Human Fibroblasts Produce Granulocyte-CSF, Macrophage-CSF, and Granulocyte-Macrophage-CSF Following Stimulation by Interleukin-1 and Poly(rI).Poly(rC)

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Electrophoretically pure human interleukin-1 (IL-1) beta was found to stimulate human fibroblasts in a monolayer culture to elaborate colony-stimulating activity (CSA). Supernatant fluids from cultures induced with increasing concentrations of IL-1 were found to stimulate colony formation of myeloid (CFU-GM), erythroid (BFU-E), and multipotent (CFU-GEMM) progenitor cells in a dose-dependent fashion. The effect on mixed colony formation, however, was less than on CFU-GM and BFU-E growth. Similar to IL-1, the synthetic double-stranded RNA poly(rI).poly(rC) also stimulated release of CSA by fibroblasts. The kinetics of IL-1- and poly(rI).poly(rC)-induced CSA release were found to be different, in that poly(rI).poly(rC)-induced CSA production occurred more slowly. Anti-IL-1 antiserum was able to completely neutralize the IL-1-induced CSA release, but had no effect on poly(rI).poly(rC)-induced CSA production, suggesting that the latter effect was mediated by other mechanisms than IL-1 in supernatant. By the use of specific immunologic assays, G-CSF, M-CSF, and GM-CSF could be identified in cell conditioned by fibroblasts treated with IL-1 or poly(rI).poly(rC). Poly(rI).poly(rC) appeared to be a better inducer for M-CSF than IL-1.

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The Netherlands), and <2% mononuclear phagocytes, as determined with alpha-naphthylbutyrate esterase staining.

**Human Diploid Fibroblasts**

Human diploid fibroblasts (E6SM, a strain of embryonic skin and muscle cells) were grown in Eagle's minimum essential medium (EMEM) with Earle's salts, supplemented with 10% FBS. Cell cultures were subcultivated in multi-well dishes (24 × 1.9 cm²; Nunc, Roskilde, Denmark) and cultured until confluency (1 week).

For induction, confluent monolayers grown in 24-well dishes were refed with induction medium (0.5 mL EMEM/well) containing 2% FBS and IL-1 or poly(rI).poly(rC) at various concentrations. After incubation for 48 hours, cell culture supernatants were collected and stored at −20°C until assayed for CSA, multi-CSA, or CSF.

**Inducing Agents**

Poly(rI).poly(rC) was purchased from P-L Biochemicals, Inc (Milwaukee, WI). Electrophoretically pure IL-1 beta was obtained from crude human lymphokine preparations by a four-step purification schedule as reported previously.24 The preparation used in all experiments contained 10,000 U/mL, as tested in a standard assay for lymphocyte-activating factor (LAF) or for antiviral activity. It did not contain other cytokine activities tested, including IL-1 alpha, IL-2, interferons,25 CSA,4 and IL-6. It was found to be free of bacterial endotoxins, as tested by a Limulus amoebocyte lysate assay, able to detect levels of endotoxin as low as 60 pg/mL. The biologic activity of the IL-1 beta preparation, as measured in an assay for antiviral activity, could be completely neutralized by an antiserum raised in sheep (National Institute for Biological Standards and Control, Hertfordshire, UK) against human recombinant IL-1 beta.

**Rabbit Anti--IL-1 Beta Antiserum**

A hetero antiserum against electrophoretically pure IL-1 beta was prepared in rabbits. At a dilution of 1:1,000, the antiserum could completely neutralize the biologic activity of IL-1 beta (100 antiviral unit of ILAF U/mL), but not that of several other lymphokines and monokines (IL-1 alpha, tumor necrosis factor, interferon alpha, beta, gamma, and interleukin-2)25 as well as CSA.4 In neutralization experiments, antiserum (1:250 dilution) and IL-1 (30 U/mL) or poly(rI).poly(rC) (100 µg/mL) were preincubated (120 minutes 37°C) before induction.

**Bone Marrow Culture Assays**

**Assay for CSA.** Cells were cultured in a medium containing 20% FBS (Reheuati, Kankakee, IL), 20% leucocyte-conditioned medium (LCM),27 or IL-1 or poly(rI).poly(rC)-conditioned medium, 30% alpha-modified Eagle's minimal essential medium (alpha-MEM), and 30% of a methylcellulose solution (3%) in alpha-MEM, in a fully humidified atmosphere of 37°C and 5% CO₂. Six replicates of 0.1 mL, each containing 0.5 × 10⁵ mononuclear cell suspension, were plated into the wells of microtiter plates. After ten days, the number of CFU-GM colonies, defined as granulocytic, monocytic, or eosinophilic aggregates of more than 20 cells, were scored. In these cultures no mixed GM colonies, containing both granulocytes and mononuclear phagocytes, were observed. Cultures to which 20% crude LCM was added as a source for CSA, were included as positive controls, determining the 100% colony growth. In the presence of 20% LCM, normal values in our laboratory for CFU-GM day 10 are 182 ± 15 (mean ± SE) per 10⁵ bone marrow mononuclear cells plated. The number of colonies obtained in the presence of LCM was within the normal limits of our laboratory. To study the effect of fibroblast-conditioned media on colony formation, LCM was replaced by IL-1, or poly(rI).poly(rC), or media conditioned by fibroblasts in the presence or absence of IL-1 or poly(rI).poly(rC). As a negative control no LCM, conditioned medium, IL-1, or poly(rI).poly(rC) was added to the culture medium.

**Assay for multi-CSA.** Quantities of 0.5 × 10⁵ mononuclear phagocyte- and T lymphocyte-depleted bone marrow cells were cultured in 1 mL medium containing 30% pooled human AB heparin plasma, 7.5% phytomenadione-leukocyte-conditioned medium (PHA-LCM), 5% 10⁻¹ mol/L 2-mercaptoethanol, 5% deionized bovine serum albumin, 5% human transferrin, 7.5% Iscove's modified Dulbecco's medium (IMDM) with 2 U human recombinant erythropoietin (kindly provided by Organon Teknika NV, Turnhout, Belgium), and 40% of a methylcellulose solution (2.8%) in IMDM, in a fully humidified atmosphere of 37°C and 5% CO₂ in 35-mm plastic dishes.28 BFU-E and CFU-GEMM, the latter defined as colonies containing at least both erythroid and myeloid cells,29 were scored on day 18. In the presence of PHA-LCM, normal values in our laboratory are 167 ± 112 for BFU-E, 7.1 ± 1.6 for mixed GM colonies, and 21.7 ± 10.9 per 10⁵ mononuclear bone marrow cells plated for CFU-GEMM. To study the effect of fibroblast-conditioned media on BFU-E, mixed-GM, and CFU-GEMM colony formation, PHA-LCM was replaced by 0.2 mL (15%) conditioned medium. Controls were similar as for the CSA assay.

**Assay for GM-CSF.** GM-CSF was quantitated using a monospecific sandwich radioimmunoassay developed by K. Kaushansky. The assay relies on two non-cross-reacting monoclonal antibodies raised against recombinant GM-CSF. The antibodies are specific for human GM-CSF and are purified by *Staphylococcus aureus* affinity chromatography. The first antibody is plated in microtiter wells, and then nonspecific protein adherence is blocked by the addition of 2% albumin. Culture supernatants are added to 10% final volume, allowed to absorb, and after washing, 125I-labeled second antibody is added. After washing the excess labeled antibody, individual wells are counted. The results are compared with standards containing recombinant GM-CSF titered for activity in a human marrow colony-forming assay (1 × 10⁴ U/mg). The assay is specific for human GM-CSF; it does not recognize G-CSF, M-CSF, or IL-3. The limit of sensitivity of the assay is 10 U/mL.

**Assay for G-CSF.** G-CSF was quantitated in a monospecific sandwich immunoassay using human recombinant G-CSF as a standard (D. Chang, M. Hockman, and B.W. Aitrock; manuscript in preparation). The specific activity of human recombinant G-CSF in a mouse CFU-GM assay is 10 U/mg. The assay is specific for G-CSF. It does not recognize GM-CSF, M-CSF, or IL-3. The limit of sensitivity of the assay used for this study was 2 ng/mL.

**Assay for M-CSF.** M-CSF (CSF-1) was quantitated using a radioimmunoassay developed by J. Allen and M. Geier (Cetus Corp, Emeryville, CA). The assay is specific for M-CSF. It does not recognize G-CSF, GM-CSF, IL-3, or denatured M-CSF. Units are defined by colony numbers in a mouse bone marrow assay, using human recombinant M-CSF (6 × 10⁵ U/mg) as a standard.11 The limit of sensitivity of the assay used for this study was 18 U/mL in two experiments, and 63 U/mL in the third experiment.

**RESULTS**

**Release of CSA by Fibroblasts Stimulated With IL-1 Beta**

Media conditioned by fibroblasts in the presence of increasing concentrations of IL-1 were tested for CSA and multi-CSA on mononuclear phagocyte- and T lymphocyte-depleted bone marrow cells. Figure 1 shows that myeloid...
almost 50% of the maximal colony growth in the presence of the PHA-LCM. As expected in T lymphocyte- and mononuclear phagocyte-depleted bone marrow cultures, IL-1 (30 U/mL) by itself did not induce colony formation, indicating that the stimulatory effect on colony growth was not due to residual IL-1 present in the fibroblast-conditioned media.

**Release of CSA by Fibroblasts Stimulated With Poly(rI).Poly(rC)**

Figure 2 illustrates that media conditioned by fibroblasts in the presence of increasing concentrations of poly(rI).poly(rC) stimulated CFU-GM (A), BFU-E (B), mixed GM (C), and CFU-GEMM (D) colony growth of media conditioned by fibroblasts in the presence of increasing concentrations of IL-1 beta. Mixed GM colonies were scored in CFU-GEMM cultures. Supernatants were tested for CSA and multi-CSA on mononuclear phagocyte- and T lymphocyte-depleted bone marrow cells. Maximal colony growth is defined by the number of colonies obtained in the presence of leukocyte-conditioned media. Results: mean ± SE of six (A) and five (B,C,D) experiments. IL-1, interleukin-1 control.

(CFU-GM), erythroid (BFU-E), and mixed colonies (mixed-GM, CFU-GEMM) were stimulated in a dose-dependent fashion by supernatant fluids from fibroblast cultures induced with increasing concentrations of IL-1. The positive effect on CFU-GM and BFU-E growth was observed at a concentration as low as 1 U/mL of IL-1. At optimal concentrations, the stimulatory effect was equal to that of the LCM or PHA-LCM control. The effect of IL-1-stimulated fibroblast-conditioned media on the formation of mixed colonies was significantly less, and reached

**Fig 1.** Stimulatory effect on CFU-GM (A), BFU-E (B), mixed Gm (C), and CFU-GEMM (D) colony growth of media conditioned by fibroblasts in the presence of increasing concentrations of IL-1 beta. Mixed GM colonies were scored in CFU-GEMM cultures. Supernatants were tested for CSA and multi-CSA on mononuclear phagocyte- and T lymphocyte-depleted bone marrow cells. Maximal colony growth is defined by the number of colonies obtained in the presence of leukocyte-conditioned media. Results: mean ± SE of six (A) and five (B,C,D) experiments. IL-1, interleukin-1 control.

**Fig 2.** Stimulatory effect on CFU-GM (A), BFU-E (B), mixed GM (C), and CFU-GEMM (D) colony growth of media conditioned by fibroblasts in the presence of increasing concentrations of poly(rI) - poly(rC). Supernatants were tested for CSA and multi-CSA as indicated in the legend to Fig 1. Results: mean ± SE of five (A) and eight (B,C,D) experiments. PIC, poly(Rl) - poly(rC) control.
PoIy(rI) PoIy(rC)-Induced Production of CSA by Human Fibroblasts
obtained in the presence of 20% LCM.

the LCM control) was observed four to eight hours after
addition of the inducer. Significant CSA production (60% of
and supernatants were harvested zero to 48 hours after

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growth was observed, except background colony formation in

CSA could be detected in culture supernatants eight hours
after initiation of poly(rI).poly(rC) induction (Fig 3B). No
colony formation in a dose-dependent fashion. As for IL-I,
no colony growth was observed, except background colony formation in
BFU-E cultures up to 20% of the PHA-LCM control.

To test whether the poly(rI).poly(rC)-induced CSA
release was mediated by induction of IL-1 beta in the
fibroblast cultures, neutralization experiments with a rabbit
anti-IL-i antiserum were performed. The data presented in Table 1 show that poly(rl).poly(rC)-induced CSA
production. Fibroblast cultures were induced for zero to
four hours with IL-1 beta (10 U/mL) or poly(rl).poly(rC)
(100 µg/mL). The cultures were then washed twice to
remove inducers, and incubated during 48 hours for CSA
production. When the cells were exposed to IL-1 beta for a
period as short as one half hour to two hours, the supernatants harvested after 48 hours contained significant colony
stimulatory activity (Table 2). An induction period of one
half hour to four hours was found to be necessary for
poly(rl).poly(rC).

**Fibroblasts Produce G-CSF, M-CSF, and G-M-CSF**

To identify the hematopoietic growth factors being pro-
duced by fibroblasts stimulated with IL-1 beta or poly(rl).poly(rC), conditioned media were assayed for
G-CSF, M-CSF, and GM-CSF using specific immunologic
assays for these growth factors. The data presented in Table 3 show that G-CSF, M-CSF, and GM-CSF could be iden-
tified in media conditioned by fibroblasts stimulated with IL-1 or poly(rl).poly(rC). In one experiment (data not shown), no
G-CSF could be detected in culture supernatants. Although
detectable levels of M-CSF were found after stimulation

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**Table 1. Effect of Rabbit Anti-IL-i Antiserum on IL-i Beta and Poly(rI) Poly(rC)-Induced Production of CSA by Human Fibroblasts**

<table>
<thead>
<tr>
<th>Condition</th>
<th>CSA* in Fibroblast-Conditioned Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1 30 U/mL</td>
<td>108 ± 15†</td>
</tr>
<tr>
<td>IL-1 30 U/mL + anti-IL-i antiserum‡</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Poly(rI) · poly(rC) 10 µg/mL</td>
<td>101 ± 12</td>
</tr>
<tr>
<td>Poly(rI) · poly(rC) 100 µg/mL</td>
<td>103 ± 17</td>
</tr>
<tr>
<td>Poly(rI) · poly(rC) 10 µg/mL + anti-IL-i antiserum</td>
<td>121 ± 29</td>
</tr>
<tr>
<td>Poly(rI) · poly(rC) 100 µg/mL + anti-IL-i antiserum</td>
<td>116 ± 17</td>
</tr>
<tr>
<td>LCM + anti-IL-i antiserum</td>
<td>84 ± 33</td>
</tr>
</tbody>
</table>

*CSA is expressed as a percentage of the total number of CFU-GM obtained in the presence of 20% LCM.
†Mean ± SD (n = 2).
‡Dilution 1:250.

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poly(rI).poly(rC) stimulate CFU-GM, BFU-E, and mixed colony formation in a dose-dependent fashion. As for IL-1,
the stimulatory effect was more pronounced on CFU-GM
and BFU-E growth than on formation of mixed colonies.
When enriched bone marrow cells were incubated in fibro-
blast growth medium or medium supplemented with IL-1
(30 U/mL) or poly(rI).poly(rC) (100 µg/mL), no colony
release was mediated by induction of IL-i beta in the
fibroblast cultures, neutralization experiments with a rabbit
anti-IL-i antiserum. These results suggest that the poly(rl).poly(rC)-
induced CSA release was mediated by other mechanisms
than release of IL-i.

**Kinetics of CSA Release After Stimulation With IL-i-beta or Poly(rI).Poly(rC)**

To define the time interval required for maximal produc-
tion of CSA, human diploid fibroblasts were incubated with
10 U/mL pure IL-i beta or 100 µg/mL poly(rl).poly(rC),
and supernatants were harvested zero to 48 hours after
addition of the inducer. Significant CSA production (60% of
the LCM control) was observed four to eight hours after
initiation of treatment with IL-i beta, and after 24 hours the
maximal stimulatory effect was reached (Fig 3A). The
kinetics of CSA production induced by poly(rl).poly(rC)
were different from those induced by IL-i beta in that no
CSA could be detected in culture supernatants eight hours
after initiation of poly(rl).poly(rC) induction (Fig 3B).

After 24 hours the levels of CSA, produced after
poly(rl).poly(rC) or IL-i beta induction, were similar.

Experiments were also done to test whether the continuous
presence of IL-i beta or poly(rl).poly(rC) was required for
CSA production. Fibroblast cultures were induced for zero to
Table 2. Production of CSA by Fibroblasts Induced by IL-1 Beta or Poly(rI) - poly(rC) During Various Time Intervals

<table>
<thead>
<tr>
<th>Induction Time (h)*</th>
<th>CSA† in Fibroblast-Conditioned Media</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>IL-1 (10 U/mL)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0 ± 0‡</td>
</tr>
<tr>
<td>1/2</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>1</td>
<td>7 ± 9</td>
</tr>
<tr>
<td>2</td>
<td>70 ± 9</td>
</tr>
<tr>
<td>4</td>
<td>53 ± 2</td>
</tr>
<tr>
<td>Poly(rI) - poly(rC) (100 μg/mL)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7 ± 8</td>
</tr>
<tr>
<td>1/2</td>
<td>17 ± 7</td>
</tr>
<tr>
<td>1</td>
<td>14 ± 9</td>
</tr>
<tr>
<td>2</td>
<td>15 ± 21</td>
</tr>
<tr>
<td>4</td>
<td>73 ± 21</td>
</tr>
</tbody>
</table>

*Fibroblast cultures were incubated for zero to four hours at 37°C with IL-1 beta or poly(rI) - poly(rC); the cells were washed (twice) and further incubated during 48 hours. Conditioned media were then harvested and tested for CSA on T lymphocyte- and mononuclear phagocyte-depleted bone marrow cells of two donors (one).
†CSA is expressed as a percentage of the total number of CFU-GM obtained in the presence of LCM.
‡Mean ± SE.

with IL-1, poly(rI).poly(rC) appeared to be a better inducer of M-CSF than IL-1.

DISCUSSION

In the present study, we demonstrate that human diploid fibroblasts are able to produce hematopoietic growth factors in response to stimulation with IL-1 beta or poly(rI).poly(rC). In vitro colony formation of myeloid progenitor cells (CFU-GM) as well as erythroid (BFU-E) and multilineage progenitors (CFU-GEMM) was stimulated progressively by media conditioned by fibroblasts in the presence of increasing concentrations of either IL-1 beta or poly(rI).poly(rC). Mixed colony formation (mixed-GM, CFU-GEMM) was clearly less stimulated than CFU-GM and BFU-E growth, possibly as a result of the limited capacity of GM-CSF to stimulate mixed colony growth.

It is well established that cell populations of the hematopoietic micro-environment, including fibroblasts, endothelial cells, and mononuclear phagocytes, produce growth factors that possess both CSA and multi-CSA.19 Early studies by Bagby et al.18,20 have indicated that soluble products of mononuclear phagocytes increase the production of CSA by T lymphocytes, fibroblasts, and endothelial cells. More recently, the monokine IL-1 has been identified as an inducer of CSA and multi-CSA by fibroblasts19 and endothelial cells.10,11 Our data confirm and extend the IL-1-inducible production of CSA and multi-CSA by fibroblasts. Concentrations as low as 1 U/mL had a significant CSA-inducing effect. At optimal concentrations of IL-1, detectable CSA levels were found between four and eight hours of incubation and maximum CSA levels occurred between eight and 24 hours of culture. The continuous presence of IL-1 appears not to be required for stimulating CSA production, ie, after a relatively short preincubation period (one half hour to four hours) with IL-1, subsequent CSA release occurred in the absence of inducers.

The double-stranded RNA poly(rI) - poly(rC) is known as an inducer of interferon beta in fibroblasts.20 Similar to IL-1, this substance has been found to be a potent inducer of IL-621 on such cells. In addition to IL-1, poly(rI) - poly(rC) was also found to act as a good inducer of CSA and multi-CSA release. As for IL-6,21 the kinetics of CSA production as induced by IL-1 beta or poly(rI) - poly(rC) were found to be different, ie, CSA was induced more slowly by poly(rI) - poly(rC) than by IL-1 beta. We considered the possibility that poly(rI) - poly(rC) induces CSA production through the intermediate release of IL-1, since poly(rI) - poly(rC) may induce IL-1 release.41 However, the antiserum, although completely abrogating IL-1-induced CSA release, had no effect on poly(rI) - poly(rC)-induced production of CSA. While these findings do not rule out a role for intracellular- or membrane-bound IL-1 in mediating the poly(rI) - poly(rC)-induced CSA release, they suggest that other mechanisms are involved.

Using specific immunologic assays, G-CSF, M-CSF, and GM-CSF could be identified in IL-1 or poly(rI) - poly(rC)-stimulated fibroblast-conditioned media. Although detectable levels of M-CSF were present in supernatants from IL-1-stimulated cultures, production of M-CSF by fibro-

Table 3. Production of G-CSF, M-CSF, and GM-CSF by Human Fibroblasts in Monolayer Cultures Stimulated With IL-1 or Poly(rI) - Poly(rC)

<table>
<thead>
<tr>
<th>Inducer</th>
<th>M-CSF (IU/mL)</th>
<th>GM-CSF (IU/mL)</th>
<th>G-CSF (ng/mL)</th>
<th>M-CSF (IU/mL)</th>
<th>GM-CSF (IU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&lt;63</td>
<td>&lt;10</td>
<td>3</td>
<td>&lt;18</td>
<td>3</td>
</tr>
<tr>
<td>Poly(rI) - poly(rC)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 μg/mL</td>
<td>227</td>
<td>27</td>
<td>300</td>
<td>101</td>
<td>28</td>
</tr>
<tr>
<td>10 μg/mL</td>
<td>209</td>
<td>10</td>
<td>ND</td>
<td>ND</td>
<td>34</td>
</tr>
<tr>
<td>1 μg/mL</td>
<td>243</td>
<td>10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>0.1 μg/mL</td>
<td>171</td>
<td>10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 U/mL</td>
<td>72</td>
<td>19</td>
<td>150</td>
<td>44</td>
<td>240</td>
</tr>
<tr>
<td>10 U/mL</td>
<td>77</td>
<td>25</td>
<td>ND</td>
<td>ND</td>
<td>140</td>
</tr>
<tr>
<td>1 U/mL</td>
<td>66</td>
<td>10</td>
<td>ND</td>
<td>ND</td>
<td>210</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.
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blasts appears to be better inducible by poly(rI) · poly(rC). Previously, we have found IL-1-inducible production of M-CSF by human marrow stromal cells in long-term culture, and on human endothelial cells. In contrast, other studies have failed to detect significant levels of M-CSF mRNA in human vascular endothelial cells or fibroblasts following stimulation by IL-1. However, in these studies recombinant preparations of IL-1 have been used that may exert different biologic activities as the native IL-1 beta, used in our studies. It is unlikely that these differences were due to contaminants in the native form, since no other cytokine activities could be detected in our IL-1 beta preparation and since the biologic activity could be completely neutralized by an antiserum against human recombinant IL-1 beta.

At present, it remains unclear whether this apparent discrepancy relates to differences in cell age and culture conditions.

In conclusion, our studies demonstrate that human fibroblasts are able to produce G-CSF, M-CSF, and GM-CSF following stimulation by IL-1 or poly(rI) · poly(rC). It is suggested that CSF production by the latter inducer is mediated by other mechanisms than induction of IL-1.

ACKNOWLEDGMENT

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