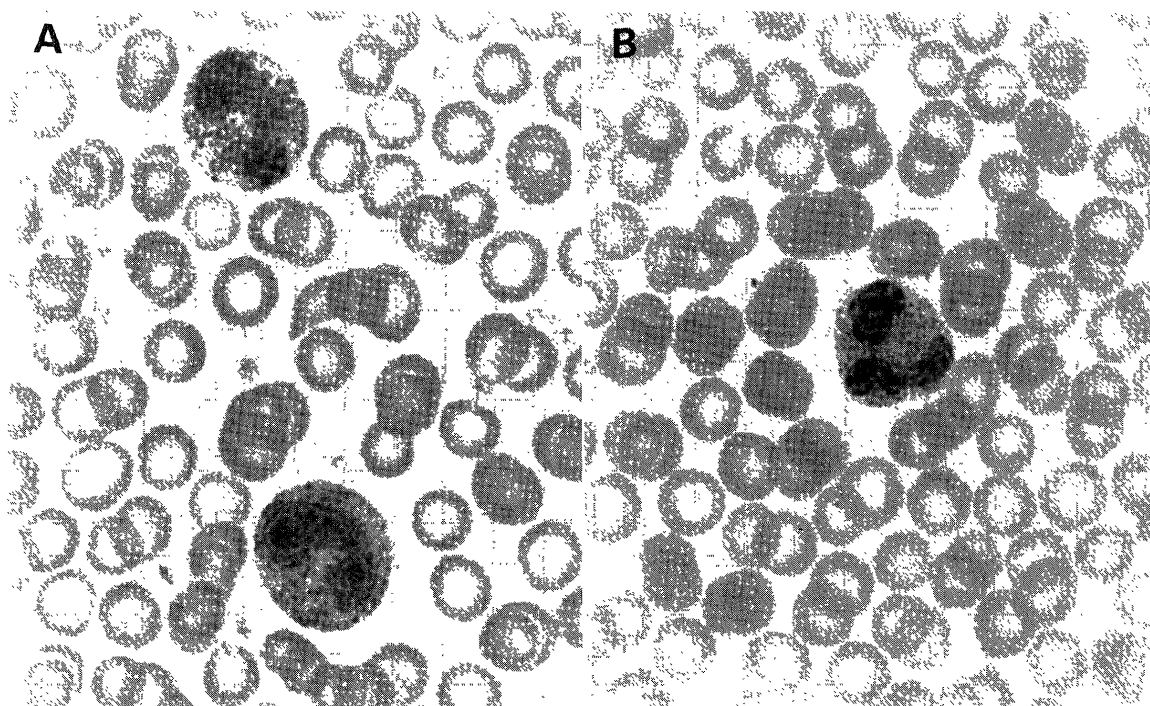


Fig. 3. (A) The atypical eosinophils from blood smears of rh GM-CSF treated animals were stained with May-Giemsa. $\times 1,056$. (B) Normal eosinophils from a control animal were similarly stained. $\times 1,056$.

the circulation 8 days after the beginning of rh GM-CSF treatment by microscopic examination of blood smears. The morphology of these cells was atypical in that they had large cytoplasmic volumes and were poorly-granulated in comparison with normal eosinophils (Figs. 3-A, B).

The PLT count also increased remarkably from a mean baseline value of $336,000/\mu\text{l}$ ($286,000\text{--}375,000/\mu\text{l}$) to a mean maximum of $775,000/\mu\text{l}$ ($643,000\text{--}946,000/\mu\text{l}$) in the rh GM-CSF-treated animals (Fig. 2-E). The increased PLT count decreased almost to pre-treatment values 8 days after the termination of treatment.

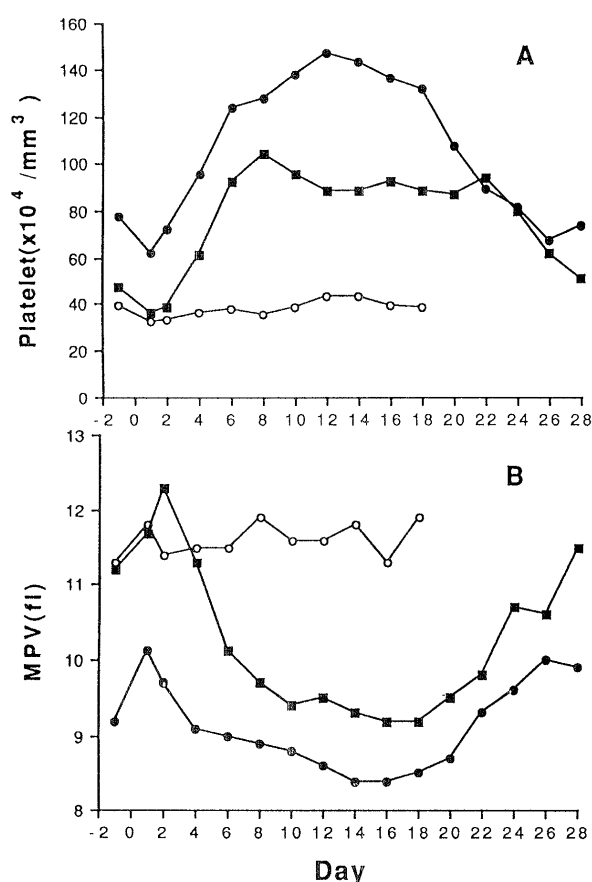


Fig. 4. Platelet counts (A) and mean platelet volume (B) of a control monkey (○) treated with vehicle for 10 days and two monkeys (●: male, ■: female) treated with rh GM-CSF (20 μ g/kg/day) for 20 days.

During the study period, RBC count, hematocrit, hemoglobin and parameters of blood biochemistry were unaffected, and no systemic toxicity was observed.

Effects of rh GM-CSF on peripheral platelets in 20-day treatment: To assess the effects of rh GM-CSF on peripheral platelets in detail, two additional animals (1 male, 1 female) was treated with rh GM-CSF (20 μ g/kg/day) for 20 days, and one animal (male) was treated with vehicle for 10 days as a control.

In the rh GM-CSF-treated animals, the PLT count increased remarkably during treatment (Fig. 4-A), while the MPV decreased (Fig. 4-B). The elevation of the PLT count was reconfirmed after supplementary evaluation by the Brecher-Cronkite method (data not shown). Both the increase in PLT count and the decrease of MPV disappeared gradually after the termination of treatment.

The effect of rh GM-CSF on peripheral WBCs was qualitatively similar to those in the 10-day treatment (data not shown). RBC count was unaffected.

DISCUSSION

This study indicates that purified rh GM-CSF is an effective multi-lineage stimulator of hematopoiesis *in vivo*

in cynomolgus monkeys. The animals receiving repeated administration of rh GM-CSF for 10 days experienced increases of peripheral WBC and PLT. The increase in WBC count was a result of increased levels of neutrophils, eosinophils, lymphocytes, monocytes and basophils. These results are similar to those of other reports [5, 12]. The increase except for lymphocytes corresponds well to *in vitro* observations [14]. On the other hand, no colony formation *in vitro* has been reported for lymphocytes, so the increase of lymphocyte count *in vivo* may represent an indirect effect of rh GM-CSF treatment.

We have a great interest in the increase of PLT count induced by the consecutive administration of rh GM-CSF for 10 days. In addition, the animals treated with rh GM-CSF for 20 days experienced the decrease of MPV during treatment and this coincided with the increase in PLT count. Although it has been documented that rh GM-CSF exhibited Meg-CSF activity *in vitro* [13], the effects of rh GM-CSF on platelet-lineage hematopoiesis *in vivo* remains to be determined. Further investigations are necessary to verify this result.

It has been reported that rh GM-CSF, in the presence of erythropoietin, can stimulate the proliferation of erythroid progenitors in simian bone marrow cells [4, 6, 18]. However, rh GM-CSF had no influence on the RBC counts in this study.

It has been also confirmed that rh GM-CSF is a releasing factor for granulocytes from the marrow reserve pool into the periphery *in vivo*. The animals receiving a single administration of rh GM-CSF at a dose of 100 μ g/kg, experienced a prompt rise of neutrophils within 24 hr. Stimulation of hematopoiesis by rh GM-CSF either at the stem cells or at the myeloblast level, however, would not contribute to the increase of circulating neutrophils until at least five days after the start of the treatment [3, 10, 11]. So the administration of rh GM-CSF would have accelerated the release of neutrophils from the marrow pool and, as a result, peripheral blood neutrophil level promptly rose. It can be concluded that rh GM-CSF acts as an important regulating factor in the release of neutrophils from the marrow pool *in vivo*.

We further compared the effects of rh GM-CSF with those of rh G-CSF in the 10-day treatment study. GM-CSF acted *in vivo* to stimulate multi-lineage hematopoiesis, while G-CSF increased neutrophils selectively. The WBC count in the rh GM-CSF-treated animals peaked four days later than that in the rh G-CSF-treated animal. The delayed response time, when compared with the effects of rh G-CSF, suggested a common hypothesis that GM-CSF acts predominantly on earlier populations than G-CSF does [14].

Unusual eosinophils, which had large cytoplasmic volume and were poorly-granulated, appeared during the treatment. This morphological change has been observed in the patients of hypereosinophilic syndrome [19] or asthma [7]. An electron microscope investigation has provided further evidence that these atypical eosinophils have irregular surface membranes and increased

mitochondria [19]. These findings are consistent with activation of eosinophils. In addition, it has been reported that GM-CSF enhances biochemical and biological properties of normal human eosinophils *in vitro* [17]. GM-CSF might be also a functional activator of eosinophils as well as a hematopoietic stimulator *in vivo*.

In this study, rh GM-CSF induced leukocytosis and thrombocytosis in cynomolgus monkeys. It can be expected that rh GM-CSF will accelerate recovery of bone marrow functions after myelosuppressive chemotherapy or bone marrow transplantation.

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