Monocyte-Derived Recruiting Activity: Kinetics of Production and Effects of Endotoxin

By Elaine McCall and Grover C. Bagby, Jr

Cultured monocytes release a factor, monocyte-derived recruiting activity (MRA), which stimulates fibroblasts, endothelial cells, and T lymphocytes to produce colony-stimulating activity (CSA). We studied the kinetics of MRA production using a technique in which MRA levels were measured in a two-stage bioassay. We used umbilical vein endothelial cells as the MRA-responsive (CSA-producing) cells, and normal colony-forming unit granulocyte-macrophage (CFU-GM)-enriched bone marrow cells (T lymphocyte- and monocyte-depleted, low density bone marrow cells) as the CSA-responsive cells. MRA stimulated a 30-fold increase in CSA production by endothelial cells. MRA production was detected in supernatants from as few as 10^6 monocytes per milliliter, required the presence of fetal calf serum, and was inhibited by cycloheximide (10 to 100 μg/mL) and puromycin (10 to 50 μg/mL). Production was detectable after 24 hours of monocyte incubation, was maintained for three days, and fell to undetectable levels by seven days. With the addition of bacterial endotoxin (lipopolysaccharide [LPS]) (50 μg per 10^6 cells), MRA was detectable after only three hours of incubation, and levels peaked at 24 hours. Further, maximum MRA levels in the supernatants of LPS-stimulated monocytes were up to ten times greater than peak levels in the supernatants of unstimulated monocytes. Endotoxin augmented monocyte production of MRA to a greater extent than it did CSA production, indicating that the stimulation of CSA production by endotoxin may be at least partly indirect. The responsiveness of MRA production to endotoxin in vitro is consistent with the notion that MRA may be a biologically relevant regulator of CSA production by cells of the hematopoietic microenvironment.

© 1985 by Grune & Stratton, Inc.

MATERIALS AND METHODS

Experiments were carried out in three steps: (1) monocytes were cultured to obtain monocyte-conditioned medium; (2) these supernatants (containing MRA) were incubated with endothelial cell cultures; (3) the CSA content of the endothelial cell supernatants was measured.

Preparation of MRA-Containing Supernatants

Monocytes were prepared as described previously. Heparinized blood from normal volunteers was diluted in RPMI 1640, layered over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) and centrifuged at 400 g for 25 minutes. The low density cells at the interface were aspirated, washed twice by centrifugation (600 g, seven minutes), resuspended in RPMI 1640, and adjusted to a concentration of 5 × 10^6 cells/mL. One milliliter was pipetted into 35-mm Petri dishes coated with lactoferrin-depleted fetal calf serum and incubated at 37°C for one hour. Nonadherent cells were removed by vigorous washing.

Generally, to produce MCM, the adherent cells were allowed to remain on the dish, and culture medium was added. When monocyte numbers were adjusted for limiting dilution studies, the adherent cells were lifted off the dishes by treatment with EDTA (0.2% wt/vol in RPMI 1640, 4°C, 20 minutes). They were washed twice and cultured in Petri dishes at the required concentration in RPMI 1640 supplemented with 2 mmol/L glutamine, 10% heat-inactivated, lactoferrin-depleted fetal calf serum, and 100 U/mL penicillin and streptomycin (monocyte culture medium) for one to seven days at 37°C in a humidified incubator (7.5% CO2 in air).

Control dishes contained culture medium alone. Test substances were dissolved in medium and added to the monocytes at the start of culture. At the end of the incubation period, the medium was aspirated from the dishes and centrifuged (200 g, five minutes) to remove any cell debris. Both monocyte-conditioned and control media were diluted before addition to the target cells. In certain experiments, supernatants were dialyzed (Spectrapor, molecular weight [mol wt] cut-off, 3,500 daltons; Spectrum Medical Indus

From the Department of Medicine, Oregon Health Sciences University, and Veterans Administration Medical Center, Portland, Ore.

Supported in part by grants from the National Institutes of Health (CA36306); the Tartar Trust; The Jackson Foundation, Portland, Oregon; and the Veterans Administration.

Presented in part at the American Society of Hematology meeting, Dec 1983.

Submitted May 15, 1984; accepted Sept 18, 1984.

Address reprint requests to Dr Grover C. Bagby, Medical Research (151), Veterans Administration Medical Center, 3710 SW US Veterans Hospital Rd, Portland, OR 97201.

© 1985 by Grune & Stratton, Inc.

0006-4971/85/6503-0025$03.00/0

Blood, Vol 65, No 3 (March), 1985: pp 689-695
tried, Los Angeles) against 0.1 mol/L phosphate-buffered saline (PBS), pH 7.4, (three changes, 4 °C) and then filtered (0.22 µm, low-binding filter; Millipore Corp, Bedford, Mass) before dilution and addition to target cells.

**Endothelial Cell Culture**

Human umbilical vein endothelial cells were prepared by a modification of the method described by Gimbrone et al. Cords were collected within eight hours of delivery, the vein was cannulated, flushed with PBS, filled with collagenase solution (Worthington, type I, 0.1% wt/vol in PBS; Millipore Corp, Laboratory Products Division, Freehold, NJ), and the cord was incubated for 20 minutes at 37 °C. The endothelial cells were rinsed free with RPMI 1640 buffered with 25 mmol/L HEPES and supplemented with 2 mmol/L glutamine, 20% heat-inactivated fetal calf serum, 100 U/mL penicillin and streptomycin, and 250 µg/mL endothelial cell growth factor (endothelial culture medium). The cells were washed, then seeded in 16-mm tissue culture wells in endothelial culture medium, and grown at 37 °C in a humidified incubator (95% air/5% CO2). Cells were subcultured by brief (five minutes, 37 °C) treatment with 0.05% trypsin/0.02% disodium EDTA (wt/vol in PBS) and were washed, resuspended in fresh endothelial culture medium, and reseeded at a 1:5 dilution in 16-mm wells coated with fibronectin (2 µg/cm²; Collaborative Research, Waltham, Mass). All cells were fed every two to three days. Both primary and subcultured endothelial cells exhibited the cobblestone morphology characteristic of endothelium, and more than 95% of cells contained fluorescent peninuclear granules visible after staining with rabbit anti-human factor VIII-related antigen and a fluorescent second antibody (Cappel Laboratories, Cochranville, Pa). In the experiments described below, endothelial cells from 1st to 15th passage were used; cell density ranged from 1 to 5 x 10⁶ cells per square centimeter.

At least 24 hours were allowed to elapse after initial seeding or subculture before an experiment was begun. The endothelial cell culture medium was replaced with monocyte-conditioned medium before an experiment was begun. The endothelial cell culture medium therefore represents activity produced by endothelial cells in response to stimulation by MRA. CSA levels in the supernatants of endothelial cells cultured in monocyte-conditioned medium incubated in the absence of endothelial cells (216 ± 56 U/mL, mean ± SEM; P < .005, Student’s t test), supernatants from endothelial cells cultured alone (77 ± 21 U/mL, mean ± SEM; P < .005, Student’s t test), or culture medium alone (less than 25 U/mL in all assays). Thus, under our experimental conditions, both cell populations alone produced only low levels of CSA. As we have previously reported, CSA production by endothelial cells alone remained consistently low at cell densities ranging from 1 to 5 x 10⁴/cm². and was similar in primary and repeatedly subcultured populations. CSA in the supernatants of endothelial cells cultured in monocyte-conditioned medium represents activity produced by endothelial cells in response to stimulation by MRA.

**Results**

In 26 experiments, supernatants from endothelial cells cultured in 50% monocyte-conditioned medium contained significantly more CSA (2,121 ± 288 U/mL, mean ± SEM) than monocyte-conditioned medium incubated in the absence of endothelial cells (216 ± 56 U/mL, mean ± SEM; P < .005, Student’s t test), supernatants from endothelial cells cultured alone (77 ± 21 U/mL, mean ± SEM; P < .005, Student’s t test), or culture medium alone (less than 25 U/mL in all assays). Thus, under our experimental conditions, both cell populations alone produced only low levels of CSA. As we have previously reported, CSA production by endothelial cells alone remained consistently low at cell densities ranging from 1 to 5 x 10⁴/cm². and was similar in primary and repeatedly subcultured populations. CSA in the supernatants of endothelial cells cultured in monocyte-conditioned medium therefore represents activity produced by endothelial cells in response to stimulation by MRA. Concentrations of MRA, as U/mL monocyte-conditioned medium, are calculated by subtracting CSA content (U/mL) of controls (monocyte-conditioned medium and endothelial cell-conditioned medium alone) from the CSA content of test medium (endothelial cells cultured in conditioned medium) and multiplying by the dilution factor of monocyte-conditioned medium in endothelial cell medium.

**Effect of MCM Concentration on CSA Production**

As we have previously reported, target (endothelial) cell CSA production was related to the amount of MRA added to the incubations. In a second series of experiments, MCM was diluted to give final concentrations ranging from 0% to 70% in the endothelial cell cultures. CSA levels in the supernatants were measured after three days’ incubation. Results are shown in Fig 1. In these experiments, CSA production by endothelial cells with no added MCM was very low, as previously observed (74 ± 43 U/mL, mean ± SEM, n = 7) but rose with 5% MCM added to the cultures (to 433 U/mL, mean of three observations), and rose further with increasing concentration, to 1,171 ± 452 U/mL (mean ± SEM, n = 6) with 20% MCM, and finally to 1,642 ± 536 U/mL (mean ± SEM, n = 7) with 70% MCM. An increase in the concentration beyond 70% did not result in any further increase in the amount of assayed CSA.

**Effect of Culture Conditions on MRA Production**

Monocyte concentration. In four experiments, monocytes at concentrations ranging from 10 to 10⁶ cells per milliliter were incubated for three days, and
Fig 1. Effect of MCM concentration on CSA production by endothelial cells. Multiple dilutions of MCM were added to endothelial cell cultures, and the resultant CSA production was assayed. Bars and vertical lines indicate mean ± SEM. The number of experiments performed in each case is given.

Fig 2. Effect of monocyte concentration on MRA production. Monocytes were incubated for three days at varying concentrations, and the resulting supernatants were assayed for MRA (open bars) and CSA (hatched bars). Bars and vertical lines indicate mean ± SEM (n = 4).

The supernatants were then added to cultures of endothelial cells and incubated for a further three days. Results, as units of MRA in the monocyte-conditioned medium, are shown in Fig 2. Levels of MRA in medium conditioned by 10 or 10^2 monocytes per milliliter were not significantly different from those found in monocyte medium controls, but MRA levels rose significantly in medium conditioned by 10^3 monocytes per milliliter (505 ± 108 U/mL, mean ± SEM; P < .005, Student's t test). MRA levels continued to rise with increasing monocyte concentration, with maximum amounts being produced by concentrations of 10^6 to 10^8 monocytes per milliliter. As can be seen in Fig 2, the production of MRA could be detected at concentrations of monocytes at which CSA could not.

Serum concentration. Monocytes were suspended at 10^2 per milliliter in culture medium containing fetal calf serum at a final concentration of 0% to 10%, and were incubated for three days in dishes that had not been previously coated with serum. Supernatants were diluted in endothelial cell culture medium whose serum concentration was adjusted to give a uniform final serum concentration of 10% in subsequent steps of the experiment. MRA levels rose from 412 ± 114 U/mL (mean ± SEM, n = 7) in the supernatants from monocytes incubated without serum, to 6,392 ± 666 U/mL in the supernatants from monocytes incubated in medium containing 10% serum. Even with the addition of 1% serum, there was a significant increase (to 2,100 ± 460 U/mL) over the values obtained in the absence of serum, and with each subsequent increase in serum concentration to 5%, there was a concomitant significant increase in MRA production. The further increase between 5% and 10% was not significant. Monocyte viability (as assessed by exclusion of trypan blue dye) was 40% in cultures without serum, and more than 90% at all other serum concentrations. Attachment of the cells to the dishes was better than 80% in all experiments.

Time course. Monocytes, 10^5 per milliliter, were incubated in replicate dishes for up to seven days. At different times, one dish was removed from the incubator, and the supernatant was aspirated and stored at 4 °C. When samples at all time points had been collected, the supernatants were assayed on the target cell population. In any set of experiments, the condi-
Table 1. Variation in MRA Production (U MRA/mL) With
Monocyte Incubation

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Days of Incubation</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>4,640</td>
<td>3,840</td>
<td>960</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>2,880</td>
<td>2,160</td>
<td>1,680</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1,380</td>
<td>2,180</td>
<td>860</td>
<td>64</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>1,280</td>
<td>1,360</td>
<td>480</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>4,640</td>
<td>3,920</td>
<td>960</td>
<td>0</td>
</tr>
</tbody>
</table>

Monocytes were incubated for up to seven days, and at each time point the supernatant was removed and assayed for MRA. Each result is the mean colony score of triplicate plates.

Levels of MRA were similar on days 1 and 3 in all experiments, with slight rises between 24 and 72 hours in two of five experiments, and slight falls in three. After five days of incubation, levels fell considerably, and this trend continued, with no detectable MRA in three of four experiments by day 7. In two further experiments in which monocytes were incubated for shorter periods, MRA was not detectable at three or 18 hours. Even after seven days of culture, no CSA was detected in the MCM.

Effect of Protein Synthesis Inhibitors on MRA Production

Cycloheximide (final concentration, 10 to 100 μg/mL) and puromycin (10 to 50 μg/mL) dissolved in RPMI 1640 were added to the monocytes at the start of the incubation. Control monocyte cultures received an equal volume of RPMI alone. Supernatants were dialyzed to remove the drugs before adding them to the endothelial cell cultures for MRA assay. In three experiments (Fig 3), addition of puromycin resulted in a dose-dependent fall in MRA levels, from a mean of 2,660 U MRA/mL in the controls, to 380 U/mL in the presence of the highest dose, a reduction of more than 85%. Addition of cycloheximide also resulted in a fall in MRA levels, but in the range tested this was not dose-related (Fig 3).

Effect of Bacterial LPS

In preliminary experiments, addition of LPS to three-day monocyte cultures resulted in a doubling of CSA levels in endothelial cell supernatants. Since LPS has been shown to stimulate CSA production by endothelial cells, it was important to establish whether the observed increase in CSA levels was due to a stimulation of monocyte MRA production or to increased CSA production by target cells. MRA content of monocyte-conditioned medium prepared in the presence of LPS (5 μg per 10⁵ cells) was compared with that of MCM to which LPS had been added subsequent to its preparation but before addition to endothelial cells. MCM alone incubated with endothelial cells resulted in 293 U CSA/mL endothelial cell supernatant; MCM prepared in the presence of LPS resulted in 2,832 U CSA/mL, and MCM to which LPS was added immediately before addition to endothelial cells resulted in 1,248 U CSA/mL (mean of three experiments in all cases). Such a carry-over effect of LPS could be prevented by dialysis: LPS (5 μg/mL) incubated three days with endothelial cell cultures resulted in formation of 1,160 U CSA/mL endothelial cell supernatant (n = 2); however, if the diluted LPS was dialyzed for 24 hours before addition to the endothelial cell cultures, production of CSA by endothelial cells was reduced to 20 U CSA/mL (n = 4). Accordingly, all LPS-containing media were dialyzed before addition to endothelial cell cultures.

In the next series of experiments, paired cultures of monocytes (10⁵ cells per milliliter) were prepared in replicate dishes, LPS (5 μg per 10⁵ cells) was added to half, and the cultures were incubated for up to seven days. In six experiments, supernatants from monocytes incubated with LPS contained up to ten times more MRA than did equivalent supernatants from control monocytes at every time point measured. Differences
between pairs were significant (Student’s paired t test) at day 1 \((P < .05, n = 5)\) and day 3 \((P < .1, n = 6)\) but not at days 5 or 7. Results from one such experiment are shown in Fig 4. While the levels of MRA are increased in the presence of LPS, the profile of production remained similar to that of unstimulated monocytes, with maximum levels being found early and then falling over the seven-day period.

The high levels of MRA observed after 24 hours of incubation led us to examine the effect of LPS on MRA production after shorter incubation periods. Addition of LPS to monocyte cultures resulted in detectable MRA levels \((40 \text{ U MRA/mL, mean of three experiments})\) after only three hours incubation, and levels then rose progressively at eight hours and 18 hours. In contrast, unstimulated monocyte cultures produced only low and inconsistent levels of MRA at any time point before 24 hours. The effect of LPS was dose-related: in a series of experiments using LPS at different concentrations, \(1 \text{ ng}\) or less added to \(10^5\) monocytes had no stimulatory effect, while \(10 \text{ ng}\) showed slight increases, \(50 \text{ ng}\) resulted in a 2.4-fold stimulation (mean of three experiments) and \(5 \mu\text{g}\) in a 5.2-fold stimulation (mean of three experiments). Further, in the presence of LPS, detectable levels of MRA were produced from as few as \(10^2\) monocytes per milliliter (data not shown). Incubation of LPS with monocytes also resulted in production of CSA (mean, \(960 \text{ U CSA/mL in nine experiments}\)) as measured by the activity of the dialyzed monocyte-conditioned medium alone in the CSA assay. LPS diluted in monocyte culture medium and dialyzed did not \((n = 2)\) have any direct stimulatory effect on bone marrow cells when tested in the CSA assay.

Puromycin \((10 \text{ to } 50 \mu\text{g/mL})\) added to cultures of monocytes and LPS reduced MRA production dose dependently; however, inhibition was not as marked as that observed when puromycin was incubated with unstimulated monocytes: mean inhibition was 35% at \(10 \mu\text{g/mL}\) \((n = 5)\), and this increased somewhat with increasing concentration of puromycin, to 48% at \(20 \mu\text{g/mL}\) \((n = 5)\) and to 59% at \(50 \mu\text{g/mL}\) \((n = 7)\). Preincubation of monocytes with puromycin for one hour before addition of LPS did not intensify the inhibitory effect of puromycin on MRA production.

**DISCUSSION**

The results described in this report demonstrate that cultured monocytes constitutively produce MRA, such production requires protein synthesis, and MRA production is stimulated by LPS. We have previously proposed that MRA may regulate steady-state granulopoiesis. Results described here add support to this hypothesis, and suggest that MRA may also regulate the granulopoietic hyperactivity induced by endotoxin.

In the experiments described here, MRA-responsive auxiliary cells have been removed from the CSA assay, and therefore colonies are formed only when an entirely exogenous source of CSA is present. In our studies, using unconcentrated conditioned media, MCM contains little or no CSA (except at high monocyte concentrations), and endothelial cells produce very little CSA in the absence of stimulation. We have designated 1 unit of MRA to be that quantity which, on stimulation of target cells, results in the production and/or release of 1 unit of CSA. Serial dilutions of MCM have confirmed that CSA production is related to concentration of MRA in the medium: with increasing dilution, progressively smaller amounts of CSA are produced by the endothelial cells. There is, however, wide variability in the amount of MRA produced by different monocyte populations; some MCM samples may be multiply diluted before there is any reduction in the ability to recruit CSA production. If only a single dilution were made of such samples, the MRA concentration would be underestimated.

Medium composition, cell concentration, and duration of culture all affected monocyte MRA production. For example, there is a requirement for some factor(s) in serum. The effect of serum concentration was only partly related to monocyte viability: while both viability and MRA production were low in the absence of serum, with the addition of as little as 1% serum,
viability was greater than 90%, and was not further improved by additional serum, while MRA production rose progressively with serum concentration. Because highly supplemented serum-free medium was not used, it is not clear whether the serum provides substrate cofactors for MRA formation or whether its action is simply to maintain the monocytes in a more functional state.

MRA production was also related to the number of monocytes in culture and the duration of the culture. In the experiments reported here, 10^3 monocytes were required in order to produce constitutively measurable quantities of MRA. In vivo, it is possible that fewer cells and smaller amounts of MRA are required. Both endothelial cells and monocytes form part of the hematopoietic microenvironment of the bone marrow, and it is reasonable to assume that in this environment, levels of MRA and CSA required to elicit responses in their respective target cells would be small. MRA levels did not rise with concentrations of cells greater than 10^3 cells per milliliter. This may be related to substrate availability, or alternatively may reflect the production of substances such as prostaglandin E2, which is known to be produced by monocytes in culture and is a potent inhibitor of macrophage progenitor growth. Similar considerations may also explain the fall in MRA production observed over a seven-day incubation period. It is also possible that MRA may be inactivated during incubation, and the decrease in MRA levels seen after three days may be related to the point at which inactivation overtakes synthesis.

When injected in vivo, LPS results in leukocytosis and increased serum levels of CSA in mice and man. In vitro, LPS causes CSA release from both murine and human mononuclear cells. These results suggest a mechanism by which granulopoiesis may be modulated during endotoxemia. In our experiments, addition of LPS to monocytes resulted in a marked increase in the amount of MRA produced by the cells, detectable levels being found after three hours of incubation. Maximal levels in both LPS-treated and control monocytes occurred between 24 and 72 hours, but LPS-treated cells produced, at any given time period, two to ten times more MRA than did controls. The time course and magnitude of this stimulatory effect is remarkably similar to previously reported data on CSA production by monocytes following LPS stimulation, and it is likely that at least part of the response previously reported to be due to CSA may in fact be ascribable to MRA (because in none of the prior studies were MRA-responsive cells removed from the system). Monocyte-derived CSA was also detectable in the LPS-treated cells, whereas levels were undetectable in controls. LPS seems, therefore, to increase both MRA and CSA production by monocytes, although unit for unit, monocytes provide more CSA by recruitment than by direct CSA production.

We have observed, and it has been reported, that LPS stimulates CSA production by endothelial cells. Endothelial cells produce CSA when incubated with LPS diluted with RPMI, whereas they did not produce CSA in response to this mixture if it was dialyzed for 24 hours before adding to the endothelial cells. Accordingly, to prevent carry-over of LPS to endothelial cells, we dialyzed the LPS-containing media, either monocyte supernatants or control media, for 24 hours before addition to the target cells (a procedure that appeared to reduce the amount of LPS bioactivity in the medium). The loss of LPS activity that occurred cannot be ascribed to its dialysis, since the mol wt of LPS is approximately 400,000, and the mol wt cut-off of the membrane is 3,500. One possible explanation is that LPS adheres to the membrane, thus reducing levels in the medium.

We have demonstrated that MRA is released by resting monocytes and that its production in vitro is related to a variety of factors, some of which may be relevant in vivo. These results lend support to our hypothesis that MRA production represents the principal mechanism by which mononuclear phagocytes may regulate granulopoiesis. It has been previously suggested that the increase in granulopoiesis observed in LPS-induced endotoxemia in vivo was a result of increased CSA production by the monocytes. Although LPS-stimulated monocytes do produce CSA, unit for unit they are better recruiters than producers. In view of these results, and our previously reported work demonstrating that unstimulated monocytes regulate CSA production by other cell types, we now propose that monocytes regulate endotoxin-induced granulopoietic hyperplasia by recruiting cells of the hematopoietic microenvironment to produce CSA.

REFERENCES

6. Bagby GC, McCall E, Layman DL: Regulation of colony-
Monocyte-derived recruiting activity: kinetics of production and effects of endotoxin

E McCall and GC Jr Bagby