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Macrophage Colony-Stimulating Factor (CSF-1) Is Expressed by Spontaneously Outgrown EBV-B Cell Lines and Activated Normal B Lymphocytes

By G. Reisbach, J. Sindermann, J.P. Kremer, L. Hültner, H. Wolf, and P. Dörmer

Human B lymphocytes activated by mitogens or infected by Epstein Barr virus (EBV) have previously been shown to release colony-stimulating activity (CSA) supporting the growth of normal human bone marrow progenitors. We established five different human EBV-B cell lines spontaneously outgrown from nonmalignant peripheral blood cells and long-term bone marrow cultures. CSA derived from all of these lines induces the growth of murine macrophage colonies, whereas virtually no human bone marrow cell progenitors were stimulated. As observed in the tumor cell line MIA PaCa-2, a 4.3-kilobase (kb) transcript was detected in all cases using a human colony-stimulating factor (CSF)-1 probe. Expression of this transcript can be further stimulated within three hours upon addition of phorbol myristate acetate (PMA). The highly purified native protein exerting macrophage colony-stimulating activity (M-CSA) exhibits a molecular size of approximately 75 to 97 Kd in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The identity of EBV-B cell derived M-CSA with human urinary CSF-1 was confirmed by a complete neutralization of macrophage CSA by an antihuman urinary CSF-1 antiserum. Normal human B lymphocytes purified from tonsils or from mononuclear blood cells also express CSF-1 upon stimulation with *Staphylococcus aureus* Cowan I. No CSF-1 expression, however, could be detected in normal resting B lymphocytes or in the Burkitt lymphoma cell line RAJI.
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COLONY-STIMULATING factors (CSFs) comprise a family of glycoproteins that promote proliferation and differentiation of hemopoietic progenitors and enhance the function of mature granulocytes and macrophages.[1-3] CSFs are expressed in activated mononuclear blood cells, endothelial cells,[4] fibroblasts,[5,6] as well as in various tumor cell lines.[7-9] Recently, they were detected in Epstein Barr virus (EBV)-transformed B lymphocytes.[10-13] Our own results suggested that among B-cell–derived CSFs there is macrophage stimulating activity,[13] which possibly points to a close interaction between B lymphocytes and macrophages.

The present study on EBV-B cell-released macrophage stimulating activity was performed to test its functional and immunologic identity with CSF-1. Furthermore, we investigated whether CSF-1 is also expressed in normal B lymphocytes. Human CSF-1 strongly promotes differentiation of myeloid progenitors to macrophage colonies[14,15] and enhances the survival[16] and function[17-19] of macrophages. To support the growth of macrophage colonies from human bone marrow progenitors, additional low doses of human granulocyte-macrophage CSF (GM-CSF) are required.[20] On the other hand, murine bone marrow progenitors are directly stimulated by human CSF-1 but not by human GM-CSF, which can be used as a sensitive discriminating bioassay.

cDNAs encoded by the single copy gene for CSF-1 have been prepared from murine and human cell lineages.[21,22] CSF-1 transcripts range in size from 1.6 to 4.5 kilobase (kb). In human monocytes activated with cytokines or phorbol myristate acetate (PMA) a 4-kb species of CSF-1 mRNA is preferentially detectable.[23-26] Furthermore, we find a single mRNA species of 4.3 kb in stimulated B lymphocytes from healthy donors as well as in EBV-transformed B lymphocytes. The molecular size of functional CSF-1 protein released from B lymphocytes suggests extensive posttranslational modification, as described for murine and human urinary CSF-1.[27]

MATERIALS AND METHODS

Human and murine cell lines. EBV-B cell lines spontaneously outgrew from long-term cultures of mononuclear peripheral blood cells (BLY 9.84 and BLY 5.85) and from long-term cultures of bone marrow cells (BLY G1, P1, and B2). None of the donors had shown hematologic abnormalities. The lines were maintained in RPMI 1640 medium (Gibco-BRL, Eggenstein, FRG) supplemented with 5% fetal calf serum (FCS) (Biochrom, Berlin, FRG). All these EBV-B lines were HLA-typed to assure different identity. For detection of EBV genomes carried by these cell lines, *Bam* HI digests of cellular DNA were transferred to nylon filters according to Southern and probed with the EBV w-fragment. The RAJI and BJAB cell lines were used as positive and negative controls, respectively. Cellular expression of the EBV-related antigens Epstein-Barr nuclear antigen (EBNA), early antigen (EA), and viral capsid antigen (VCA) was detected by specific human antisera using standard immunofluorescent techniques.[28] The cell lines HL-60, U937, MIA-PaCa-2, and L929 were obtained from the American Type Culture Collection, Rockville, MD, and grown in RPMI 1640 medium supplemented with 10% FCS. The cloned murine cell line BAC1.2F5,[29] responsive to CSF-1 or GM-CSF, was obtained from J.N. Ihle, NCI, Frederick, MD. L929 cell conditioned medium (LCCM) containing 1% FCS was concentrated 20-fold, extensively dialyzed against phosphate-buffered saline (PBS), and used for maintaining the growth of the BAC1.2F5 line. Cells were seeded at a density of 3×10^4/mL and starved for 24 hours prior to titration of CSF-1 activity. Cell growth was quantitated by a colorimetric assay of tetrazolium salt (MTT) (Serva, Heidelberg, FRG) reduction and read on a scanning multiwell spectrophotometer, as described by Mosmann.[30]

B-lymphocyte preparation and stimulation. Peripheral blood cells (PBL) were obtained from healthy volunteers and tonsils from tonsillectomized patients. Mononuclear cell suspensions were separated by Percoll density gradient centrifugation, and monocytes were depleted by plastic adherence (2 × 1 hour, 37°C). T lympho-

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cytes were removed by E rosetting twice. The percentage of B and T lymphocytes in all cell preparations was monitored by indirect immunofluorescence staining with the CD 19 (M740, DAKOPATTS, Hamburg, FRG) and CD 2 (M720, DAKOPATTS) monoclonal antibodies. The resultant B-cell population (90% to 95% CD 19 positive) contained less than 5% T cells and less than 1% monocytes, as shown by staining with a CD 14 monoclonal antibody (Becton Dickinson, Heidelberg, FRG). These B-cell fractions were used for the preparation of total cellular RNA, either without any prior treatment or activated by *Staphylococcus aureus* Cowan I at a final dilution of 0.005% (vol/vol) (Sigma, Munich, FRG) for three or four days in RPMI 1640 supplemented with 5% FCS.

Preparation of cellular RNA and Northern blot analysis. Total cellular RNA was isolated as described[31] by lysing cells in guanidine isothiocyanate and centrifuged through cesium chloride. Glyoxylated RNA samples were electrophoresed through a 1.0% agarose gel and blotted onto a nylon membrane (Hybond-N; Amersham, Braunschweig, FRG). The membranes were baked at 80°C for two hours. RNA on the filters was visualized by staining with 0.04% methylene blue (Merck, Darmstadt, FRG) in 0.5 mol/L Na-acetate, pH 5.2, for five minutes and subsequent washing in distilled water for ten minutes. Prehybridization was performed at 42°C for four hours (50% formamide, 5x SSC (sodium chloride–sodium citrate), 1x Denhardt's solution, 0.2% sodium dodecyl sulfate [SDS], 100 μg/mL salmon sperm DNA, 13 μg/mL *Escherichia coli* tRNA) and hybridized at 42°C for 24 hours to a 1.8-kb human CSF-1 cDNA insert isolated from plasmid pcDBCSF-4 (generously provided by P. Ralph, Cetus Corp, Emeryville, CA). The probe was oligonucleotide random primed and labeled with ^{32}P-dCTP (Amersham) to a specific activity of 1×10^9 cmp/μg cDNA. The membranes were washed for one hour in 1× SSC and 0.5% SDS at 55°C, and subsequently for one hour in 0.1× SSC and 0.1% SDS at 55°C. Autoradiography was performed with X-ray film and intensifying screens at −70°C for three to seven days.

Purification of human EBV-B cell-derived M-CSA and bone marrow colony assay. For harvesting of the EBV-B cell-conditioned media (BCM) on days 7 to 9, the cell lines were seeded at densities between 0.5 and 1×10^6/mL and grown in RPMI 1640 medium containing 1% FCS. Macrophage colony-stimulating activity (M-CSA) of crude BCM or of chromatography fractions was titrated in agar cultures of bone marrow cells (BMC) from C3H mice. One unit was defined as the activity producing one colony in 5×10^4 nucleated BMC and was calculated by extrapolation from the point of half maximum activity. Agar cultures of murine bone marrow progenitors were performed as described.[13]

For assaying human bone marrow progenitors, the cells were separated on a Percoll gradient,[32] washed twice with PBS, and depleted from adherent cells by incubation for 2 × 2 hours in plastic flasks in RPMI 1640 medium containing 20% FCS (Biochrom). Nonadherent cells were plated in 35-mm Petri dishes in 1 mL of Iscove's modified Dulbecco medium (IMDM) containing 20% FCS, 0.3% Bacto-agar (Difco, Detroit), and supplemented with 0.2 mL of the test sample.

M-CSA BCM was concentrated by ultrafiltration of BCM with YM 10 membranes (Amicon Co, Danvers, MA) and bound to a Q sepharose gel (Pharmacia, Freiburg, FRG) equilibrated with 50 mmol/L Tris/HCl at pH 7.5. M-CSA was eluted using a linear gradient of 0.6 to 1.5 M NaCl (step 1). Active fractions were equilibrated with 1 mol/L ammonium sulfate, buffered with 10 mmol/L sodium phosphate at pH 6.8, and applied to a phenylsepharose gel (Pharmacia). The activity was eluted at 0.7 to 0.4 mol/L ammonium sulfate in a decreasing gradient (step 2). Subsequently,

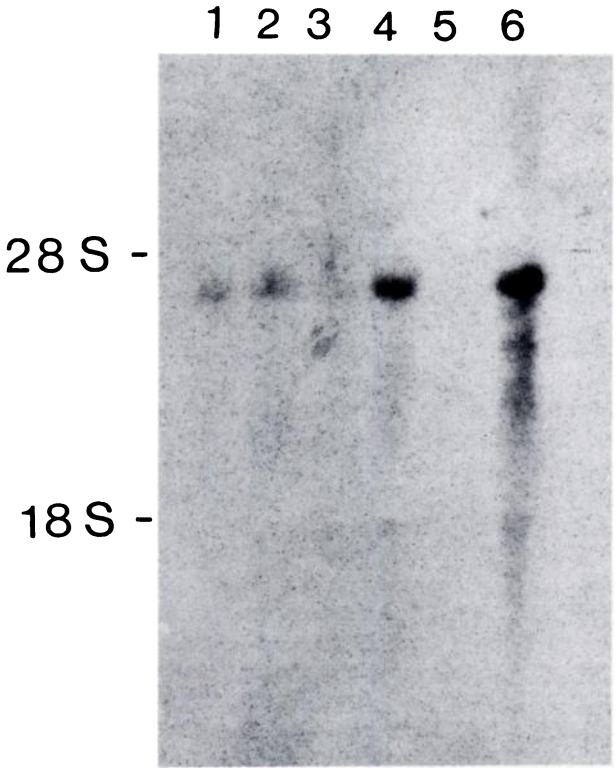

Fig 1. (A) Northern blot analysis of CSF-1 mRNA expression in human EBV-B cell lines. Tumor cell lines U937 and MIA-PaCa-2 were used as controls. Twenty micrograms of total cellular RNA from each sample were fractionated on an agarose gel. Lane 1 shows BLY P1; lane 2, BLY 5.85; lane 3, BLY B2; lane 4, BLY G1; lane 5, U937; lane 6, MIA-PaCa-2.

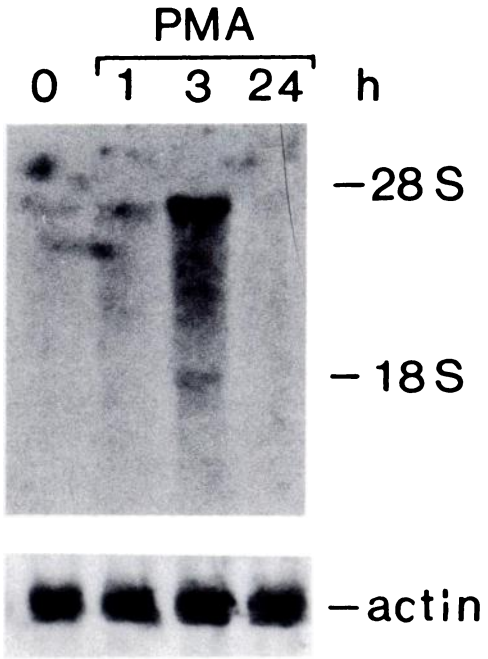

Fig 2. Northern blot analysis of CSF-1 expression in BLY9.84. Cells had been treated with 500 nmol/L PMA for different times as indicated. Twenty micrograms of total cellular RNA were electrophoresed. β-Actin expression is shown below.

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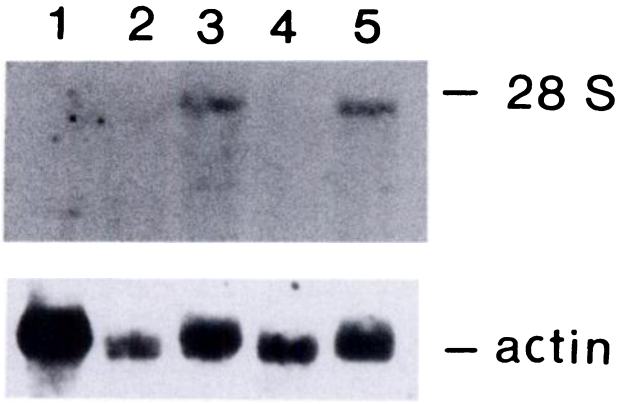

Fig 3. Northern blot analysis of CSF-1 mRNA in Burkitt lymphoma cell line RAJI and normal B lymphocytes. Twenty micrograms of cellular RAN were analyzed. Lane 1: poly A(+) mRNA from Burkitt lymphoma line RAJI. Lanes 2 and 4: total cellular RNA from peripheral B lymphocytes and tonsillar B cells, respectively. Lanes 3 and 5: SAC-treated B lymphocytes as in lanes 2 and 4.

in step 3, the active fractions were equilibrated with 50 mmol/L Na-acetate, pH 5.2, applied to an S-sepharose gel (Pharmacia), and retrieved in the run-through fraction. Molecular size determination was done with step 3 material using 0.2% SDS-PAGE without reducing agents according to Laemmli.[32a] Samples were denatured by heating at 60°C for 30 minutes in 2% SDS and applied to the SDS-PAGE gel. After electrophoresis, one lane was sliced in equal parts. Slices were eluted with 4 mol/L urea in PBS, then dialysed extensively against PBS, and each fraction was tested in the murine bone marrow colony assay for CSF-1 activity. A second lane was silver stained to analyze contaminating proteins in step 3 material. Apparent molecular weights (mol wts) were estimated by extrapolation from the positions of stained marker proteins.

RESULTS

Transcription of the CSF-1 gene in human B lymphocytes. From all five spontaneously outgrown EBV-B cell lines, M-CSA is released constitutively and shows heterogeneity in molecular size,[13] an observation that prompted our present studies on CSF-1 expression. A human CSF-1 cDNA probe was hybridized to total cellular RNA from EBV-B cells and tumor cell lines U937 and MIA PaCa-2. A 4.3-kb transcript, although only weakly expressed in BLY B2 cells, was detected in all lymphoblastoid lines (Fig 1). In order to test for the stability of expression of the CSF-1 transcript under different culture conditions, poly(A)+ enriched RNA from EBV-B cell line BLY 9.84 was prepared 24 hours after medium exchange and on day 5 of culture, respectively. There was significantly more CSF-1 mRNA detectable in exponentially growing cells as compared with cells in the stationary phase of growth on day 5 without medium exchange (data not shown). To enhance levels of CSF-1 transcripts, the cell line BLY 9.84 was induced by PMA (500 nmol/L) for various periods of time up to 24 hours. Increased levels of CSF-1 RNA were obtained after 3 hours and declined thereafter below constitutive expression (Fig 2). Similar results were found in cell lines BLY 5.85 and BLY B2 (data not shown).

Table 1. Expression of EBV-Encoded Antigens and Detection of EBV Genome in Spontaneously Outgrown B-Lymphoblastoid Cell Lines

Cell line	BLY 9.84	BLY 5.85	BLY G1	BLY P1	BLY B2
EBNA	+	+	+	+	+
Early antigen	+	(+)	(+)	–	–
Viral capsid antigen	(+)	+	–	–	–
w-fragment	+	+	+	+	+

EBNA, early antigen, and viral capsid antigen are determined by immunofluorescence. w-Fragment of the EBV gemone was detected in each cell line by Southern blots of *Bam*HI-digested cellular DNA. Parentheses indicate weak expression of EBV-encoded antigens.

In order to prove if normal B lymphocytes from healthy donors also express CSF-1 upon stimulation, B cells enriched from mononuclear blood cells or minced suspended tonsillar cells were exposed to *S aureus* Cowan I. Analysis of total cellular RNA harvested at day 3 or 4 revealed a 4.3-kb message for CSF-1. In resting B lymphocytes, however, as well as in polyA + mRNA from RAJI cells, CSF-1 expression was not detectable (Fig 3).

Expression of EBV-related antigens in spontaneously outgrown B-cell lines. Each of the five EBV-B cell lines investigated was characterized with respect to antigens related to the EBV cycle (Table 1). Lines BLY P1 and B2 exhibit a latent EBV cycle, whereas in subpopulations from cell lines BLY 9.84 and 5.85, a lytic cycle of EBV is initiated. Obviously, the lytic cycle did not significantly alter the release of CSF-1, since comparable levels of activity were detectable in concentrated supernatants from all cell lines (data not shown).

Secretion of CSF-1 by EBV-B cell lines. M-CSA-stimulating murine progenitor cells were detected in concentrated supernatants of our EBV-B cell lines. EBV-B cell-released M-CSF was purified (Table 2). However, even with step 3 material, the growth of only a few colonies or clusters was

Table 2. Chromatographic Purification of Human M-CSA Released From EBV-B Cells

Fraction	Total Protein (mg)	Total Activity (Colonies × 10^3)	Specific Activity (Colonies/mg)	Yield (%)	Purification Factor
BCM 20x	767	110	143	100	1
Q-sepharose	1.70	41.0	2.41×10^4	37	169
Phenylsepharose	0.31	36.3	1.17×10^5	33	818
S-sepharose	0.02	25.9	1.3×10^6	24	9.090

Three liters of supernatant (BCM) from EBV-infected B-cell line BLY 9.84 containing 1% FCS were harvested on days 7 to 9. Protein concentration was determined by the method of Bradford.[41] One colony defines one unit of activity as described in Materials and Methods, using murine BMCs from C3H mice at a density of 5×10^4 cells/mL in agar cultures. Supernatants from other EBV-B cell lines were fractionated by Q-sepharose columns (step 1) only. M-CSA eluted reproducibly in same fractions for all EBV-B lines.

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stimulated in agar cultures of human BMC, as shown with three different BMC samples in independent experiments (Table 3). Inhibitory activity was not present in step 3 material as shown by the addition of giant cell tumor-conditioned medium (GCT-CM). At least partial structural identity of EBV-B cell-derived M-CSA with human CSF-1 was proved by the use of a specific antihuman urinary CSF-1 rabbit antiserum (rabbit 53) (kindly provided by E.R. Stanley). The antiserum by itself did not induce colony growth. Furthermore, colony-stimulating activity from spleen cell-conditioned medium was not blocked by this antiserum as judged by the granulocyte-macrophage colony-forming cells (GM-CFC) assay (Table 4), excluding the possibility of unspecific growth inhibition.

It was considered that the biologic activity of EBV-B cell-derived M-CSA asayed in agar cultures might be secondary and mediated or enhanced by other cytokines.[33,34] Therefore, we used the cloned macrophage line BAC-1.2F5[29] as target cells. Titration of EBV-B cell-derived M-CSA resulted in a growth response close to that caused by CSF-1 released by murine L929 cells (Fig 4). Application of a three-step chromatography to EBV-B cell-derived M-CSA was necessary in this assay because crude supernatants showed cytotoxic effects on BAC-1.2F5 cells. To determine the approximate mol wt of the EBV-B cell-derived M-CSA, more than 9000-fold purified M-CSA samples (Table 2) were electrophoresed on a gradient polyacrylamide gel under denaturing conditions. About 50% of colony-stimulating activity could be recovered from gel slices. The elution profile was determined by the murine bone marrow assay. Two maxima of M-CSA were observed at an apparent M_r of 75 to 97 Kd and 130 to 190 Kd as single peaks (Fig 5). The larger molecular species may represent a CSF-1 precursor protein or simply aggregates of CSF-1.

DISCUSSION

Human-activated B lymphocytes purified from peripheral blood or tonsils as well as neoplastic B lymphocytes or lymphoblastoid cell lines have been shown to release colony-stimulating activities[10-11] that were not further specified. Our studies demonstrate that one of these cytokines expressed by EBV-infected or activated B lymphocytes from healthy donors is identical to CSF-1.

Obviously, quite different events may induce production of CSF-1 by B lymphoblasts, such as staphylococcus aureus Cowan (SAC)-induced activation or growth transformation by EBV infection of resting lymphocytes. Infection results in virus-encoded antigen expression characteristic of different stages of the EBV cycles.[35] The antigen state of lines BLY P1 and B2 indicates that latent infection with EBV is sufficient for the induction of constitutive CSF-1 transcription. It remains to be clarified, however, how latency relates to the

Table 3. Colony Formation by Human and Murine BMCs in the Presence of Purified (Step 3) Human M-CSA From EBV-B Cells

Test Sample (20% vol/vol)	Human BMC (10^5 cells/plate)	Murine BMC (5×10^4 cells/plate)
RPMI 1640	0	0
M-CSA (step 3)		
1:1	2	180
1:2	Clusters	150
1:4	0	110
GCT-CM + RPMI 1640	130	nd
GCT-CM + M-CSA* 1:2	127	nd

Murine colonies grown in the presence of M-CSA (step 3) were identified as macrophages on day 14 in May-Grünwald-Giemsa-stained cytocentrifuge preparations. Human BMCs stimulated by GCT-CM containing human GM-CSF served as a positive control. The test samples were added at 20% (vol/vol) to the bone marrow agar cultures. Colonies were scored on day 14. Data represent mean values of three independent experiments.

Abbreviation: nd, no data.

*Step 3.

Table 4. Inhibition of EBV-B-Cell Line-Derived M-CSA by Antihuman Urinary CSF-1 Antiserum

Supernatant Derived From	Colonies/Plate: No Anti-CSF-1	Dilution of Anti-CSF-1 1:48	Antiserum 1:12
BLY 9.84	50 ± 7	34 ± 8	0 ± 0
BLY 5.85	42 ± 1	23 ± 3	0 ± 0
BLY P1	26 ± 7	nd	0 ± 0
BLY G1	36 ± 5	nd	0 ± 0
BLY B2	21 ± 2	nd	0 ± 0
MIA-PaCa-2	42 ± 4	19 ± 2	0 ± 0
Murine spleen	63 ± 10	76 ± 8	78 ± 5

Crude supernatants and antihuman CSF-1 antiserum were mixed in petri dishes prior to agar culture at equal volumes (0.1 mL each) using different dilutions (as indicated) of antihuman CSF-1 antiserum. Twenty percent (vol/vol) of each sample was added to 1 mL of agar culture of murine C3H BMC (5×10^4 cells/dish). Data represent mean numbers of colonies ± SD, scored on day 14. Conditioned media derived from cell line MIA-PaCa-2 or pokeweed mitogen-stimulated murine spleen cells were used as positive controls of human CSF-1 and murine hematopoietic growth factors, respectively.

Abbreviation: nd, no data.

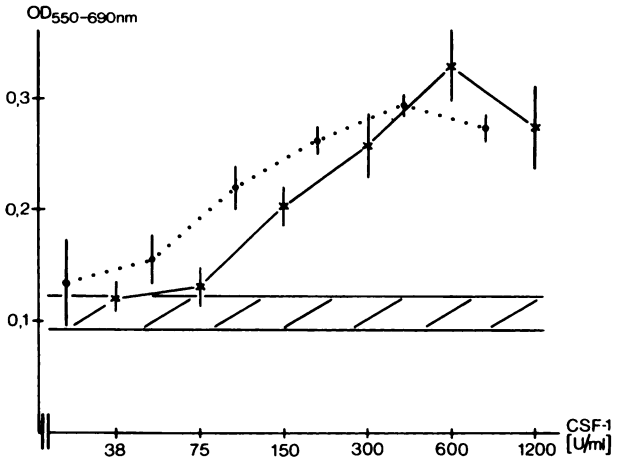

Fig 4. Dose-response of the murine cell line BAC-1.2F5 stimulated with serial dilutions of M-CSA (CSF-1) purified from EBV-B line 9.84 (step 3 material) (×—×) or crude murine LCCM CSF-1 (● · · · ●). Hatched area indicates control with culture medium only. Growth was measured on day 5 by the MTT tetrazolium salt asasy.

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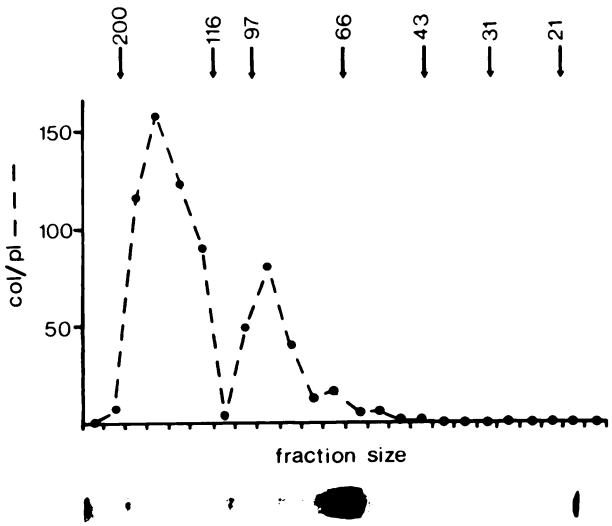

Fig 5. Electrophoretic analysis of M-CSA derived from EBV-B cells. Aliquots of purified M-CSA (step 3) were denatured in 2% SDS at 60°C for 30 minutes and electrophoresed in an SDS-polyacrylamide gradient gel (6% to 18%). The first lane was sliced in equal parts and each slice eluted in 4 mol/L urea. After equilibration in PBS, bioactivity of M-CSA was determined for each slice in a murine colony assay. Contaminating proteins in step 3 material (lane below elution profile) and mol wt standards were detected by silver staining. Myosin 200 Kd, β-galactosidase 116 Kd, phosphorylase b 97 Kd, bovine albumin 66 Kd, ovalbumin 43 Kd, carbonic anhydrase 31 Kd, trypsin inhibitor 21 Kd.

activation of the CSF-1 gene, as the EBNA expressing Burkitt lymphoma cell line RAJI does not express CSF-1.

The CSF-1 message obviously is not maximally expressed in EBV-transformed B lymphocytes. Stimulation of the BLY 9.84 cell line by phorbol ester induced a higher level of CSF-1 message within three hours and a significant down-regulation after 24 hours. This is in accordance with PMA-stimulated CSF-1 expression in normal human monocytes[23,25] and parallels the activity of protein kinase C[36] induced in different cell lines[37] by PMA. Since it has been shown that B cells can also be activated in the absence of protein kinase C,[38] there is possibly more than one pathway of signal transduction for CSF-1 expression. In this context, it is of interest that the HL 60 cells induced by PMA also show a protein kinase C unrelated delayed expression of CSF-1[23] (unpublished observations).

Our results indicate a preferential expression of a 4.3-kb CSF-1 message in activated B lymphocytes. Nevertheless, there is heterogeneity in the apparent molecular size of released CSF-1 protein. Native human CSF-1 protein has first been purified from urine and described as a glycoprotein of heterogenous size with an M_r of 45 to 60[27] or 70 to 90 kd,[22,39] composed of two disulfide-bonded subunits.[27] We found CSF-1 protein species with molecular sizes of 75 to 97 Kd and 130 to 190 Kd using SDS-PAGE under nonreducing conditions to recover bioactivity.

These CSF-1 molecules share antigenic determinants, since the total activity could be blocked by rabbit antihuman urinary CSF-1 antiserum. Quite similar molecular sizes have been obtained by expressing a 4-kb cDNA encoding urinary CSF-1 in CHO cells,[22] which are secreted as a 70- to 90-Kd recombinant CSF-1 besides a protein species of 200 Kd. Recent cloning of human CSF-1 from the pancreatic tumor cell line MIA-PaCa-2 and an SV40 transformed trophoblast cell line also resulted in full-length cDNA copies of different sizes. They corresponded to a calculated protein size of 26 Kd (pcCSF-17), 61 Kd (p3ACSF-69), and 60.5 Kd (pcDBCSF-4) for a monomeric CSF-1 subunit.[21,22,40] Thereafter, an alternative splicing mechanism can be concluded.[40] However, since EBV-B cells express only one single CSF-1 transcript, it can be concluded that in this case posttranslational protein modification considerably contributes to the final size heterogeneity.

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