Modulation of Spontaneous Outgrowth of Epstein-Barr Virus Immortalized B-Cell Clones by Granulocyte-Macrophage Colony-Stimulating Factor and Interleukin-3

By Cassandra C. Paul and Michael A. Baumann

Spontaneous outgrowth of immortalized Epstein-Barr virus (EBV) infected B-cell clones will occur from cultures of peripheral blood mononuclear cells (PBMCs) of some persons with a history of EBV infection. We determined that outgrowth of such clones may be reproducibly modulated by supplementation of cultures with the hematopoietic growth factors GM-CSF and interleukin-3 (IL-3). Continuous supplementation of cultures with GM-CSF facilitates emergence of immortalized B-cell clones, whereas supplementation with IL-3 completely prevents their emergence. The effect of GM-CSF may be direct, at least in part, as the proliferation of pure clones of EBV-transformed B lymphocytes was augmented in response to GM-CSF. An indirect mechanism appears to be responsible for the inhibition of transformed B-cell outgrowth in response to IL-3, as IL-3 had no inhibitory effect on proliferation of pure transformed B-cell clones and IL-3-mediated inhibition could be eliminated by antibody neutralization of gamma interferon (gamma-IFN) or tumor necrosis factor-alpha (TNF-alpha) in culture. The mechanisms of these effects deserve further study and may have clinical relevance to use of hematopoietic growth factors for support of bone marrow (BM) function in immunocompromised patients.

THE AVAILABILITY of recombinantly produced hematopoietic growth factors has facilitated detailed study of the activity of each factor. Two factors that possess multilineage hematopoietic activities are granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3). These molecularly distinct glycoproteins have several overlapping activities, including the support of proliferation of multipotential (CFU-GEMM), eosinophil, and GM colonies. Each growth factor also possesses distinct activity, such as enhancement of mature neutrophil function by GM-CSF. These effects have resulted in considerable interest in use of these agents for amelioration of bone marrow (BM) hypoplasia complicating intensive chemotherapy, hastening of marrow transplant engraftment, and treatment of primary BM failure.

Early studies of recombinantly produced IL-3 and GM-CSF suggested that these factors were devoid of activity on lymphoid cells. However, more recently normal and malignant B-cell precursors were reported to proliferate in response to IL-3, and IL-3 receptors have been demonstrated on malignant pre-B cells. No such effects have yet been reported for GM-CSF, but it is noteworthy that most clinical trials of GM-CSF have shown that absolute lymphocyte counts increase in a dose-dependent manner.

We describe a differential effect of human recombinant GM-CSF and IL-3 on the emergence of Epstein-Barr virus (EBV)-transformed B-cell lines from peripheral blood mononuclear cell (PBMC) suspension cultures. Continuous exposure of cultures to GM-CSF enhances emergence of EBV-transformed cell lines, whereas IL-3 consistently prevents their emergence. These observations may be of importance to the understanding of lymphohematopoietic growth regulation and may have clinical relevance to use of these multipotent growth factors for hematologic support in conditions complicated by severe immune compromise.

MATERIALS AND METHODS

Growth factors. Recombinant human IL-3 was a gift from Dr Steven Clark (Genetics Institute, Cambridge, MA). Recombinant human GM-CSF was a gift from Dr Christopher Henney (Immunex, Seattle, WA). Both factors were supplied in phosphate-buffered saline (PBS), pH 7.2, and were used as units of activity per milliliter. All dilutions of the factors were made in complete medium as defined.

Anticytokine antibodies. Murine monoclonal antibodies (neutralizing MoAbs) to human GM-CSF and gamma interferon (gamma-IFN) were obtained from Genzyme, Boston, MA. Neutralizing rabbit anti-rabbits to human tumor necrosis factor-alpha (TNF-alpha) was obtained from Genzyme.

PBMC cultures. After patients gave informed consent, their PB was obtained on several occasions from each of two subjects with known previous exposure to EBV. We had determined previously that spontaneous EBV-transformed B-cell lines would emerge from suspension cultures of PBMCs of these subjects. The mononuclear fraction was isolated after centrifugation of theuffy layer over Ficoll-Hypaque. Cells were washed twice by centrifugation at 200 g for 10 minutes in RPMI 1640 containing 5% fetal calf serum (FCS) and were resuspended at 4 x 10^6/mL in complete medium, consisting of RPMI 1640 containing 7.5% FCS, 2 mmol/L l-glutamine, 1 mmol/L sodium pyruvate, 5 x 10^-5 mol/L 2-mercaptoethanol, and 40 mg/mL gentamicin. Suspension cultures were established in either 25- or 75-cm^2 tissue culture flasks were fed by replacement of one third of the medium with an equal volume of fresh complete medium. Parallel cultures were established containing either 40 U/mL GM-CSF or 20 U/mL IL-3. When these cultures were fed, the replacement medium contained the same concentration of the appropriate growth factor. Viability determinations were made periodically by the trypsin blue exclusion method. Composition of the cultures was carefully monitored daily by inspection on an inverted microscope and by periodic microscopic analysis of Wright-stained cytospin preparations. During a repeat experiment, flow-cytometric analysis of cell-surface differentiation-related antigens was performed periodically on aliquots taken from the parallel cultures. In one experiment, IL-3-supplemented cultures were similarly established, but to parallel cultures either anti-TNF-alpha sufficient to neutralize 20 U/mL TNF activity or
anti-γ-IFN in an amount sufficient to neutralize 25 U/mL was added. Antibodies were again added to cultures every 2 days.

_3H-Thymidine uptake proliferation assay._ EBV-transformed B-cell lines to be analyzed were cultured in replicate in complete medium (2 x 10^6 cells in a total volume of 0.2 mL) in 96-well tissue culture plates for 24, 48, 72, 96, 120, and 144 hours at 37°C in a humidified 5% CO₂ atmosphere. At the end of each culture period, the wells were pulsed with 0.5 μCi ^3H-thymidine and incubated for 16 hours more before being harvested onto glass-fiber filters in a semiautomatic cell harvester for liquid scintillation counting. Results are expressed as mean cpm ± SD of replicate cultures. Parallel cultures were incubated similarly, but with the addition at culture initiation of varying concentrations of GM-CSF or IL-3.

**Statistical analysis.** All analyses were made by two-tailed t test with the Tadpole program (Elsevier-Biosoft, Cambridge, England).

### RESULTS

**Effect of growth factors on emergence of EBV-transformed B-lymphoblastoid cell lines.** Suspension cultures of PBMNCs maintained in complete medium alone progressively decreased in viability until 7 to 8 weeks, at which time a population of morphologically lymphoblastoid cells invariably emerged. These cells were capable of replication in unsupplemented complete medium, with doubling times of 56 to 72 hours. Immunophenotyping of these cells by indirect immunofluorescent labeling and flow-cytometric analysis consistently demonstrated a CD20⁺, CD22⁺, CD23⁺, sIg⁺ phenotype, consistent with EBV-transformed mature B lymphocytes. The presence of EBV in the cells was confirmed by immunofluorescent labeling with anti-EBNA (Chemicon International, El Segundo, CA). In the parallel PBMNC cultures containing IL-3 or GM-CSF, vigorous proliferation of myeloid elements with a similar differential composition by Wright's-stained cytoreparation morphology occurred in the early weeks of culture. A marked discrepancy in emergence of lymphoblastoid cells was evident, however, between the GM-CSF- and IL-3-supplemented cultures. In cultures supplemented with GM-CSF, emergence of morphologically recognizable lymphoblastoid populations occurred at 5 to 6 weeks (2 weeks earlier than in parallel cultures maintained without supplemental growth factors). Phenotypical analysis of these cells demonstrated that they were identical to the later-emerging cells in unsupplemented cultures and were also EBNA⁺. In contrast, lymphoblastoid cells never emerged in IL-3-supplemented cultures, and these cultures invariably contained no viable elements by week 12 (Fig 1). Identical results were obtained in parallel cultures initiated in 12 different experiments using PBMNCs from two subjects: EBV-transformed B-cell lines emerged earlier in cultures supplemented with GM-CSF but never emerged in IL-3-supplemented cultures.

We found that the inhibitory effect of IL-3 on emergence of EBV-transformed B cells is absolutely dependent on continued maintenance of IL-3 supplementation. Failure to supplement with IL-3 for as brief a period as one 4-day feeding cycle during the first 4 weeks of incubation will permit emergence of EBV-transformed cell lines (data not shown). To ascertain whether the inhibitory effects of IL-3 might be caused indirectly by induction of cytokines with known inhibitory activity against EBV-transformed B cells, such as γ-IFN or TNF-α, parallel IL-3-containing cultures were supplemented with neutralizing antibodies to these cytokines, as described. EBV-transformed B cells emerged from the culture containing IL-3 and anti-γ-IFN and from the culture containing IL-3 and anti-TNF-α, but not from the culture containing IL-3 alone.

**Effect of hematopoietic growth factors on spontaneous emergence of EBV-transformed B-cell lines.** To ascertain whether modulation of emergence of EBV-transformed B-cell lines is a direct effect of GM-CSF or IL-3 on proliferation of the cells, five EBV-transformed B-cell lines obtained after long-term culture of PBMNCs from the two subjects were assayed in standard ^3H-thymidine uptake studies during culture in either complete medium alone or in medium with GM-CSF or IL-3 added. Purity of the populations was ascertained by flow-cytometric analysis and, in three cases, by demonstration of clonal immunoglobulin gene rearrangements by Southern analysis. Each of these three cell lines had a distinct rearrangement (Dr A. Fishleder, personal communication, March 1989). IL-3 had no significant inhibitory effect on proliferation of the B lymphoblasts regardless of time of culture or concentration up to 100 U/mL, but GM-CSF reproducibly caused a significant increase in proliferation of all five cell lines that was most evident at 96 hours after a single addition of the growth factor and was maximal at a concentration of between 25 and 50 U/mL (Table 1, Figs 2 and 3). Preincubation of GM-CSF with a neutralizing murine anti-human GM-CSF MoAb for 4 hours at 20°C completely prevented stimulation of proliferation (Fig 4).

### DISCUSSION

We showed that IL-3 and GM-CSF may have profound effects on spontaneous emergence of EBV-transformed B-cell lines from suspension cultures of PBMNCs isolated from subjects known to have been previously infected by EBV.
Table 1. Effect of GM-CSF and IL-3 on Proliferation of EBV-Transformed B-Cell Clones

<table>
<thead>
<tr>
<th>No Growth Factor</th>
<th>GM-CSF (50 U/mL)</th>
<th>P</th>
<th>IL-3 (100 U/mL)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 3,019 ± 770</td>
<td>4,624 ± 664</td>
<td>.003</td>
<td>3,294 ± 711</td>
<td>.58</td>
</tr>
<tr>
<td>2. 4,987 ± 1,366</td>
<td>8,381 ± 2,390</td>
<td>.01</td>
<td>5,798 ± 1,220</td>
<td>.36</td>
</tr>
<tr>
<td>3. 18,792 ± 920</td>
<td>23,686 ± 2,581</td>
<td>.001</td>
<td>18,467 ± 2,511</td>
<td>.81</td>
</tr>
<tr>
<td>4. 7,002 ± 660</td>
<td>8,225 ± 1,030</td>
<td>.03</td>
<td>6,581 ± 362</td>
<td>.26</td>
</tr>
<tr>
<td>5. 10,459 ± 1,480</td>
<td>19,786 ± 1,001</td>
<td>&lt;.00005</td>
<td>10,331 ± 4,113</td>
<td>.96</td>
</tr>
</tbody>
</table>

Results are mean cpm ± SD of replicates of six cultures incubated for 96 hours before being labeled with 3H-thymidine.

Addition of GM-CSF facilitates outgrowth of transformed B cells, whereas IL-3 completely prevents their emergence. These observations warrant a more careful consideration of possible direct or indirect effects of these hematopoietic growth factors on lymphoid cells.

Cell lines established from human T-cell and B-cell lymphomas lack receptors for GM-CSF, and GM-CSF does not cause proliferation of EBV-negative lymphomas in vitro. Despite this, in many clinical trials of GM-CSF, total lymphocyte counts increased in a dose-dependent manner after administration of the growth factor. The mechanism of this increase has not been established. A murine IL-2-dependent T-cell line proliferated in response to GM-CSF. In our system, the augmented proliferation of EBV-transformed B cells after addition of GM-CSF appears to be at least in part a direct effect because increased proliferation is consistently observed in cultures after attrition of all elements other than transformed B cells. A possible indirect effect mediated by accessory cells was further excluded by flow-cytometric analysis and immunoglobulin gene rearrangement study verifying the purity of the studied populations. Immortalization of B lymphocytes by EBV results in expression of several "activation" antigens, at least one of which is believed to function as a growth factor receptor. EBV immortalization may result in a change that permits B lymphocytes to respond to GM-CSF.

Normal and malignant pre-B cells (CD10+) proliferate in response to IL-3 and bear IL-3 receptors. No direct proliferative effect of IL-3 on mature lymphocytes has yet been established, although a recent report suggested that IL-3 may be capable of augmenting IgG secretion by mature B cells. The inhibition of emergence of EBV-transformed B cells that we observed after addition of IL-3 is most likely an indirect effect, as IL-3 did not inhibit proliferation of pure cultures of transformed B cells.

Most humans are infected by EBV by adulthood. Persons who have been infected by EBV are known to harbor small populations of EBV-transformed B lymphocytes persistently. The proliferation of these clones is believed to be tightly controlled by cytotoxic T lymphocytes that may recognize "foreign" antigens expressed on the surface of infected cells. In murine systems, IL-3 has been reported to augment in vitro survival of cells that mediate natural cytotoxicity. The inhibitory effects of IL-3 on transformed B cells observed in our system may have resulted indirectly from an effect of IL-3 on cytotoxic T cells present early in culture. An indirect role for IL-3 is further supported because the inhibitory effect was eliminated in cultures supplemented with antibodies to either γ-IFN or TNF-α. Cytotoxic suppressor...
GM-CSF AND IL-3 MODULATE EBV-B-CELL OUTGROWTH

Fig 3. Time course of stimulation of three EBV-transformed B-cell clones by 50 U/mL GM-CSF. Results are the mean of replicates of six cultures expressed as a percentage of the mean of parallel cultures not supplemented with GM-CSF. Error bars are omitted for clarity. Maximum stimulation was observed at 96 hours.

Fig 4. Removal of stimulation by GM-CSF of proliferation of transformed B cells after preincubation of GM-CSF with a neutralizing antibody. B Cells alone (A), B cells with 50 U/mL GM-CSF (B), B cells with 50 U/mL GM-CSF preincubated with neutralizing antibody (C). Cultures were performed in replicates of six for 96 hours.

sort cells produce γ-IFN, and TNF-α is a product of natural cytotoxic cells, T cells, and macrophages. Both lymphokines are capable of suppressing growth of EBV-transformed B cells in vitro. Thus, IL-3 may exert its effect by causing induction of secretion of one or both of these lymphokines by accessory cells present early in our culture system.

Highly lethal EBV-associated lymphoproliferative diseases complicate conditions of severe congenital or acquired immunodeficiency and have occurred often in allograft recipients. In BM transplantation, the syndrome appears most likely to complicate T-cell-depleted non–HLA-matched grafts, occurring in up to 24% of cases. In addition, increasing evidence suggests that some of the lymphomas that complicate acquired immunodeficiency syndrome (AIDS) may be at least partially induced by EBV in an impaired immunoregulatory system. There is currently great interest in use of hematopoietic growth factors to hasten BM transplant engraftment, to lessen the period of BM hypoplasia after intensive chemotherapy, to treat conditions of primary BM failure, and to ameliorate the severe BM suppression which often occurs in AIDS. Our observations of differential effects of GM-CSF and IL-3 on in vitro emergence of EBV-transformed B-cell clones may be of some relevance to these clinical situations. Further study of the mechanisms of these actions is warranted.

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REFERENCES


granulocyte-macrophage colony-stimulating factor in patients with

11. Champlin RE, Nimer SD, Ireland P, Oette DH, Golde DW: 
Treatment of refractory aplastic anemia with recombinant human 

12. Vadhan-Raj S, Keating M, LeMaistre A, Hittleman WN, 
McCreed K, Trujillo JM, Broxmeyer HE, Henney C, Gutterman 
JU: Effects of recombinant human granulocyte-macrophage colony-
stimulating factor in patients with myelodysplastic syndromes. N 

13. Wilson W, Rubin M, Mckinnaud F, Mertins S, Walsh T, 
Magrath I, Londo D, Pizzo PA: The effect of recombinant human 
(rh) GM-CSF on the proliferation of human B-cell lymphomas. 
Blood 70:145a, 1987 (abstr)

14. Park LS, Friend D, Gillis S, Urbdl DL: Characterization of 
the cell surface receptor for human granulocyte/macrophage colony 

San Diego, CA, Academic, 1988, p 219

interleukin-3 induces proliferation of normal and leukemic human 
B cell precursors. Blood 70:190a, 1987 (abstr)

17. Uckun FM, Gesner TG, Song CW, Myers DE, Mufson A: 
Leukemic B-cell precursors express functional receptors for human 

18. Groopman JE, Mitsuyasu RT, DeLeo MJ, Oette DH, Golde 
DW: Effect of recombinant human granulocyte-macrophage colony-
stimulating factor on myelopoiesis in the acquired immunodeficiency 

19. Thorley-Lawson DA, Nadler LM, Bhan AK, Schooley RT: 
Blast-2 (EBVCS), and early cell surface marker of human B cell 

20. Sullivan JL: Epstein-Barr virus and lymphoproliferative disor-

ontogenic precedence of suppressor T cell functions in the human 

22. Hasler F, Bluestein HG, Zvaifler NJ, Epstein LB: Analysis of 
the defects responsible for the impaired regulation of Epstein-Barr 
 virus-induced B cell proliferation by rheumatoid arthritis lympho-
cytes. I. Diminished gamma interferon production in response to 

interleukin 2/interleukin 4-dependent T cell line induced by granulo-
138:4288, 1987

24. Woods A, West J, Rasmussen R, Bottomly K: Granulocyte-
macrophage colony stimulating factor produced by cloned L3T4a', 
class II-restricted T cells induces HT-2 cells to proliferate. J 
Immunol 138:4293, 1987

analysis suggests distinct functional roles for the blast-1 and 

26. Thorley-Lawson DA: Basic virological aspects of Epstein-
Barr virus infection. Semin Hematol 25:247, 1988

27. Sudgen B, Metzenberg S: Characterization of an antigen 
whose cell surface expression is induced by infection with Epstein-


29. Sullivan JL: Epstein-Barr virus and lymphoproliferative disor-

30. Lattime EC, Pecoraro GA, Stutman O: The activity of 
natural cytotoxic cells is augmented by interleukin 2 and interleukin 

31. Zoumbos NC, Gasc6n P, Djeu JY, Tost SR, Young NS: 
Circulating activated suppressor T lymphocytes in aplastic anemia. 

32. Donnuijsen-Ant R, Abken H, Bornkamm G, Donnuijsen K, 
Grosse-Wide H, Neumann-Haefelin D, Westerhausen M, Wiegand 
H: Fatal Hodgkin and non-Hodgkin lymphoma associated with 
persistent Epstein-Barr virus in four brothers. Ann Intern Med 
109:946, 1988

JE, Mulder C: Epstein-Barr virus-induced lymphoproliferation. 
Implications for antiviral chemotherapy. N Engl J Med 311:1163, 
1984

34. Shapiro RS, McClain K, Frazier G, Gajl-Peczalska KJ, 
Kersey JH, Blazar BR, Arthur DC, Patton DF, Greenberg JS, 
Burke B, Ramsay NKC, McGlave P, Filippovich AH: Epstein-Barr 
virus associated B cell lymphoproliferative disorders following bone 

35. Shearer WT, Ritz J, Finegold MJ, Guerra IC, Rosenblatt 
HM, Lewis DE, Pollack MS, Taber LH, Sumaya CV, Grunet FC, 
Clery ML, Wavne K, Sklar J: Epstein-Barr virus-associated B-cell 
proliferations of diverse clonal origins after bone marrow transplan-
tation in a 12-year-old patient with severe combined immunodefi-

36. Knowles DM, Inghirami G, Ubriaco A, Dalla-Favera R: 
Molecular genetic analysis of three AIDS-associated neoplasms of 
uncertain lineage demonstrates their B-cell derivation and the 
possible pathogenetic role of the Epstein-Barr virus. Blood 73:792, 
1989