Enhanced Hematopoietic Growth Factor Production in an Experimental Myeloproliferative Syndrome

By Marie-Caroline Le Bousse-Kerdiles, Michèle Souyri, Florence Smadja-Joffe, Vincent Praloran, Claude Jasmin, and Hermann J. Ziltener

The murine myeloproliferative syndrome induced by the myeloproliferative sarcoma virus (MPSV) has numerous similarities to human primary myelofibrosis. We have shown that medium conditioned by spleen cells of MPSV-infected mice has the capacity to support the growth of primitive blast cell colonies. The detection of this activity associated with MPSV infection stimulated us to characterize the hematopoietins responsible for this activity. Northern blot analysis showed a large increase, or induction, of interleukin-6 (IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage-CSF (CSF-1), and granulocyte-CSF (G-CSF) transcripts in the hematopoietic organs of MPSV-infected mice; however, no IL-3 transcript could be detected in either MPSV-infected or normal mice. Significant levels of IL-1α, IL-6, G-CSF, and CSF-1 bioactivities were found in the serum of MPSV-infected mice, but not in controls. Additionally, analysis of medium conditioned by spleen cells of MPSV-infected mice showed the presence of tumor necrosis factor α bioactivity. The increased production of cytokines that are able to stimulate pluripotent hematopoietic stem cells corroborates the hypothesis of a possible involvement of hematopoietic growth factors in the development of some myeloproliferative disorders.

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MATERIALS AND METHODS

Mice

Inbred specific pathogen-free, 8-week-old DBA/2 male mice were purchased from Ifla-Credo (Institut Merieux, L’Arbresle, France) and maintained in a protected area in our animal facilities.

Virus

The MPSV was prepared and injected on day 0 as previously described.

Serum Collection

Blood of four normal or MPSV-infected mice (harvested at day 11, 18, or 25 after MPSV injection) was collected with heparin by heart puncture. After centrifugation, serum was removed and kept at -70°C.

Spleen Cell Conditioned Media

In three separate experiments, spleens from 10 normal or MPSV-infected mice per experiment were aseptically removed on day 11, 18, or 25 after MPSV injection and homogenized with a Potter homogenizer in RPMI 1640 medium. Conditioned media were prepared by incubating total spleen cells (5 × 10⁴ cells/mL)
in RPMI 1640 containing 5% fetal calf serum at 37°C for 3 days, in the absence of lectin stimulation, as already described.\textsuperscript{11}

**Blast Cell Colony Assay**

Methylcellulose cell culture was performed in 35-mm dishes (Falcon, no. 1008; ATGC, St. Maur, France) as described by Nakahata and Ogawa.\textsuperscript{12} One milliliter of culture containing 5 × 10\(^{5}\) bone marrow cells or 3 × 10\(^{6}\) spleen cells from 5-fluorouracil (5-FU)-treated mice (150 mg/kg, intravenously, 4 days before harvest), 2X Dulbecco's modified Eagle's medium, 0.8% methylcellulose (Fluka-Coger, Mulhouse, France), 1% deionized bovine serum albumin (fraction V; Sigma, La Verpillière, France), 30% fetal calf serum (FCS; Flow Laboratories, Puteaux, France), 1 × 10\(^{-4}\) mol/L mercaptoethanol, 15% MPSV-spleen cell conditioned medium (MPSV-CM), and 0.6 IU/mL of purified pork plasma erythropoietin (Epo; CNTS, Les Ulis, France) were incubated for 12 to 18 days at 37°C in a humidified atmosphere flushed with 5% CO\(_2\) in air.

The highly multipotential primitive blast cell colonies (BL-CFU) that were supported by MPSV-CM and that had no morphologic feature of differentiation, were individually replated to test for the capacity to give rise to secondary colonies of all myeloid lineages (CFU-GEMM, granulocyte/macrophage colony [CFU-GM]; mast cell colony [CFU-Mast]; erythroid bursts [BFU-E]). Individual colonies that were supported by MPSV-CM and that had multipotential capacity to give rise to secondary colonies of all myeloid lineages (BFU-E) were individually replated to test for the capacity to differentiate, were individually replated to test for the ability to give rise to secondary colonies of different types was counted 7 to 12 days after, according to criteria described previously.\textsuperscript{12} The number of secondary colonies of different types was counted 7 to 12 days after, according to criteria described previously.\textsuperscript{12}

**Lymphokine Assays**

**Factor-dependent cell lines.** Growth factor responsiveness of the factor-dependent cell lines used is as follows: FDC-2: IL-2, IL-3, and IL-4; R6X: IL-3 and IL-4; FDCP-1: IL-3 and GM-CSF; NSF-60: IL-3, GM-CSF, and G-CSF; B9: IL-6\textsuperscript{26}; and WEHI-274.3: IL-3, GM-CSF, CSF-1, and IL-4.\textsuperscript{29} The cells were grown in RPMI-1640 medium with 10% FCS, mercaptoethanol (5 × 10\(^{-3}\) mol/L), L-glutamine (2.8 × 10\(^{-3}\) mol/L), penicillin (100 U/mL), and streptomycin (100 µg/mL). All cell lines were grown in the presence of their appropriate growth factors. Two percent of concentrated WEHI-3-conditioned medium was used as a source of IL-3 for the cell lines FDCP-1, FDC-2, R6X, NSF-60, and WEHI-274.3. The B9 cell line was cultivated in the presence of 2% of 10-fold concentrated 3T3 conditioned medium as a source of IL-6 and the cell line EL4.NOB1 was grown in medium without growth factor. Primary mast cells used for measuring steel activity\textsuperscript{31} were produced by cultivating bone marrow cells, obtained from C57BL/6 × DBA/2 F1 hybrid mice, for a minimum of 2 weeks in 2% of 10-fold concentrated WEHI-3-conditioned medium and 1% of conditioned medium obtained from the myeloma X.653 cell line transfected with a cDNA-encoding murine IL-4.\textsuperscript{32}

**Cytokines and anticytokine antibodies.** IL-4 was used as conditioned medium from the myeloma X.653 transfected with the cDNA for murine IL-4 and was provided by F. Melchers (Basel Institute of Immunology, Basel, Switzerland).\textsuperscript{33} Chemically synthesized murine IL-3, GM-CSF, and steel factor\textsuperscript{34} were the kind gifts of I. Clark-Lewis (The Biomedical Research Centre, Vancouver, BC, Canada). L-cell conditioned medium served as a source of CSF-1 and conditioned medium from the human bladder carcinoma 5637 was used as source of G-CSF. Antiserum against IL-3, IL-6, and GM-CSF were raised in rabbits against the chemically synthesized cytokine. Antiserum were raised in sheep against IL-1α and in a goat against IL-1β. Recombinant IL-1α was kindly provided by P. Lomedico (Hoffmann-LaRoche, Nuttley, NY) and recombinant IL-1β was kindly provided by R. Newton (Dupont, Philadelphia, PA). The monoclonal antibody S1A\textsuperscript{35} neutralizes murine CSF-1 and was kindly provided by Hsiu-san Lin (Mallinckrodt Institute of Radiology, St Louis, MO).

**Thymidine incorporation assays.** These assays were performed by the addition of 500 of the appropriate target cells per well of a Terasaki plate (Flow Laboratories) in a final volume of 15 µL of medium.\textsuperscript{36} Samples were diluted in twofold steps in medium in 96-well trays and 5-µL aliquots of the dilutions were transferred, in triplicate, to the Terasaki trays. When appropriate, 5 µL of an antiserum dilution were added to each well. Trays were incubated at 37°C for 2 or 3 days and were pulsed with 6 hours with [\(^{3}H\)]thymidine, harvested onto glass fiber filters, washed, and then counted in a liquid scintillation counter. Data were analyzed using a computer program that fits a “four parameter logistic model” to the dose-response curve.\textsuperscript{37} One unit of biologic activity was defined as the quantity of factor per milliliter giving a 50% to maximal response in the assay.

The thymidine incorporation assay for IL-1 is a cocultivation assay and was adapted to the Terasaki microculture system from a method described elsewhere.\textsuperscript{38} Briefly, 1,000 EL4.NOB1 cells and 500 FDC-2 cells were coincubated in the well of a Terasaki tray in the presence of serial dilutions of samples in a total volume of 15 µL. After 2 days of incubation, [\(^{3}H\)]thymidine incorporation was measured as described above. This assay is based on the fact that EL4.NOB1 cells are stimulated by IL-1 to release IL-2, which serves as growth factor for the FDC-2 cells. Because EL4.NOB1 cell are deficient in thymidine kinase, only FDC-2 proliferation is measured by [\(^{3}H\)]thymidine incorporation. In our biologic assay, one IL-1 unit corresponds to 0.25 pg IL-1.

**TNFa Activity Assay**

TNFa activity was defined by the L929 fibroblast cell lytic assay.\textsuperscript{39} The L929-TNFa-sensitive subclone, derived from the original L929 cell line, was a gift from A. Lasfargues (Faculté d’Orsay, Orsay, France). Briefly, 1.25 × 10\(^4\) L929 cells were seeded in wells of flat-bottom microfilter plates at 37°C for 24 hours in 5% CO\(_2\) in air. On day 1, 100 µL of serially diluted test samples or purified TNFa and actinomycin D (1 µg/mL) was added to the cells and incubated for an additional 24 hours. In some experiments a 1/500-fold dilution of anti-TNFa antibody (Genzyme, TEBU, Le Perray en Yvelines, France) was also added to the cells. On day 2, plates were washed and cell lysis was determined by staining the plates with 100 µL of crystal violet (0,5%) in methanol-water (1:4) for 15 minutes. After four washes, the remaining dye of each well was solubilized with 100 µL of sodium dodecyl sulfate (SDS) (1%) and the dye uptake was calculated by an automatic micro enzyme-linked immunosorbent assay (ELISA) autoreader (570-nm filter). One TNF unit was defined as the reciprocal of the dilution lysing 50% of the target cells.

**RNA Preparations and Northern Blot Analysis**

Total RNA was purified using the guanidium thiocyanate/CS\(_2\)Cl\(_2\) procedure.\textsuperscript{40} In each of the three experiments performed, RNA was extracted from individual or pooled frozen tissues (in liquid Nitrogen) from 10 to 20 normal control or MPSV-infected mice (11 and 20 days after virus infection). Polyadenylated (Poly A\(^{+}\)) RNAs were then selected by oligo (dT) cellulose column chromatography,\textsuperscript{41} precipitated with ethanol, and kept at −80°C.

For Northern blot analysis, 5 µg of poly A\(^{+}\) RNA was denatured in glyoxal buffer according to McMaster and Carmichael\textsuperscript{42} and
subsequently electrophoresed in 1.1% agarose gels in 10 mmol/L Na+/Na2 phosphate buffer. RNA blotting was performed overnight on Gene Screen nylon membrane (NEN, Dupont de Nemours, Paris, France) with 25 mmol/L phosphate buffer. After baking (2 hours at 80°C), the membrane was prehybridized for 4 to 5 hours at 42°C in 50% formamide, 1× Denhardt, 50 mmol/L Tris-HCl, pH 7.5, 1 mol/L NaCl, 1% SDS, 0.1% Na Pyrophosphate, 5% dextran sulfate, and 100 μg/mL herring sperm DNA. 32P-labeled probe (1.5 × 10^6 cpm) was then added, and hybridization was performed overnight at 42°C. After washing, the membrane was exposed at -70°C to Hyperfilm-MP (Amersham, Les Ulis, France) with an intensifying screen.

Probes

Mouse (m) IL-1α probe was a 420-bp Pst I-Pvu II fragment inserted into the Sma I-Pst I site of pSP 645 (kindly provided by S. de Kossodo, C.M.U., Geneva, Switzerland, through G. Milon, Institut Pasteur, Paris, France). m.IL-3 probe was a 309-bp Pst I-HindIII fragment from IL-3 cDNA corresponding to the 5’ end, inserted in pSP 645. m.IL-6 was the complete cDNA clone pHP IB5 kindly provided by Dr Van Snick. m.GM-CSF was a 750-bp BamHI-EcoRI fragment from cDNA inserted in pJL4 vector. m.CSF-1 probe was a 3.9-kb EcoRI cDNA inserted in pGEM 2 vector. m.G-CSF was a 1-kb BamHI-EcoRI fragment inserted in pBR 327.46

RESULTS

Conditioned Medium From MPSV-Infected Splenocytes Supports Primitive Blast Cell Colony Formation

In the presence of Epo, MPSV-CM stimulated the growth of huge colonies from primitive CFU-GEMM, BFU-E, and CFU-GM from bone marrow cells as well as from spleen cells of 5-FU–treated mice. In the experimental system we described here, we now show that MPSV-CM was capable of inducing the formation of multipotent blast cell colonies composed of approximately 300 to 500 round and refringent cells that were very homogeneous in size. Formation of those blast colonies could also be induced by either ultracentrifuged (100,000g for 1 hour at 4°C) or heat-treated (56°C for 0.5 hours) MPSV-CM, indicating that the virus was not directly responsible for such blast cell colony formation (Table 1).

To confirm the early nature of the blast cell colonies, we have replated individual blast cell colonies induced by MPSV-CM in secondary cultures containing WEHI-3B conditioned medium and 0.6 IU/mL Epo. As shown on Table 1, primary blast cell colonies supported by MPSV-CM were capable of proliferating and differentiating into secondary colonies of all myeloid lineages, ie, CFU-GEMM, BFU-E, CFU-GM, and CFU-Mast. However, blast cell colonies showed variable replating efficiency. Some of the colonies (nos. 2 and 4, Table 1) presented a very high replating efficiency close to 100% because in the presence of WEHI-3 CM and Epo, one blast cell colony (no. 4) composed of approximately 600 cells could give rise to about 140 CFU-GEMM, 350 BFU-E, and to a confluent number of CFU-GM and CFU-Mast. It is worth noting that, under the same experimental conditions, 5 × 10^4 normal bone marrow cells generate a maximum of 5 to 10 CFU-GEMM and 10 to 20 BFU-E (data not shown). In MPSV-Infected Mice

We studied the level of expression of genes coding for IL-1α, IL-3, IL-6, GM-CSF, G-CSF, and CSF-1 in different organs of MPSV-infected and noninfected control mice, by Northern blot analysis of poly A+ messenger RNA (mRNA). The results shown are obtained from two separate experiments in which the same amount of mRNA per organ was loaded, as measured by expression of the PDGAF'DH gene (data not shown). Cytokine expression was similar in each experiment, whether organs were studied individually or in pools.

An increase in IL-6 and GM-CSF transcripts could be observed in the spleen, liver, and thymus of MPSV-infected mice compared with normal mice (Fig 1). CSF-1 transcripts were increased in the liver and thymus, but decreased in the spleen of infected mice (Fig 1), whereas G-CSF transcripts were only weakly increased in the spleen (data not shown).

Finally, IL-1α and IL-3 transcripts could not be detected either in spleen, liver, or thymus from MPSV or from control mice.

Increased Production of Biologically Active Cytokines in MPSV-Infected Mice

Cytokines have been shown to be active at very low concentrations. We therefore decided to complete this study by measuring their biologic activity in the sera and in the organs of MPSV-Infected Mice.

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Table 1. Number of Secondary Colonies Obtained by Replating Blast Cell Colonies Initiated With MPSV-Spleen Cell Conditioned Medium

<table>
<thead>
<tr>
<th>CM Colony No.</th>
<th>CFU-GEMM</th>
<th>BFU-E</th>
<th>CFU-GM</th>
<th>CFU-Mast</th>
</tr>
</thead>
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<tr>
<td>MPSV-spleen</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>57</td>
</tr>
<tr>
<td>cell CM</td>
<td>2</td>
<td>73</td>
<td>107</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>4</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>140</td>
<td>350</td>
<td>Confluent</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>26</td>
<td>18</td>
<td>21</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>33</td>
<td>25</td>
<td>83</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>5</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>MPSV-spleen</td>
<td>8</td>
<td>1</td>
<td>82</td>
<td>6</td>
</tr>
<tr>
<td>cell CM (ultra-centrifuged)</td>
<td>9</td>
<td>2</td>
<td>6</td>
<td>176</td>
</tr>
<tr>
<td>10</td>
<td>19</td>
<td>18</td>
<td>26</td>
<td>31</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>3</td>
<td>189</td>
<td>10</td>
</tr>
<tr>
<td>MPSV-spleen</td>
<td>12</td>
<td>2</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>cell CM (56°C, 1/2 h)</td>
<td>13</td>
<td>1</td>
<td>2</td>
<td>192</td>
</tr>
<tr>
<td>14</td>
<td>12</td>
<td>8</td>
<td>147</td>
<td>2</td>
</tr>
<tr>
<td>15</td>
<td>1</td>
<td>17</td>
<td>18</td>
<td>57</td>
</tr>
<tr>
<td>16</td>
<td>5</td>
<td>24</td>
<td>27</td>
<td>132</td>
</tr>
<tr>
<td>17</td>
<td>9</td>
<td>62</td>
<td>34</td>
<td>36</td>
</tr>
</tbody>
</table>

Blast cell colonies were obtained by incubating, for 12 to 18 days, 5 × 10^5 5-FU–treated hematopoietic cells in the presence of 15% MPSV-spleen cell conditioned medium and 0.6 IU/mL Epo in methylcellulose medium. They were individually replated, for 7 to 12 days, into methylcellulose culture containing 10% WEHI 3B-CM and 0.8 IU/mL Epo. Blast cell colony nos. 2 and 4 contained about 400 and 600 cells, respectively.
Cytokine Levels in the Serum

Serum taken from controls and MPSV-infected mice at days 11, 18, and 25 was tested for several cytokines (Fig 2). Small but significant levels of IL-1 activity were found in the sera of MPSV-infected mice, but not in the control sera. The level of IL-1 activity was highest at day 18 of the infection. Anti–IL-1α antibody was able to neutralize IL-1 bioactivity, establishing the nature of the cytokine (data not shown).

IL-6 was also detected in MPSV sera samples using the hybridoma B9. No IL-6 activity was found in the control sera. IL-6 titres increased with the progression of MPSV infection. The B9-stimulating activity could be neutralized using anti–IL-6 antibody, thus positively identifying this activity to be IL-6 (data not shown).

High levels of CSF-1 activity were also found in the serum of MPSV-infected mice, whereas the control sera again showed no activity. The cell line WEHI 274.3, used to identify CSF-1, also responds to IL-3 and GM-CSF. Although we could not detect GM-CSF or IL-3 in any of the sera samples, as indicated below, the CSF-1 bioassay was still performed in the presence of neutralizing amounts of anti–IL-3 and anti–GM-CSF antibody to exclude any possible synergistic effects that small amounts of these cytokines could have. Using the anti–CSF-1 monoclonal antibody 5A1 in neutralizing experiments, the nature of the WEHI 274.3 stimulating activity was confirmed as CSF-1 (data not shown).

Bioassays performed with NSF-60 cells that are reported to respond to IL-3, GM-CSF, and G-CSF also resulted in significant titres of bioactivity in the MPSV serum samples, but not in the controls. Because we could not detect IL-3 or GM-CSF activity in any of the serum samples and anti–IL-3 antibody together with anti–GM-CSF antibody did not reduce the NSF-60 stimulating activity, we therefore concluded that the activity found in the MPSV sera samples using NSF-60 cells was G-CSF. Control experiments show that the NSF-60 cells used in our laboratory do not respond to IL-6 or CSF-1 and respond to chemically synthesized IL-3 and GM-CSF and to G-CSF present in the conditioned medium of the human bladder carcinoma 5637. This latter activity can be specifically inhibited by neutralizing antihuman G-CSF antibody (data not shown).

Neither IL-2, IL-3, nor IL-4 could be detected in the serum samples, as judged by the lack of detectable stimulation of FDC-2 or R6X cells. Lack of response of FDCP-1 cells to any of the serum samples confirms the absence of IL-3 and also of GM-CSF. Using primary bone marrow-derived mast cells, steel factor, a recently described factor able to stimulate HSC[^15] was also not detectable in the serum samples.

Cytokines in Conditioned Medium of Splenocytes

Conditioned medium from cultures of spleen cells obtained from control and from MPSV-infected mice were tested for cytokine activity. Significant levels of IL-1 activity were detected in the conditioned medium of control and day 11 spleen, whereas much less IL-1 activity was detected in medium obtained from day 18 and day 25 spleen (Table 2). The IL-1 activity could be neutralized by anti–IL-1α antiserum, thus establishing the nature of the IL-1 and also excluding the presence of IL-2, IL-3, or IL-4 that could directly stimulate the FDC-2 cells used in the EL4.NOB1/FDC-2 costimulation assay. Considerable levels of CSF-1 were found in conditioned medium of spleen cells from MPSV-infected mice, but not in the control sample. G-CSF activity was detected in the spleen cell conditioned medium of control as well as of day 11 and day 18, but not day 25 MPSV-infected mice. Whereas IL-6 bioactivity was also
weakly present in control, its amount was significantly increased 11 days after MPSV infection. GM-CSF activity was detectable in small amounts in the conditioned medium of spleen cells obtained from control mice, whereas only trace amounts (day 11) or no activity could be detected in the conditioned medium of spleen cells from MPSV-infected mice. No IL-2, IL-3, IL-4, or steel activity could be detected in either sample of spleen cell conditioned media.

TNFα activity was also evaluated in conditioned media from spleen of normal or MPSV-infected mice. As shown on Fig 3, TNFα cytolytic activity on L929 cells was about 10-fold increased in the spleen 20 days after virus infection, in comparison with normal control mice. Anti-TNFα antibody treatment completely inhibited the MPSV-CM-induced L929 lysis, confirming the presence of TNFα in these conditioned media (Fig 3).

DISCUSSION

The murine myeloproliferative syndrome induced by MPSV is characterized by a huge increase of the pluripotent and committed hematopoietic progenitor cell compartments.4-7 We earlier proposed that this myeloproliferation might result from a hematopoietin capable of stimulating the proliferation of pluripotent HSC (CFU-GEMM and CFU-S).8,9,10 In the present report we show that MPSV-spleen cell conditioned medium can stimulate the formation of colonies of primitive blast cells, which are, until now, the earliest precursors detected in vitro in semisolid culture.12 It is interesting to note that 2 of the 17 replated blast cell colonies (nos. 2 and 4, Table 1) give rise to very high number of secondary clones, including numerous CFU-GEMM, indicating the very early stage of these colonies. We therefore studied the occurrence of cytokines known to stimulate directly or indirectly early hematopoiesis, such as steel factor, IL-1, IL-3, IL-6, GM-CSF, CSF-1, and TNFα, in the MPSV-induced syndrome by measuring (1) cytokine mRNA levels in several tissue sources and (2) cytokine activities in serum samples and in medium conditioned by cells obtained from the spleen.

The mRNA of two cytokines studied, IL-6 and GM-CSF, were expressed at increased levels in the spleen, thymus, and liver of MPSV-infected mice. CSF-1 transcripts were also increased in the thymus and liver, but not in the spleen, where they were decreased. G-CSF transcripts were only increased in the spleen. The measurement of cytokine mRNA indicated not only the nature of cytokine involved in the MPSV syndrome but also the in vivo site of production. In the normal liver, there was no expression of IL-6,

**Table 2. Cytokine Titres in Spleen Cell Conditioned Medium**

<table>
<thead>
<tr>
<th>Day of Serum Collection</th>
<th>IL-1 (U/mL) (95% confidence limits)</th>
<th>CSF-1 (U/mL) (95% confidence limits)</th>
<th>G-CSF (U/mL) (95% confidence limits)</th>
<th>IL-6 (U/mL) (95% confidence limits)</th>
<th>GM-CSF (U/mL) (95% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>183 (110-305)</td>
<td>0</td>
<td>5.0 (3.5-7.3)</td>
<td>1,710 (1,008-2,858)</td>
<td>12.4 (9.7-16.0)</td>
</tr>
<tr>
<td>Day 11</td>
<td>305 (182-508)</td>
<td>13.0 (8.7-19.7)</td>
<td>9.2 (6.3-13.6)</td>
<td>11,700 (6,876-18,400)</td>
<td>1.2 (0.9-1.6)</td>
</tr>
<tr>
<td>Day 18</td>
<td>24.3 (14.3-40.7)</td>
<td>8.5 (5.8-12.6)</td>
<td>4.5 (3.1-6.5)</td>
<td>7,610 (4,481-5,295)</td>
<td>0</td>
</tr>
<tr>
<td>Day 25</td>
<td>24.5 (13.8-42.3)</td>
<td>4.9 (3.2-7.4)</td>
<td>0</td>
<td>4,710 (2,780-3,275)</td>
<td>0</td>
</tr>
</tbody>
</table>

Medium conditioned by spleen cells obtained from normal or from mice infected with MPSV (on day 11, 18, or 25 after virus infection) was tested for cytokine activities as described in Materials and Methods.
GM-CSF, and CSF-1 and cytokine gene expression seemed to be induced rather than augmented by MPSV infection. This agrees with the observation of Troutt and Lee, who showed that with an even more sensitive technique (S1-nuclease analysis) no IL-6, GM-CSF, or CSF-1 transcript could be detected in the liver of normal mice, even after in vivo lipopolysaccharide stimulation. The lack of IL-1 or IL-3 mRNA detection in tissues from normal or MPSV-infected mice could reflect a very low expression of such cytokines undetectable by Northern blot analysis.

For most of the cytokines studied, the increased level of cytokine transcripts correlates with an enhanced production of the corresponding bioactive proteins that were detected in the serum of MPSV-infected mice and in spleen cell conditioned medium. However, in the case of the CSF-1 measured in the spleen, there was a discrepancy between the decrease level of transcript and the increased protein production. This result could suggest a reduction in the half-life of CSF-1 mRNA and/or a posttranscriptional regulatory mechanism, as has already been reported for the human CSF-1. Furthermore, for GM-CSF, although the expression of the gene was increased in hematopoietic organs of infected mice, the biologically active molecule could not be detected in the circulation. GM-CSF is known to be rapidly cleared from the circulation. Moreover, GM-CSF could be sequestered in the extracellular matrix of the organs in which it was produced. The recent demonstration by Smadja et al (manuscript in preparation) that the synthesis of heparan sulfate, which is capable of binding GM-CSF in vitro, was greatly increased in the stroma of MPSV-infected spleen corroborates this hypothesis.

These results highlight the difficulty of comparing results obtained with methods that have some fundamental differences. Measurement of mRNA levels in tissue samples reflects the potential in vivo site of cytokine production but does not prove that biologically active cytokine is secreted. Measurement of cytokine bioactivity in the serum confirms the presence of cytokines in vivo at the actual time point a sample is taken and, combined with the mRNA determination, best reflects the situation in vivo. However, it is often difficult to evaluate the low levels of cytokines occurring in vivo, not only because they have generally very short serum half-lives, but also because cytokines are in general also thought to act locally in a paracrine rather than in an endocrine manner. Thus, the in vitro production of cytokines and their detection in medium conditioned by cells obtained from organs that are likely sources of cytokines is often used as an additional tool to study in vivo cytokine induction. However, it has to be kept in mind that the production of different cytokines in vitro could be higher or lower than in vivo, reflecting the absence of stimulating mechanisms present in the whole organ in vivo or the presence of new stimuli in the culture system. The results presented in Table 2 are, therefore, to be assessed with caution. Data showing that conditioned medium obtained from spleen cells of noninfected mice contains significant levels of IL-1a, IL-6, GM-CSF, and G-CSF activity probably reflect in vitro activation of the cellular sources.

There was evidence from mRNA studies that all the cytokines detected in the serum of MPSV-infected mice were produced in the hematopoietic tissues that are the main organs in which the pathologic process is observed. Indeed, after MPSV infection, spleen and thymus are progressively depleted in lymphocytes and are invaded, as the liver, by mature and immature cells from the granulocytic and erythroid series. These organs, the myeloproliferation is always associated with the proliferation of cells belonging to the hematopoietic microenvironment, also called stromal cells, especially fibroblasts, monocytes-macrophages, and endothelial cells. Several arguments point to an involvement of cells from the stroma in the MPSV-induced secretion of cytokine. (1) Whereas, in the mouse, IL-6 and GM-CSF could be produced by both stromal cells as well as activated T cells, G-CSF, CSF-1, and TNFa are secreted essentially by stromal cells. (2) IL-6 transcripts were greatly increased in the adherent spleen cell population mainly composed of macrophages (70%) and fibroblasts (data not shown). (3) GM-CSF and MPA bioactivities were present in the supernatant of cultures of macrophages or fibroblasts infected by MPSV either in vivo or in vitro. Moreover, T lymphocytes did not seem to be necessary for production of the cytokines we detected. First, no modification in the constitutive or induced level of growth factor release was detected in T lymphocytes that were infected in vitro with MPSV; second, no increased production of specific T lymphokines such as IL-2 or IL-4 was observed in MPSV-infected mice (these results); third, the total number of T lymphocytes decreased in the thymus of MPSV-infected mice, representing no more than 60% of the thymic cell population, 20 to 25 days after MPSV infection. Finally, athymic nude mice are sensitive to MPSV infection and develop a myeloproliferative syndrome similar to that induced in wild mice.

Troutt and Lee recently reported that, after intravenous injection of bacterial endotoxin, growth factor mRNAs...
were increased in the spleen, kidney, heart, and lung, but not in the liver or thymus. In MPSV-infected mice, we observed a different pattern of cytokine mRNA expression (spleen, liver, and thymus), suggesting that the cytokines detected in our system were not increased in response to a bacterial infection. Viral infections, including several pathogenic viruses such as Sendai, Ectromelia, Hepatitis, lymphocytic choriomeningitis virus, and Adenovirus, can also increase growth factor production. However, our mice and the MPSV stock are free of these viruses.

Such an in vivo production of cytokines, correlated by a huge increase in HSC concentration, has not yet been reported in other murine virus-induced leukemias, raising the possibility of a specific role of MPSV in the in vivo disorganization of hematopoiesis via cytokine production. We have shown that cells from hematopoietic organs of MPSV-infected mice and especially cells involved in the cytokine production, ie, splenic fibroblasts and macrophages, are infected by MPSV and produced infectious viral particles (F. Smadja-Joffe, in preparation). However, the molecular mechanism by which this virus induces the hyperproduction of hematopoietins is not yet known. In contrast to other oncogenes, the mos oncogene does not modify the growth factor requirements of hematopoietic precursors. A pan-activation mechanism of multiple hematopoietin genes, via trans-activating factors, could occur in stromal cells infected by MPSV, as proposed in Abelson virus-transformed myeloid cells.

In conclusion, our data show that IL-1α, IL-6, GM-CSF, G-CSF, CSF-1, and TNFα are produced in MPSV-infected mice. This production of cytokines is either amplified in organs in which myelopoiesis is increased (spleen) or in organs in which metaplasia of the myeloid series is described (liver and thymus). These results suggest the existence of a relationship between the sites of in vivo growth factor production and the dramatic increase of the pluripotent and nonlymphoid committed progenitor compartments observed in the hematopoietic organs of MPSV-infected mice. Our hypothesis on the role of hematopoietic growth factor is the establishment of the MPSV-induced myeloproliferation is strengthened by studies of Johnson et al and Wong et al that show that lethal myeloproliferative syndromes, similar to the MPSV-induced hemopathy, occur in mice infected by retroviral vectors bearing growth factor genes. Several recent studies reported an increase in the hematopoietic growth factor production in patients with myeloproliferative syndromes, indicating a possible involvement of these factors in such human pathologies. In that respect, the MPSV-induced myeloproliferative syndrome could serve as a model to study new therapeutic approaches, such as the use of neutralizing antibodies against hematopoietic growth factors, for the treatment of some myeloproliferative disorders.

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REFERENCES


Enhanced hematopoietic growth factor production in an experimental myeloproliferative syndrome

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