Generation of Functional Murine Macrophage Lines Employing a Helper-Free and Replication-Defective SV40-Retrovirus: Cytokine-Dependent Growth

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Key words: SV40/retroviral vector/recombinant virus/macrophage

ABSTRACT. By using a helper-free and replication-defective recombinant retrovirus encoding the SV40 early antigens (MV40), we have established continuous macrophage (Mφ) lines. All of the lines were nonproducer Mφ's with differentiated Mφ functions such as phagocytosis, cytotoxicity, and IL-1 and TNF production. To determine the effects of several cytokines on growth of mature Mφ's, the responsiveness of these established Mφ lines to various cytokines was investigated in methylcellulose culture. Their response patterns to several cytokines alone and in combination were different, implying that there might be mature Mφ subpopulations with distinct growth profiles regulated by several cytokines. On the other hand, all of the lines efficiently yielded a number of colonies in response to interleukin-4 (IL-4) alone. Moreover, IL-4 cooperated with interleukin-3 (IL-3) to enhance colony formation of all the lines. A similarly synergistic effect was observed in combination of IL-4 and macrophage-colony stimulating factor (M-CSF) in almost all the lines. Similar results were obtained with colony formation of fresh thioglycolate-induced Mφ's. These observations suggested that IL-4 was involved in growth of mature Mφ's.

Our present results suggest that the helper-free and replication-defective MV40 is of use to obtain continuous and functional cell lines from primary Mφ's.

The macrophage/monocyte plays primitive and indispensable roles in the primary host defense, the succeeding immunoreaction, and hematopoiesis in vivo (12, 24). These mononuclear phagocytes are generally considered to consist of heterogenous populations, the functions and phenotypes of which vary depending upon the distribution in the body (27, 28). Although recent studies show that several cytokines including interleukin-3 (IL-3), interleukin-4 (IL-4), granulocyte-macrophage-colony stimulating factor (GM-CSF), macrophage-colony stimulating factor (M-CSF), and interferon-γ (IFN-γ) have effects on macrophage (Mφ) function (4, 21, 22, 31, 32), their possible involvement in the growth of mature Mφ has been poorly understood. Several reports suggest that growth requirements of mature Mφ's differ depending on their origins and differentiation stages (1, 3).

To analyze the growth and function of Mφ's in vitro, it would be of help to obtain a number of clonal and functional macrophage/monocyte lines. Some tumor viruses such as SV40 and wild-type retroviruses have often been utilized to establish cell lines in mice (14, 23, 25). Murine Mφ's are not efficiently immortalized by SV40 because of its low infectivity. In the case of wild-type retroviruses, progeny viruses propagated in established cell lines affect the further biological examination and confuse the analysis. Recent improvement in the recombinant viral vector system has provided a helper-free and replication-defective virus stock (13). This virus infects cells, integrates into their genomes, and expresses an inserted gene efficiently. Moreover, no progeny of the virus are produced. Here, employing a helper-free and replication-defective SV40-retrovirus encoding the SV40 early antigens (MV40) (19, 10), we have immortalized and characterized several murine continuous Mφ lines. The established lines did not produce any virus and retained several Mφ functions. To clarify effects of various cytokines on growth of murine

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Abbreviations used: MV40, SV40-retrovirus; Mφ, macrophage; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage-colony stimulating factor; M-CSF, macrophage-colony stimulating factor; IFN-γ, interferon-γ; IL-1, interleukin-1; IL-2, interleukin-2; IL-3, interleukin-3; IL-4, interleukin-4; TNF, tumor necrosis factor; TG, thioglycolate; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PHA, phytohemagglutinin; SRBC, sheep red blood cells; LPS, lipopolysaccharide; kb, kilobase pairs; kd, kilodaltons; ADCC, antibody-dependent cellular cytotoxicity

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mature Mφ, the cytokine responsiveness of the cell lines was investigated. Their patterns of cytokine-dependent growth were different, implying that there might be murine Mφ subpopulations whose proliferation was regulated by several cytokines.

MATERIALS AND METHODS

Mice. All mice were purchased from Japan SLC Inc. (Shizuoka, Japan).

Cells. ¥2EVXT14, which produced helper-free and replication-defective retroviruses encoding the SV40 early antigens (MV40) (9), J774-1, which was kindly provided by Dr. S. Natori at Tokyo University, Japan, EL4, P815, and WEHI3 cells were maintained with RPMI1640 (Gibco Laboratories, Grand island, NY, USA) containing 50 µM 2-ME, 50 µg/ml streptomycin (Meiji Seika Kaisha, Ltd., Tokyo, Japan), 50 U/ml penicillin (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan), 10% FCS (Hyclone, defined, Hyclone Laboratories, Inc., Logan, UT, USA) (complete RPMI). L929 cells, kindly provided by Dr. S. Natori, were maintained with Eagle’s MEM containing 50 µM 2-ME, 50 µg/ml streptomycin, 50 U/ml penicillin, 5% FCS. FDC-P2 (IL-3-dependent cells kindly provided by Dr. K. Kumagai at Tohoku University, Japan), IC2/GM (GM-CSF-dependent cells kindly provided by Dr. S. Koyasu at the Tokyo Metropolitan Institute of Medical Science, Japan), and NFS60/GM (GM-CSF-dependent cells kindly provided by Dr. T. Suda at Jichi Medical School, Japan), were maintained with the complete RPMI containing 30% conditioned media of WEHI3 cells as murine IL-3 or 50 U/ml recombinant murine GM-CSF.

Cytokines and other reagents. Recombinant factors were as follows: recombinant human (rh) interleukin-2 (IL-2) (6); recombinant murine (rm) IL-3 (pcDMIL-3, 6); rmIL-4 (pSRamIL-4 constructed from pSP6BSF (17), a kind gift of Drs. T. Hirano and T. Kishimoto at Osaka University, Japan), rmGM-CSF (pHSgm-CSF, 8); and rm granulocyte-colony stimulating factor (G-CSF) (pLSVmG-CSF). Supernatants of COS cells transfected with each factor CDNA expressed in pSRamIL-4 constructed from pSP6BSF (17), a kind gift of Drs. T. Hirano and T. Kishimoto at Osaka University, Japan), rmGM-CSF (pHSgm-CSF, 8); and rm granulocyte-colony stimulating factor (G-CSF) (pLSVmG-CSF). Supernatants containing 10⁶ units/ml recombinant murine GM-CSF were less than 0.1%. PCDL-SRa296 vector-transfected cells were kindly provided by Dr. S. Koyasu at the Tokyo Metropolitan Institute of Medical Science, Japan, and NFS60/GM (GM-CSF-dependent cells kindly provided by Dr. T. Suda at Jichi Medical School, Japan), were maintained with the complete RPMI containing 30% conditioned media of WEHI3 cells as murine IL-3 or 50 U/ml recombinant murine GM-CSF.

Cytokine assay. IL-3, GM-CSF and G-CSF were assayed by proliferation of factor-dependent cell lines using MTT (16). Briefly, samples serially diluted were cultured with 10⁴ cells in 100 µl of the complete RPMI in a flat-bottom 96-well plate for 18 hours. Each well was filled with 10 µl of 5 mg/ml MTT, further incubated for 4 hours at 37°C, and then filled with 150 µl of 0.04 N HCl/isopropanol. After the blue crystals dissolved, the plates were read on a Titertek Multiscan MCC (Flow Laboratories, McLean, VA, USA) at 590 nm. One unit represents the activity that induced half-maximum proliferation response. Usual units were normalized on the basis of a factor laboratory reference.

Interleukin-1 (IL-1) activity was assayed essentially as described above. Samples were cultured with 1.5 x 10⁶ LPS-unresponsive thymocytes of 5-week-old C3H/HeJ mice in the complete RPMI containing 1 µg/ml PHA for 72 hours at 37°C. Proliferation was assessed by the MTT assay. One unit and usual units represent the same activity as described above.

Tumor necrosis factor (TNF) was assayed by L929 cells as indicator cells. Samples serially diluted were incubated with 10⁶ cells in 100 µl of the culture media containing 1 µg/ml anti-IL-2 mAb in a flat-bottom 96-well plate for 12 hours at 37°C. Thereafter, the plate was stained with crystal violet and read on the Titertek Multiscan MCC at 590 nm. Units represent the inverse of the dilution that induced half-maximal killing and corresponded to a TNFa reference control.

M-CSF (colony-forming unit/ml) was assayed by colony formation in soft agar of fresh bone marrow cells (15). Triplicate samples diluted were cultured with 10⁵ of fresh nonadherent bone marrow cells of C57BL/6 mice in 1 ml of α-MEM (nucleic acid-free, Gibco Laboratories) containing 0.3% noble agar and 20% FCS in a 35-mm culture dish (Corning 25000, Iwaki Glass, Tokyo, Japan) for 7 days at 37°C. Formed colonies (>40 cells) were counted in the inverted microscope.

Phagocytosis. Latex beads (Polymer Science Inc., Warrington, PA, USA) and anti-sheep red blood cell (SRBC) antibody (Cappel, Organon Teknika corp., The Netherlands)-sensitized SRBC were incubated with cells for 0.5 hours at 37°C. After incubation, the cells were washed with the complete RPMI and then microscopically observed.

Immunological marker. Mac-1 (M1/70, Ia (M5/114), and Ly-5 (M1/9.3 HL) (Hybritech Inc., San Diego, CA, USA) were detected by fluorescein-conjugated goat anti-rat IgG (ab’2 fragments (Cappel), and then analyzed by Epics CS (Coulter Electronics, Hialeah, FL, USA).

Immunoprecipitation. Cells were washed methionine-free RPMI1640 (Met (-) RPMI, Gibco Laboratories), incubated in Met (-) RPMI containing 2% dialyzed FCS (Gibco Laboratories) for 2.5 hours, and further incubated in 1 ml of Met (-) RPMI containing 100 µCi [³⁵S]-methionine (Amersham corp., Arlington Heights, IL, USA) and 2% dialyzed FCS for 3 hours. After washing with prewarmed PBS containing 2 mg/ml methionine, the cells were lysed with 1 ml of 50 mM Tris-HCl, pH 8.0, 120 mM NaCl, and 0.5% NP40 for 0.3 hours on ice and centrifuged. Aliquots of the lysates contain-
ing equivalent quantities of incorporated radioactivity were precipitated by a monoclonal antibody against the SV40 early antigens (Clone 419, Oncogene Science, Inc., Mineola, NY, USA) and protein A (Pharmacia, Uppsala, Sweden). The precipitates were solubilized, separated by 10% SDS-PAGE, dried up, and subjected to autoradiography.

**Cytotoxicity.** Antitumor activity was detected by $^{51}$Cr-release assay. Briefly, EL4 and P815 cells were labeled with 100 μCl Na$_2^{51}$CrO$_4$ (Amersham Corp.) for 1 hour at 37°C, and washed 3 times with the complete RPMI. The $10^5$ labeled target cells were mixed with effector cells at different effector-to-target ratios (10:1, 3:1, 1:1, and 0.3:1) in 200 μl medium in U-shaped 96-well plates. After incubation for 18 hours at 37°C and then centrifugation, 100 μl supernatants were collected from each well and counted in a gamma counter. All tests were carried out in triplicate. The percentage specific $^{51}$Cr release was calculated by the formula:

$$\frac{\text{specific} \text{ } ^{51}\text{Cr} \text{ release of sample} - \text{specific} \text{ } ^{51}\text{Cr} \text{ release in medium}}{\text{specific} \text{ } ^{51}\text{Cr} \text{ release in 1% TritonX-100} - \text{specific} \text{ } ^{51}\text{Cr} \text{ release in medium}} \times 100.$$

Antibody-dependent cellular cytotoxicity (ADCC) was assayed with EL4 as described above in the presence of anti-Thyl.2 monoclonal antibody (Cappel).

**Southern blot hybridization.** Genomic DNA was extracted as previously described (20). The digested DNA's were electrophoresed on a 0.8% agarose gel, transferred to a nitrocellulose membrane filter, and hybridized with $^{32}$P-nick-translated SV40 DNA. After washing under stringent conditions, the dried filter was subjected to autoradiography.

**Morphology.** Cells were subjected to cytospin smear (Cytospin Centrifuge, Shandon Southern Products Ltd., Cheshire, UK) and stained with May-Giemsa.

**Preparation of peritoneal exudate cells.** One ml per mouse of 10% thioglycolate (TG, Difco Laboratories) solution was intraperitoneally injected. After 3 days, peritoneal exudate cells obtained from the mouse were washed and incubated onto tissue culture dishes (Nunclon 150288 A/S Nunc, Roskilde, Denmark) for 2 hours at 37°C. Nonadherent cells were washed off. At least 97% of the adherent cells were Mφ's judged by morphologic and phagocytic criteria.

**Methylcellulose culture.** $10^3$ or $10^4$ Mφ's were cultured in 1 ml of 0.88% methylcellulose/the complete RPMI in triplicate 35-mm petri dishes (Falcon 1008, Becton Dickinson & Co., Mountain View, CA, USA) for 2 weeks at 37°C. Formed colonies (>10 cells) were scored in the inverted microscope.

**RESULTS**

**Generation of continuous cell lines by infection with a helper-free and replication-defective MV40.** 100 whole embryo cells at gestation day 9 of C57BL/6 mice were cocultured with $^{92}$EVXT14 cells (3 $\times$ 10$^5$ cells) producing helper-free and replication-defective MV40 in a 35-mm petri dish (Corning 25020) overnight. Nonadherent cells were harvested and further cultured in a 100-mm petri dish (Corning 25020). In a few weeks, the growing cells became apparent and such cells could be expanded. Adult bone marrow adherent cells of Balb/c mice were cultured in a 100-mm petri dish (Corning 25020) in the presence of 20% conditioned media of L929 cells overnight. The medium was removed and replaced with fresh supernatants of $^{92}$EVXT14 cells sup-

**Fig. 1.** Phenotypical features of a continuous cell line. TME5 cells were cultured on a plastic culture flask (A), were stained with May-Giemsa (B), ingested latex beads (C), and formed a rosette with IgG-sensitized SRBC and ingested it (D).

**Fig. 2.** Integration of MV40 proviruses in the genomes of Mφ lines. DNAs (10 μg) of normal mouse bone marrow cells (N) and cell lines (TME1, TME3, TME5, TME6, TME8, TME9, TME18, TME30, and BABT91) were digested with Hpa I, which cuts only one site within the MV40 provirus, electrophoresed on a 0.8% agarose gel, transferred to a nitrocellulose filter, and hybridized with $^{32}$P-labeled SV40 DNA.
plemented with 20% conditioned media of L929 cells and 5 μg/ml polybrene (Aldrich, WI, USA). After overnight culture, the adherent cells were washed and cultured in the presence of 20% conditioned media of L929 cells. In a few weeks, the growing cells were collected by ring-trypsinization and expanded. In both experiments, the growing cells were cloned by limiting dilution. TME lines were established from the embryo cells. A BABT9I line was obtained from the bone marrow cells and could be propagated without conditioned media of L929 cells. All of the lines were maintained over a year with the complete RPMI. Phenotypical features of the TME5 line as a representative are shown in Fig. 1. All continuous cell lines were strongly adherent to a plastic culture flask (Fig. 1, A). With May-Giemsa stain, they showed typical morphological features as Mφ (Fig. 1, B): they had round nuclei, and large and pale cytoplasms. They ingested latex beads and IgG-sensitized SRBC (Fig. 1, C and D, respectively), and formed rosettes with IgG-sensitized SRBC (Fig. 1, D). Their immunological surface markers were the same, namely Mac-1+, Ia+, Ly-5+, andFcγR+.

Integration of MV40 provirus into the genomic DNA’s of established Mφ lines. Figure 2 shows the results of Southern blot analysis with SV40 DNA. Since MV40 provirus has only one Hpa I site (10), the appearance of many SV40-specific hybridization bands in all TME lines suggested that each TME line harbored multiple proviral copies. In contrast, two Hpa I fragments were detected in BABT9I, suggesting that only one complete MV40 provirus was integrated into BABT9I genome.

Expression of the SV40 early antigens in the immortalized Mφ lines. Lysates prepared from 35S-methio-nine-labeled cells were quantitatively precipitated by the monoclonal antibody against the SV40 T/t antigens, followed by 10% SDS-PAGE (Fig. 3). This antibody specifically immunoprecipitated the large T antigen (94 kd) and its associated p53 (53 kd), none of which could be detected by normal mouse IgG (Fig. 3, A). All of the lines expressed the SV40 large T antigen and the p53 (Fig. 3, B). The small t antigen (17 kd) was detected by longer exposure of autoradiography (data not shown). These immunoprecipitation analyses revealed that the integrated MV40 proviruses were active.

In spite of integration and expression of the MV40 proviruses, no progeny virus could be detected in the culture fluids of all the lines by focus formation assay on rat fibroblasts (data not shown). These observations indicated that the lines did not produce any MV40.

Characterization of the established cell lines as Mφ’s. As shown in Fig. 4, the growth of almost all the lines except for TME3 and BABT9I was significantly inhibited by LPS. Simultaneously, all the LPS-stimulated lines were larger in cytoplasm and much more adherent to the culture dish (data not shown). These observations indicated that all of the lines were sensitive to LPS. We then investigated their antitumor activity against EL4
Table I. IL-1 and TNF production of Mφ lines.

<table>
<thead>
<tr>
<th>Mφ lines</th>
<th>IL-1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TNF&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TME3</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>TME5</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>TME6</td>
<td>&lt;2</td>
<td>21</td>
</tr>
<tr>
<td>TME9</td>
<td>&lt;2</td>
<td>64</td>
</tr>
<tr>
<td>TME30</td>
<td>&lt;2</td>
<td>20</td>
</tr>
<tr>
<td>BABT9I</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

<sup>a</sup> 3 x 10⁵ cells of each line were cultured in a 12-well culture plate (Corning 25815) in 1 ml of the complete RPMI with (LPS) or without (None) 1 µg/ml LPS. After overnight culture, supernatants were harvested.

Cytokine-dependent growth of fresh and established mature Mφ's. To know the response of fresh Mφ's to cytokines, we investigated cytokine-induced growth of fresh TG-induced peritoneal Mφ’s in methylcellulose culture (Table II). GM-CSF, IL-3, and IL-4 alone induced a number of colonies, whereas G-CSF and M-CSF alone yielded a small number of colonies. Combination of IL-3 and IL-4 generated colonies more effectively than each alone. M-CSF, on the other hand, appeared to inhibit the GM-CSF colony formation of fresh TG-induced Mφ’s. Since fresh TG-induced Mφ’s were mixed populations, it was difficult to know whether individual Mφ's could respond to single or multiple factors. To clarify this point, using the Mφ lines including TME and J774-1 lines, we examined their growth property in terms of responsiveness to cytokines. These results are summarized in Table III. When cultured without any cytokine, these lines except for TME3 and J774-1

Table II. Colony formation of fresh TG-induced peritoneal Mφ’s<sup>b</sup>.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Number of colonies per dish&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>IL-3</td>
<td>251±15</td>
</tr>
<tr>
<td>IL-4</td>
<td>349±13</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>327±5</td>
</tr>
<tr>
<td>M-CSF</td>
<td>143±10</td>
</tr>
<tr>
<td>G-CSF</td>
<td>104±8</td>
</tr>
<tr>
<td>IL-3+IL-4</td>
<td>536±12</td>
</tr>
<tr>
<td>IL-3+M-CSF</td>
<td>251±23</td>
</tr>
<tr>
<td>IL-4+M-CSF</td>
<td>304±6</td>
</tr>
<tr>
<td>GM-CSF+M-CSF</td>
<td>219±17</td>
</tr>
</tbody>
</table>

<sup>b</sup> 10⁵ cells of fresh peritoneal Mφ’s obtained from TG-injected C57BL/6 mice were seeded into methylcellulose culture supplemented with the indicated cytokines (100 U/ml) in triplicate petri dishes. After 2 weeks, formed colonies were counted.

<sup>c</sup> Each value represents the mean number (±SD) of colonies from triplicate cultures.

<sup>d</sup> COS cells were transfected with pcDL-SRα296 vector alone. The supernatants were harvested after 3 days and used as control at 10% final concentration.
Table III. Effects of various cytokines on the growth of Mφ lines in methylcellulose culture.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>TME3</th>
<th>TME5</th>
<th>TME6</th>
<th>TME9</th>
<th>TME30</th>
<th>J774-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controlb)</td>
<td>336± 8</td>
<td>86± 6</td>
<td>24± 4</td>
<td>66± 4</td>
<td>10± 2</td>
<td>268±12</td>
</tr>
<tr>
<td>IL-2</td>
<td>332±12</td>
<td>82± 9</td>
<td>30± 2</td>
<td>68± 8</td>
<td>38± 2</td>
<td>234±15</td>
</tr>
<tr>
<td>IL-3</td>
<td>375±32</td>
<td>87± 3</td>
<td>56± 1</td>
<td>102±4</td>
<td>8± 1</td>
<td>596±36</td>
</tr>
<tr>
<td>IL-4</td>
<td>344± 4</td>
<td>120±4</td>
<td>173±5</td>
<td>102±4</td>
<td>94± 2</td>
<td>448± 8</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>349±15</td>
<td>436±4</td>
<td>328± 7</td>
<td>120±4</td>
<td>152±16</td>
<td>500±12</td>
</tr>
<tr>
<td>M-CSF</td>
<td>390± 6</td>
<td>284±11</td>
<td>30± 2</td>
<td>136± 8</td>
<td>70±14</td>
<td>352±8</td>
</tr>
<tr>
<td>G-CSF</td>
<td>340±10</td>
<td>172±20</td>
<td>57± 7</td>
<td>70± 4</td>
<td>10± 2</td>
<td>267± 5</td>
</tr>
<tr>
<td>IL-3+IL-4</td>
<td>330±18</td>
<td>238±6</td>
<td>296±4</td>
<td>132±4</td>
<td>206±14</td>
<td>480± 8</td>
</tr>
<tr>
<td>IL-3+M-CSF</td>
<td>308± 4</td>
<td>161±1</td>
<td>48± 8</td>
<td>135±3</td>
<td>30± 2</td>
<td>400± 4</td>
</tr>
<tr>
<td>IL-4+M-CSF</td>
<td>332± 4</td>
<td>320±4</td>
<td>348±20</td>
<td>192± 4</td>
<td>245± 7</td>
<td>294±6</td>
</tr>
<tr>
<td>GM-CSF+M-CSF</td>
<td>203± 1</td>
<td>410±14</td>
<td>78±5</td>
<td>78±2</td>
<td>52± 3</td>
<td>296±16</td>
</tr>
</tbody>
</table>

a) 10³ cells of each line, but 10⁴ cells in TME9, were seeded into methylcellulose culture supplemented with the indicated cytokines (100 U/ml). Formed colonies were scored after 14 days.
b) Each value represents the mean number (±SD) of colonies from triplicate cultures.
c) Mock-transfected supernatants of COS-1 cells were obtained as described in the footnote to Table II and used as control at 10% final concentration.

hardly generated colonies. In contrast, GM-CSF and IL-4 alone significantly enhanced colony formation of almost all the lines but TME3 as observed in fresh TG-Mφ's. In addition, TME5 and TME6 lines were responsive to G-CSF. TME5, TME9, TME30, and J774-1 lines were responsive to M-CSF. The cytokine responsiveness of each Mφ line was shown to be totally different. Moreover, in various combinations of two cytokines, synergistically promoting effects were observed in TME5, TME6, and TME30 with IL-3 plus IL-4, and in TME6 and TME30 with IL-4 plus M-CSF. However, almost all the lines did not form in GM-CSF and M-CSF combination, as observed in the fresh TG-Mφ's. M-CSF-induced colonies of TME5 and TME30 appeared to be inhibited by IL-3. A similar inhibitory effect was observed in culture of J774-1 with IL-4 plus M-CSF. No significant effects of mock-supernatants, IL-2, or other cytokine combinations tested were observed.

These methylcellulose culture experiments revealed that each Mφ line could respond to multiple cytokines and that the cytokine-dependent growth properties differed among the Mφ lines. These results may imply that there are murine mature Mφ subpopulations whose responsiveness to several cytokines is distinct and that their growth is controlled by several cytokines. It should be noted, on the other hand, that Mφ growth was supported by GM-CSF, G-CSF, IL-3, and IL-4. These findings suggest that these factors partly act as mature Mφ growth factors.

DISCUSSION

To analyze their growth and function in vitro, clonal Mφ lines with differentiated functions are convenient because of Mφ heterogeneity (27, 28) and the difficulty of obtaining a homogenous population. Several Mφ lines have been used under biologically restricted conditions since these lines are tumorigenic, less functional, or produce wild-type retroviruses. Particularly, the progeny of these viruses often affect biological examinations. Here, employing the helper-free and replication-defective MV40 (9, 10), murine continuous Mφ lines have been immortalized. These lines established here carried MV40 proviruses, but produced no progeny of MV40. Judging from our observation that no myeloid except for the Mφ line could be obtained (data not shown), MV40 appeared to immortalize rather selectively murine Mφ's, like raf/mil retrovirus (2).

The established Mφ lines expressed several functions such as phagocytosis, antitumor activity, ADCC, and IL-1 and TNF production, although to a somewhat different extent. The TME lines produced IL-1 and TNF upon LPS stimulation, whereas the BABT9I line did not. This difference might be due to the different origins; TME lines were derived from embryos while BABT9I came from bone marrow. However, BABT9I constitutively expressed IL-1α, IL-1β, and TNF mRNA's detected by northern blot analysis (data not shown). BABT9I might have produced these factors whose levels were not detected by our present biological assays. Although flow cytometric analysis showed that 41-74% of cells of several Mφ lines expressed Ia antigen, these lines hardly exhibited antigen presenting activity (data not shown). The reason for this deficiency in antigen presenting activity is uncertain. This might be due to the origin, the influence of the SV40 T antigens, or both. These results suggested that the nonproducer lines retained several Mφ functions except for antigen
presenting activity.

We examined cytokine-dependent growth of fresh TG-induced peritoneal Mφ's. IL-3, IL-4, GM-CSF, M-CSF, and G-CSF induced their growth to generate colonies in semisolid medium. However, the fine responsiveness of mature Mφ's to cytokines remains unclear because of their heterogeneity. To clarify this point, we examined growth properties of the established Mφ lines. The different response patterns of the Mφ lines to several cytokines were observed. These results may imply that mature individual Mφ's respond to multiple cytokines and that there are distinct subpopulations of Mφ's which have different growth profiles to several cytokines. The differences in cytokine responsiveness of the lines might not be due to the SV40 T antigens since these expression levels were not correlated with the cytokine response pattern. However, the possibility could not be ruled out that the SV40 T antigens modified cytokine-dependent growth of the Mφ lines.

It is established that IL-4 is a B-cell stimulating factor (11, 19), and that IL-4 acts as a mature mast cell growth factor (11). However, IL-4 receptors are found on not only B and mast cells but also T cells and Mφ's (17). Moreover, IL-4 modifies the antigen presenting ability of Mφ's (32). In our experiments, IL-4 supported colony growth of the TG-induced Mφ's and all of the Mφ lines. Furthermore, IL-4 enhanced Mφ colony formation in the presence of IL-3 or M-CSF. These observations suggested that IL-4 had a promoting effect on mature Mφ proliferation. It is presumed that IL-4 secreted by activated T cells might induce mature Mφ growth and simultaneously regulate functions of Mφ's and other cells in host defense reactions such as inflammation and primary immunoreaction. Mature Mφ growth promoting effect was also observed in IL-3 (a mast cell growth factor, 29), G-CSF, and GM-CSF, although these factors stimulate bone marrow progenitor cells to generate various types of hematopoietic colonies (5, 7, 18). These findings suggested that these factors could also induce mature Mφ proliferation.

M-CSF is well known to stimulate Mφ colony formation of bone marrow progenitor cells in soft agar and growth of mature Mφ's. Recently, it was reported that op/op (osteopetrosis) mutant mice lacking production of biologically active M-CSF are severely deficient in mature Mφ's (30). This finding strongly suggests that M-CSF also plays a major role in Mφ differentiation. Here, M-CSF induced colony formation of fresh TG-induced Mφ's and the lines efficiently. However, M-CSF nonresponsive Mφ lines (TME3 and TME6) and the potential Mφ growth activity of GM-CSF, IL-3, IL-4, and G-CSF raise the possibility that GM-CSF, G-CSF, IL-3, and IL-4 partly play a role in mature Mφ growth and function. This possibility is supported by the observation that growth of murine pulmonary alveolar Mφ is supported by IL-3, not M-CSF (3). In addition, in op/op mice, there are M-CSF-responsive cells in spite of the abnormality of M-CSF (30).

In the present experiments, M-CSF was inhibitory to GM-CSF-induced colony formation of almost all the Mφ lines and mature Mφ's. Similar inhibitory effects were observed in culture of TME5 and TME30 with IL-3 plus M-CSF, and J774-1 with IL-4 plus M-CSF. On the other hand, IL-3 plus IL-4 and IL-4 plus M-CSF had synergistically promoting effects on mature Mφ's. It seemed that the proliferation and/or function of mature Mφ subpopulations might be regulated through several cytokines in normal tissues or inflammation.

Our present results suggest that the helper-free and replication-defective MV40 would be of help in obtaining a number of clonal and functional cell lines from primary Mφ's. The established Mφ lines with distinct characteristics would be of use in analyzing the growth and function of Mφ in vitro.

Acknowledgement. We thank Drs. R. Mulligan, M. Botchan, S. Natori, K. Kumagai, S. Koyasu, T. Suda, Y. Takebe, T. Hirano, and T. Kishimoto for cells and reagents, and Dr. H. Narliuchi at the Institute of Medical Science, Tokyo University for valuable advice and help with the antigen presentation test.

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(Received for publication, July 11, 1991 and in revised form, September 14, 1991)