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Identification of Interleukin-6 as an Autocrine Growth Factor for Epstein-Barr Virus-Immortalized B Cells

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Autocrine growth factors are believed to be important for maintenance of an immortalized state by Epstein-Barr virus (EBV), because cell-free supernatants of EBV-immortalized cell lines promote the proliferation of autologous cells and permit their growth at low cell density. In this study, we provide evidence for the existence of two autocrine growth factor activities produced by EBV-immortalized lines distinguished by size and biological activities. Much of the autocrine growth factor activity in lymphoblastoid cell line supernatants resided in a low-molecular-weight (<5,000) fraction. However, up to 20 to 30% of the autocrine growth factor activity resided in the high-molecular-weight (>5,000) fraction. While the nature of the low-molecular-weight growth factor activity remains undefined, the high-molecular-weight growth factor activity was identified as interleukin-6 (IL-6). Culture supernatants from six EBV-induced lymphoblastoid cell lines tested contained IL-6 activity, because they promoted proliferation in the IL-6-dependent hybridoma cell line B9. In addition, a rabbit antibody to human IL-6 neutralized the capacity of the high-molecular-weight (>5,000) fraction of a lymphoblastoid cell line supernatant to promote growth both in autologous EBV-immortalized cells and in B9 cells. Similarly, this high-molecular-weight autocrine growth factor activity was neutralized by a monoclonal antibody to human IL-6. Furthermore, characteristic bands, attributable to IL-6, were visualized in supernatants of each of four EBV-induced lymphoblastoid cell lines after immunoprecipitation with a rabbit antiserum to human IL-6. Thus, in addition to its previously reported properties, IL-6 is an autocrine growth factor for EBV-immortalized B cells cultured under serum-free conditions.

Epstein-Barr virus (EBV) is a unique herpesvirus that latently infects a proportion of human B lymphocytes and induces their immortalization (8). The mechanisms responsible for initiation and maintenance of B-cell immortalization by EBV are largely unknown. Certain EBV genes that are consistently expressed in B cells immortalized in vitro (EBNA-1, EBNA-2, and BNLF-1) are believed to play a role in these processes (for a review, see reference 23). However, the precise function of these viral genes is incompletely understood, and it is suspected that additional gene products, also expressed in B lymphocytes latently infected with EBV, may also be required for B-cell immortalization.

It has been observed that the vigorous proliferation of EBV-immortalized B cells cultured at low density is dependent upon growth factors found in culture supernatants of either EBV-immortalized B cells or activated monocytes (3, 6, 7, 27). Such findings have suggested that autocrine and/or paracrine growth factors may have a role in the maintenance of an immortalized state by this virus. In the case of monocyte supernatants, interleukin-6 (IL-6), a multifunctional cytokine produced in a variety of cell types, was recently identified as being the molecule responsible for the stimulation of growth of EBV-immortalized B cells (27, 30). Thus, IL-6 can act as a paracrine growth factor for EBV-immortalized cells.

The molecular nature of the factor(s) responsible for autocrine growth stimulation of EBV-immortalized B cells is still controversial. 3B6 IL-1, a structurally novel protein with IL-1 bioactivity, was reported to be an autocrine growth factor for EBV-immortalized B cells (21, 35). Recently, 3B6 IL-1 was found to belong to a family of reducing enzymes known as thioredoxins (26; J. Bertoglio, E. Vollman, L. Rimsky, and D. Fradelizi, Letter, J. Immunol. **141**:2869–2970, 1989; H. Wakasugi, N. Wakasugi, T. Turz, Y. Tagaya, and J. Yodoi, Letter, J. Immunol. **142**:2569–2570, 1989). Recombinant 3B6 IL-1, however, while exhibiting thioredoxin activity, lacked a number of the biological activities attributed to 3B6 IL-1, including IL-1 activity (Bertoglio et al., J. Immunol., 1989). Thus, it was proposed that 3B6 IL-1 might not be an autocrine growth factor for EBV-immortalized B cells, but a copurified contaminant (Bertoglio et al., J. Immunol., 1989). More recently, affinity-purified, soluble CD23, a B-cell activation antigen expressed at high levels in EBV-immortalized cells that also functions as a low-affinity receptor for immunoglobulin E (IgE) (12, 37), was reported to promote growth in EBV-immortalized B cells and in anti-IgM-stimulated B cells (24). In other studies, however, soluble CD23, while preserving a variety of biological activities attributed to CD23, failed to induce growth in IgM-costimulated B cells (31) and in EBV-immortalized B cells (G. Tosato, J. Tanner, and S. E. Pike, FASEB J., vol. 3, p. A497, 1989). Thus, while there is evidence suggesting that 3B6 IL-1 and soluble CD23 might be autocrine growth factors for EBV-immortalized B cells, additional studies will be required to clarify some of the controversial issues.

In this study, we provide evidence for the existence of two autocrine growth factor activities produced by EBV-immortalized B cells, distinguishable by size fractionation and the spectrum of biological activities. Much of the autocrine growth factor activity in lymphoblastoid cell line supernatants resided in a low-molecular-weight (<5,000) fraction. However, up to 20 to 30% of the autocrine growth factor activity resided in the high-molecular-weight (>5,000) fraction. This high-molecular-weight growth factor activity was attributed to IL-6 by a variety of criteria. Thus, under the

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serum-free culture conditions employed, IL-6 appears to act as an autocrine growth factor for EBV-immortalized B cells.

MATERIALS AND METHODS

Cells, cell lines, and reagents. Monocyte-enriched populations were obtained from peripheral blood, as described elsewhere (27). Eight lymphoblastoid cell lines were studied. Six of these lines (VDS-O, RY, Rb, Ma, TB, and TI) were obtained by EBV (B95-8 strain) immortalization of normal peripheral blood B cells, as described previously (28). One of these six cell lines (VDS-O) fails to secrete immunoglobulin (28). Two of the lines (3/22 and La) were produced by spontaneous in vitro outgrowth of B cells from healthy EBV-seropositive donors (28). The hybridoma cell line B9 was a gift of R. Nordan (1). All cell lines were mycoplasma-free. T lymphocytes were purified from peripheral blood as previously described (29).

A rabbit antiserum to highly purified, *Escherichia coli*-derived IL-6 (17) (a gift of L. T. May and P. B. Sehgal, Rockefeller University, New York, N.Y.) was used as such or following purification with protein A-Sepharose (Pharmacia, Inc., Piscataway, N.J.). Control rabbit serum consisted of a rabbit antiserum to myoglobin (a gift of I. Berkower, National Institutes of Health, Bethesda, Md.); control rabbit antibody consisted of rabbit IgG against *E. coli*-derived IL-1β (33) (a gift of J. Giri and P. Newton, Du Pont Glenolden Laboratory, Glenolden, Pa.). A mouse monoclonal antibody to *E. coli*-derived IL-6 was purified with protein A-Sepharose (Pharmacia) (20). Control mouse monoclonal antibodies included an anti-CD2 antibody (Ortho Diagnostics, Inc., Raritan, N.J.) and an anti-IL-2 receptor monoclonal antibody, anti-Tac (32) (a gift of T. A. Waldmann, National Institutes of Health). Recombinant IL-2 was obtained from Cetus Corp., Emeryville, Calif. One unit of IL-2 was defined by comparison with an IL-2 standard preparation (Biologic Response Modifiers BRM standard).

Preparation of lymphoblastoid cell line supernatants. All cell lines were maintained in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Reheis, Armour Pharmaceutical Co., Kankakee, Ill.), 2 mM L-glutamine (GIBCO), and 5 μg of gentamicin per ml (Sigma Chemical Co., St. Louis, Mo.). Exponentially growing lymphoblastoid cells were extensively washed in RPMI 1640 medium and incubated for 24 h at 37°C in RPMI 1640 medium supplemented with 1 mg of bovine serum albumin (BSA) per ml (Miles Laboratories, Inc., Kankakee, Ill.) and 2.5 μg of transferrin per ml (Sigma) at a cell density of 1.5×10^6 cells per ml. After incubation, cell-free supernatants were supplemented with 10 μg of aprotinin per ml (Sigma), sterile filtered (0.45-μm membranes), and stored at 4°C. Control medium consisted of RPMI 1640 medium supplemented with 1 mg of BSA per ml and 2.5 μg of transferrin per ml incubated for 24 h at 37°C; after incubation, the medium was supplemented with 10 μg of aprotinin per ml (Sigma), sterile filtered, and stored at 4°C.

Assay for autocrine growth factor activity. The assay for autocrine growth factor activity was performed essentially as described by Gordon et al. (6). Exponentially growing lymphoblastoid cells were washed extensively in RPMI 1640 medium and then incubated for 24 h in RPMI 1640 medium supplemented with 1 mg of BSA per ml at a cell density of 0.5×10^6/ml (starvation regimen). After incubation, the cells were washed in RPMI 1640 medium and then suspended in RPMI 1640 medium supplemented with 1 mg of BSA per ml and 2.5 μg of transferrin per ml. To assay for growth factor activity, 0.1 ml of serially diluted test culture supernatants or control medium was added in triplicate to flat-bottom microdilution plates (Costar, Cambridge, Mass.) containing 3×10^3 starved lymphoblastoid cells in 0.1 ml; culture medium consisted of RPMI 1640 medium supplemented with BSA (1 mg/ml) and transferrin (2.5 μg/ml). Cultures were pulsed with [^{3}H]thymidine (net 027; New England Nuclear Corp., Boston, Mass.; specific activity, 6.7 Ci/mmol) during the final 18 h of a 3-day incubation. Results were expressed as counts per minute per culture, and the arithmetic mean counts per minute of triplicate cultures were calculated. Each experiment included a 50×-concentrated culture supernatant of the lymphoblastoid cell line VDS-O that yielded maximal stimulation at the dilution of 1:4.

Assay for IL-6 activity. B9 cells were used in a standard assay for IL-6 activity (1). Exponentially growing B9 cells (a gift of R. Nordan, National Institutes of Health) were washed free of IL-6 and suspended in RPMI 1640 medium supplemented with 20% FCS and 10^{-4} M 2-mercaptoethanol (2ME) (Sigma) at a cell density of 2×10^4/ml. Test supernatants or medium control was added in 0.1-ml volumes to triplicate flat-bottom microdilution plates (Costar) containing 2×10^3 B9 cells in 0.1 ml of RPMI 1640 medium supplemented with 20% FCS and 10^{-4} M 2ME. Cultures were pulsed with [^{3}H]thymidine during the final 4.5 h of a 3- to 4-day culture. Results are expressed as mean counts per minute of triplicate cultures. One unit of IL-6 activity in this assay is defined as the activity inducing half-maximal proliferation of B9 cells. One unit of IL-6 activity in this assay corresponds to approximately 20 pg of *E. coli*-derived IL-6 used throughout as a laboratory standard (a gift of Genetics Institute, Boston, Mass.).

Gel filtration chromatography. Cell line culture supernatant, prepared as described above, was first fractionated by ultrafiltration through a membrane with a 5,000-molecular-weight cutoff (YM5; Amicon, W. R. Grace and Co., Danvers, Mass.). A sample of the concentrated (25×) supernatant (3 ml) was applied to a preequilibrated Sephadex G-75 (Pharmacia) gel filtration column (1.5 by 75 cm; Bio-Rad Laboratories, Richmond, Calif.). The column was eluted with 0.1 M Tris (pH 7.5) at a flow rate of 10 ml/h; 3.0-ml fractions were collected. Molecular weight standards included BSA (67,000), ovalbumin (43,000), chymotrypsinogen A (25,000), and RNase A (13.7,000) (Pharmacia). In addition, a sample (120 ml) of ultrafiltered cell line supernatant (YM5 filter; Amicon), lyophilized and suspended in 4.5 ml of distilled water, was applied to a preequilibrated Bio-Gel P2 (Bio-Rad) gel filtration column (1.5 by 50 cm; Bio-Rad). The column was eluted with 0.1 M Tris (pH 7.5) at a flow rate of 4 ml/h; 1.9-ml fractions were collected. Molecular weight standards included vitamin B_{12} (1,350) and phenol red (376).

Radiolabeling, immunoprecipitation, and polyacrylamide gel electrophoresis. Extensively washed peripheral blood monocytes (15×10^6) and lymphoblastoid cells (15×10^6) were cultured for 24 h in methionine-deficient minimal essential medium (GIBCO) supplemented with 1 mg of BSA per ml, 2 mM L-glutamine (GIBCO), and 100 μCi of [^{35}S] methionine per ml (New England Nuclear; 100 μCi/mmol) at a cell density of 1.5×10^6/ml. Monocyte cultures were additionally supplemented with 1 μg of lipopolysaccharide (*E. coli* O127:B8; Sigma). After incubation, cell-free supernatants were precipitated at 4°C with a saturated solution of ammonium sulfate (50%, vol//vol) and centrifuged at 4,000 × *g* for 30 min (SS34 rotor; Ivan Sorvall, Inc., Norwalk,

Conn.), and the resulting pellets were suspended in 0.4 ml Tris-buffered saline (phosphate-buffered saline, 25 mM Tris [pH 7.4]) containing 2 mM phenylmethylsulfonyl fluoride and 150 μg of *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) per ml. The immunoprecipitation was performed essentially as described previously (5). A sample (0.1 ml) of labeled precipitate that had been precleared five times by using 100 μl of a 10% (wt/vol) suspension of heat-killed and Formalin-treated *Staphylococcus aureus* Cowan 1 (Calbiochem-Behring, La Jolla, Calif.) was incubated for 18 h at 4°C with either 2 μl of a rabbit antiserum to human IL-6 (17) or 2 μl of a control rabbit antiserum. After incubation, a sample (0.1 ml) of a 10% (wt/vol) *S. aureus* suspension was added, and the tubes were further incubated for 1 h at 4°C. The bacteria were then washed three times with a cold (4°C) immunoprecipitation buffer (phosphate-buffered saline, 25 mM Tris [pH 7.4], 1% Triton X-100, 2mM phenylmethylsulfonyl fluoride, 150 μg of TPCK per ml, 2.5 M KCl). After two additional washes in isotonic immunoprecipitation buffer at 4°C, the bacteria pellets were suspended in 40 μl of 2× gel sample buffer (0.125 M Tris [pH 6.8], 4% sodium dodecyl sulfate, 10% 2ME, 20% glycerol), boiled for 10 min, centrifuged, and then electrophoresed through a 12.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (15). After electrophoresis, the gels were fixed, fluorographed by treatment with Enlightening (New England Nuclear), dried and autoradiographed at −70°C.

RESULTS

Size heterogeneity of autocrine growth factor activity produced by EBV-immortalized B cells. To maximize autocrine growth factor production, exponentially growing EBV-immortalized cells that had been extensively washed in RPMI 1640 medium were incubated for 24 h at a cell density of 1.5×10^6 cells per ml in RPMI 1640 medium containing 1 mg of BSA per ml and 2.5 μg of transferrin per ml. Autocrine growth factor activity in these supernatants was assessed by a 3-day culture of lymphoblastoid cells that had been starved by incubation for 24 h at a cell density of 5×10^5/ml in RPMI 1640 medium supplemented with 1 mg of BSA per ml. An example of autocrine growth factor production under these conditions is shown in Fig. 1. As shown, the addition of increasing amounts of autologous supernatant resulted in increased levels of [^{3}H]thymidine incorporation by the starved B cells, up to 27-fold over background when 50% of the culture medium consisted of the supernatant of autologous cells prepared as described above. Increases in [^{3}H]thymidine incorporation were accompanied by comparable increases in cell numbers.

Supernatants of the lymphoblastoid cell line VDS-O, prepared in this manner, were sized fractionated by ultrafiltration through a membrane with a 5,000-molecular-weight cutoff (YM5; Amicon). As shown in a representative experiment of five performed, most of the autocrine growth factor activity was recovered in the ultrafiltrate, a fraction containing predominantly molecules with molecular weights lower than 5,000 (Fig. 2). However, a proportion (20 to 30%) of the autocrine growth factor activity was found in the concentrate, a fraction containing predominantly molecules with molecular weights higher than 5,000. The biological activity measured in each of these supernatant fractions was due to conditioning of the culture medium by the lymphoblastoid cells, because control medium incubated without the cells and then fractionated by ultrafiltration (YM5; Amicon) consistently failed to promote growth in EBV-induced lymphoblastoid cells.

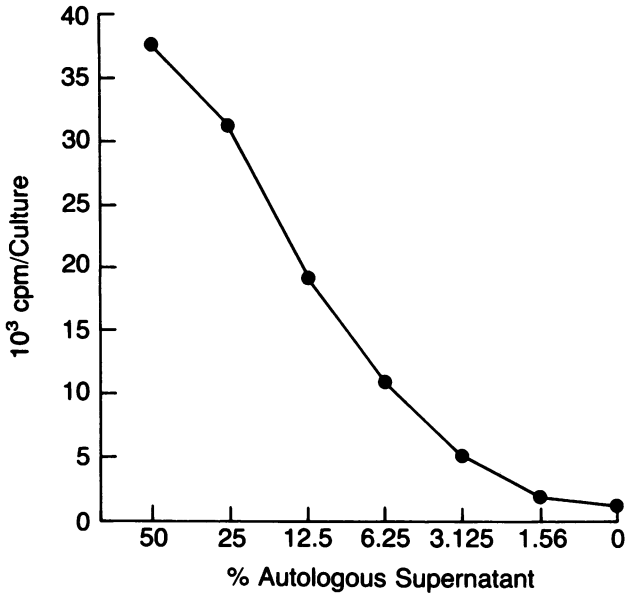

FIG. 1. Autocrine growth stimulation by supernatant of a lymphoblastoid cell line. Starved cells from the EBV-immortalized B-cell line VDS-O (3×10^3 cells per well) were cultured in 0.2 ml of culture medium (RPMI 1640 medium plus 1 mg of BSA per ml and 2.5 μg of transferrin per ml) supplemented with various amounts of autologous culture supernatant (prepared by 24-h culture of VDS-O cells at 1.5×10^6 cells per ml in RPMI 1640 medium with 1 mg of BSA per ml plus 2.5 μg of transferrin per ml).

To estimate the relative molecular weights of these autocrine growth factor activities, concentrate and ultrafiltrate of the VDS-O cell line, obtained by size fractionation over a membrane with a 5,000-molecular-weight cutoff, were individually subjected to gel filtration chromatography. When the concentrate of VDS-O cell line supernatant was fractionated by using a precalibrated Sephadex G-75 column, the autocrine growth factor activity eluted with a relative molecular weight higher than 25,000 (Fig. 3A). When the filtrate of the same supernatant was fractionated with a precalibrated Bio-Gel P-2 polyacrylamide column, the autocrine growth factor activity eluted with a relative molecular weight lower than 1,300 (Fig. 3B). These findings suggested that supernatants of the EBV-induced lymphoblastoid cell line VDS-O contained at least two autocrine growth factor activities that differed markedly in size.

Supernatants of EBV-immortalized B cells display IL-6-like activity. We have previously shown that monocyte-derived as well as *E. coli*-derived IL-6 promotes growth in EBV-immortalized B cells (27, 30). We next tested whether IL-6 was present in the supernatant of the EBV-immortalized B cells and whether it could act as an autocrine growth factor for these cells. The IL-6-dependent hybridoma cell line B9 proliferated in response to both the unfractionated supernatant of the VDS-O cell line and the ultrafiltered concentrate containing predominantly molecules of molecular weights higher than 5,000 (Fig. 4). In contrast, the ultrafiltrate, which contained predominantly molecules of molecular weight lower than 5,000, failed to promote growth of B9 cells despite the fact that this fraction contained autocrine growth factor activity. In the example shown, the unfractionated supernatant of the VDS-O cell line contained approximately 80 B9 growth factor units per ml of supernatant compared with a standard IL-6 preparation.

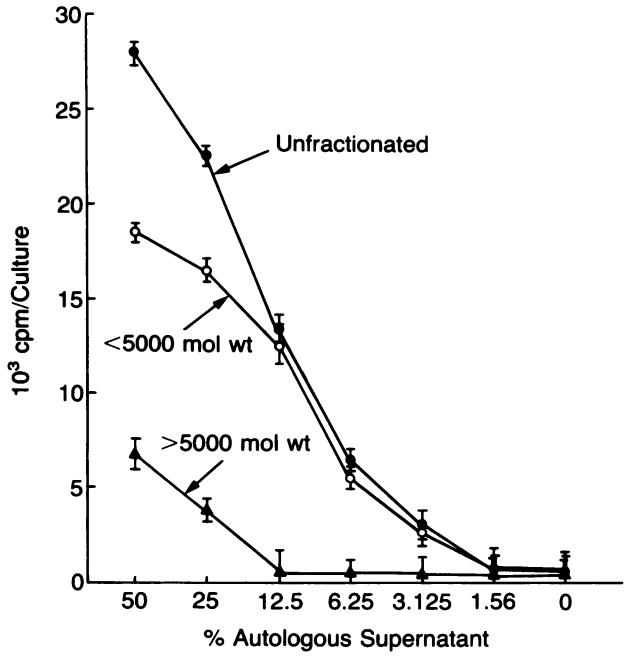

FIG. 2. Heterogeneity of autocrine growth factor activity produced by EBV-immortalized B cells. Starved cells from the EBV-immortalized B-cell line VDS-O (3×10^3 cells per well) were cultured in 0.2 ml of culture medium (RPMI 1640 medium plus 1 mg of BSA per ml and 2.5 μg of transferrin per ml) supplemented with various amounts of autologous culture supernatant either unfractionated or fractionated by ultrafiltration through YM5 membranes (Amicon).

Supernatants from seven additional EBV-induced lymphoblastoid cell lines were examined in the same manner. All unfractionated supernatants had variable contents of IL-6 activity (assayed on B9 cells), ranging from 0.5 to 17.5 U/ml (Table 1). Similarly, following ultrafiltration, each of the concentrates (containing predominantly molecules of molecular weights higher than 5,000) induced growth in B9 cells. In contrast, all supernatant filtrates (containing mostly molecules of molecular weights lower than 5,000) demonstrated no IL-6 activity (Table 1). It should be noted that unfractionated supernatants of each of these seven lymphoblastoid cell lines contained autocrine growth factor activity (not shown). When fractionated by ultrafiltration (YM5 filter; Amicon), each of these supernatants demonstrated autocrine growth factor activity both in the high- (>5,000) and in the low (<5,000)-molecular-weight fractions, with a distribution that was comparable to that of the VDS-O cell line (not shown). Together, these findings suggested that culture supernatants of EBV-immortalized cell lines contain both a high- and a low-molecular-weight autocrine growth factor activity as well as IL-6 like activity.

IL-6 is produced by EBV-immortalized B cells and acts as an autocrine growth factor for these cells. To assess whether IL-6 is responsible for the autocrine activity in the ultrafiltrate concentrate, neutralization experiments were performed, first with a rabbit polyclonal antibody to recombinant, *E. coli*-derived, human IL-6 (17). Control or anti-IL-6 antibody was incubated for 1 h at 37°C in microdilution plates with medium, IL-6, or supernatant of the lymphoblastoid cell line VDS-O that had been concentrated by ultrafiltration (YM5 filter; Amicon) to contain predominantly molecules of molecular weights higher than 5,000. After incubation, either starved VDS-O cells or B9 cells were added to the wells, and the plates were further incubated for 3 days. As expected, the anti-IL-6 rabbit heteroantiserum neutralized growth stimulation of B9 cells induced by recombinant IL-6 (Fig. 5A). In parallel assays, the anti-IL-6 rabbit antibody neutralized the growth-promoting activity of high-molecular weight VDS-O supernatant both in B9 cells (Fig. 5B) and autologous cells (Fig. 5D). The same anti-IL-6 rabbit antibody had little or no effect on T-cell proliferation in response to exogenous IL-2, while an anti-IL-2 receptor monoclonal antibody, anti-Tac, was markedly inhibitory in this assay (Fig. 5C). Finally, this neutralization by the anti-IL-6 rabbit antibody was reversed by a mean of 68% when the anti-IL-6 antibody was incubated with 500 ng of *E. coli*-derived IL-6 prior to the addition of VDS-O cell line supernatant (not shown).

Similar experiments were then performed, using a mouse monoclonal antibody to human IL-6 (20). This antibody neutralized the autocrine growth factor activity of VDS-O cell line supernatant that had been fractionated to contain predominantly molecules with molecular weights higher than 5,000 (Fig. 6). In contrast, this monoclonal antibody failed to neutralize autocrine growth factor activity in ultrafiltrates (<5,000 molecular weight) of VDS-O cell line supernatants (not shown). These findings suggested that IL-6 was present in a size-fractionated (>5,000-molecular-weight) culture supernatant of the VDS-O cell line and that IL-6 was responsible for the autocrine stimulation of B cells by this size-fractionated supernatant. IL-6, however, appeared not to be involved in autocrine growth stimulation by culture supernatants size fractionated to contain predominantly molecules with molecular weights lower than 5,000.

To more directly assess whether EBV-immortalized B cells produce IL-6, immunoprecipitations were done. We had previously established that the maximum amount of IL-6 secreted by any of the lymphoblastoid cell lines tested reached 80 U/ml, approximately 100-fold less than the average amount of IL-6 produced by lipopolysaccharide-activated monocytes (mean, 7,700 U/ml), as determined in the B9 assay. To maximize production of radiolabeled IL-6 by the EBV-immortalized lines, exponentially growing cells were cultured for 24 h in methionine-free minimal essential medium supplemented with 1 mg of BSA per ml, 2.5 μg of transferrin per ml, and 100 μCi of [^{35}S]methionine per ml. To concentrate radiolabeled IL-6, the supernatants were precipitated with ammonium sulfate, and the resulting pellets were used for immunoprecipitation. In parallel experiments, monocytes were cultured in a similar medium except that 1 μg of lipopolysaccharide per ml was added; the resulting radiolabeled monocyte supernatants were processed identically to lymphoblastoid cell line supernatants. Two bands at molecular weights of 20,000 and 26,000 were visualized upon immunoprecipitation of a monocyte supernatant (control) with an anti-IL-6 serum (Fig. 7). In addition, similarly migrating bands were visualized upon immunoprecipitation of four lymphoblastoid cell line supernatants (VDS-O, La, Rb, and 3/22) with the same anti-IL-6 reagent (Fig. 7, lanes b) but not with a control rabbit antiserum (Fig. 7, lanes a). The detection in four lymphoblastoid cell line supernatants of material that reacted specifically with an anti-IL-6 antiserum and had an electrophoretic mobility similar to that of IL-6 strongly suggests that these supernatants contain IL-6.

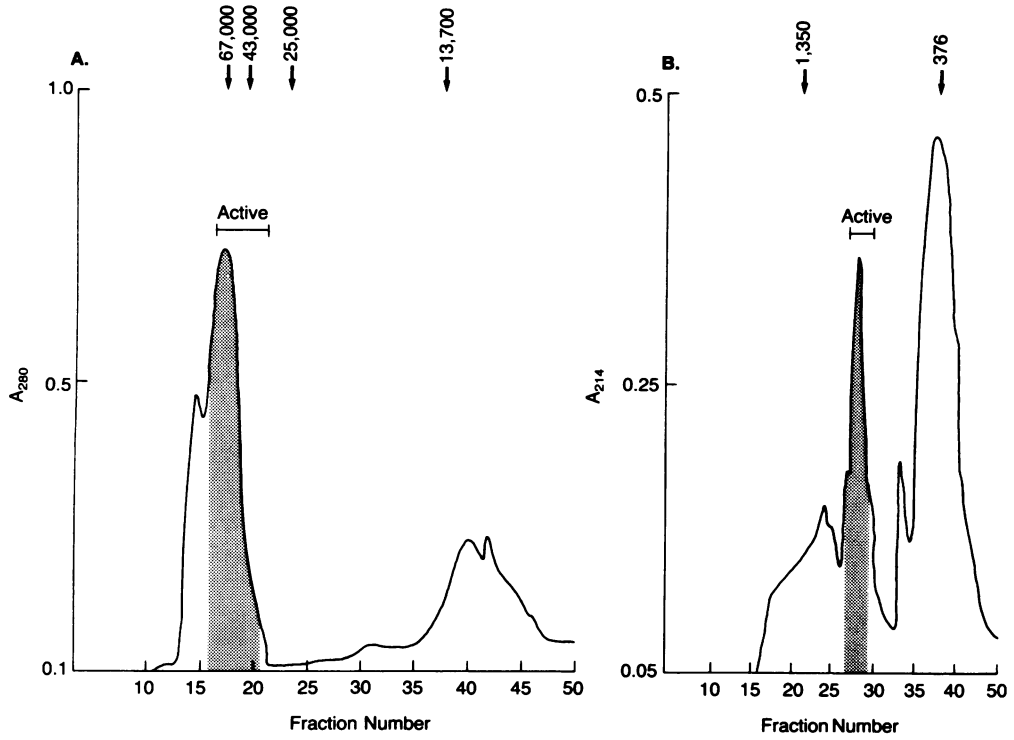

FIG. 3. Gel filtration chromatography of lymphoblastoid cell line-derived autocrine growth factors. (A) Supernatant of the lymphoblastoid cell line VDS-O, prepared as described in Materials and Methods, and concentrated 25-fold by ultrafiltration (YM5 filter; Amicon) was applied (3 ml) to a preequilibrated gel filtration column (Sephadex G-75, 1.5 by 75 cm) and eluted in 0.1 M Tris (pH 7.5; flow rate, 10 ml/h). Each fraction was assayed for autocrine growth factor activity as described in Materials and Methods. Shaded area denotes the active fractions. (B) A sample (120 ml) of supernatant of the VDS-O cell line, prepared as described in Materials and Methods, ultrafiltered through a 5,000-molecular-weight membrane (YM5; Amicon), lyophilized, and then suspended in 4.5 ml of distilled water, was applied to a preequilibrated Bio-Gel P-2 gel filtration column (1.5 by 50 cm) and eluted with 0.1 M Tris (pH 7.5; flow rate, 4 ml/h). Each fraction was tested for autocrine growth factor activity. Shaded area denotes active fractions. Molecular weights are indicated at the top.

DISCUSSION

IL-6, a phosphoglycoprotein originally identified in fibroblasts stimulated by poly(I) · poly(C), is increasingly being recognized as a multifunctional cytokine induced by a variety of signals (22, 36). In humans, it regulates the synthesis of many acute-phase plasma proteins in liver cells (4), acts as an accessory signal for mitogen-costimulated T lymphocytes (16, 29), serves as a growth factor for myeloma cells (11, 14), and supports colony formation in hematopoietic progenitor cells (10). In addition, IL-6 has been shown to promote both growth and immunoglobulin secretion in EBV-infected B cells (9, 27, 30). Monocytes, fibroblasts, endothelial cells, keratinocytes, endometrial stromal cells, and human T-cell lymphotropic virus type 1-infected T-cell lines have all been reported to produce IL-6 either constitutively or in response to appropriate induction (9, 13, 17, 19, 20, 25, 31–34). However, IL-6 has not previously been shown to either be produced by EBV-immortalized B lymphocytes or to represent an autocrine growth factor for these cells.

In the present study, we show that supernatants of each of eight EBV-immortalized cell lines tested display IL-6-like activity, in that they support the growth of the IL-6-dependent hybridoma cell line B9. This activity is specifically neutralized by a rabbit antibody (17) to human recombinant IL-6. In addition, by using an anti-IL-6 rabbit serum, but not a control serum, the characteristic 20,000- and 26,000-molecular-weight bands, attributable to IL-6, could be visual-

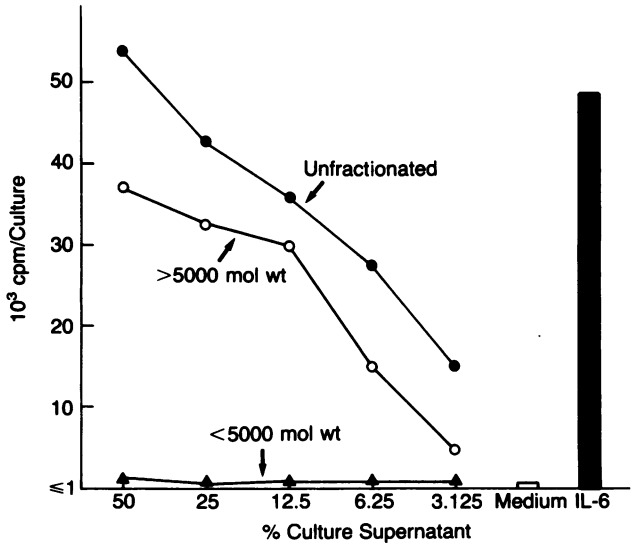

FIG. 4. Supernatant of a lymphoblastoid cell line displays IL-6 activity. Supernatant of the EBV-immortalized cell line VDS-O was tested for IL-6 activity, as described in Materials and Methods, either as unmanipulated supernatant (unfractionated) or following size fractionation through a YM5 membrane (Amicon) with a 5,000-molecular-weight cutoff. Recombinant IL-6 (200 ng/ml) was used as a positive control.

TABLE 1. Distribution of IL-6 bioactivity in the culture supernatants of EBV-immortalized cell lines

Cell line[a]	IL-6 bioactivity (U/ml)[b] in supernatant	
	Unfractionated	>5,000 mol wt
TB	1.6	0.83
La	14.4	14.0
TI	4.4	3.2
RY	0.5	1.2
Rb	17.5	3.35
3/22	9.7	6.1
TI	0.6	1.2

[a] Culture supernatants from the indicated cell lines (prepared as described in Materials and Methods) were size fractionated by ultrafiltration through a membrane with a 5,000-molecular-weight cutoff (YM5; Amicon). Unfractionated and size-fractionated supernatants were individually tested for IL-6 bioactivity, as described in the text.

[b] Units of activity per milliliter of supernatant. For concentrated (>5,000-molecular-weight) supernatants, the results are corrected for the concentration factor. No IL-6 bioactivity as found in supernatants with molecular weights lower than 5,000.

ized in immunoprecipitates of ^{35}S-labeled supernatants of each of four B-cell lines examined (2, 17, 18).

Moreover, the results provide substantial evidence that the IL-6 secreted into the culture supernatants of EBV-induced lymphoblastoid cell lines acts as a growth factor for the cells producing it (i.e., an autocrine growth factor). In particular, supernatants of EBV-immortalized cells that had been fractionated to contain mostly molecules with molecular weights higher than 5,000 enhanced the proliferation of autologous EBV-immortalized B cells by 20- to 30-fold, and this growth-promoting effect was specifically neutralized both by a polyclonal and by a monoclonal antibody to human IL-6.

How much IL-6 is produced by EBV-immortalized cells under the culture conditions employed? Using induction of growth in B9 cells to quantitate IL-6 in a defined culture system, we found that 1 U of IL-6 activity corresponds to approximately 20 pg of a recombinant IL-6 preparation used as a laboratory standard. By using this conversion factor, IL-6 production by EBV-immortalized B-cell lines should range between 10 pg/ml and 1.6 ng/ml. This may represent an overestimate, because other laboratories, using the same target B9 cells, have reported that 1 U corresponds to 1 or 5 pg of IL-6 (1, 35). By using the same biological assay for IL-6, lipopolysaccharide-stimulated monocyte supernatants were found to contain a mean of 154 ng of IL-6 per ml, at least 96-fold more IL-6 than that detected in supernatants of the lymphoblastoid cell lines.

IL-6 did not appear to be the sole autocrine growth factor for EBV-immortalized cell lines. Indeed, the majority of the autocrine growth factor activity appeared to lie in a low-molecular-weight (<5,000) fraction in each of eight cell line supernatants tested. Biologically, this low-molecular-weight autocrine growth factor activity is distinguishable from IL-6, in that it failed to support growth in the IL-6 dependent cell line B9; structurally, this low-molecular-weight autocrine growth factor is at present undefined, and additional studies are under way to define this factor further. In addition, even in the high-molecular-weight (>5,000) fraction of the lymphoblastoid cell line supernatants, autocrine B-cell growth stimulation was generally greater than that achieved with purified IL-6 from either natural or recombinant sources (28, 30). This may be due to the manipulations used to purify IL-6 resulting in functional impairment of the molecule. Alternatively, it is possible that IL-6 produced by EBV-immortalized cells has structural differences from monocyte- or *E. coli*-derived IL-6 that make it more potent. Finally, contamination with low-molecular-weight (<5,000) growth factor or other molecules may contribute to the autocrine growth

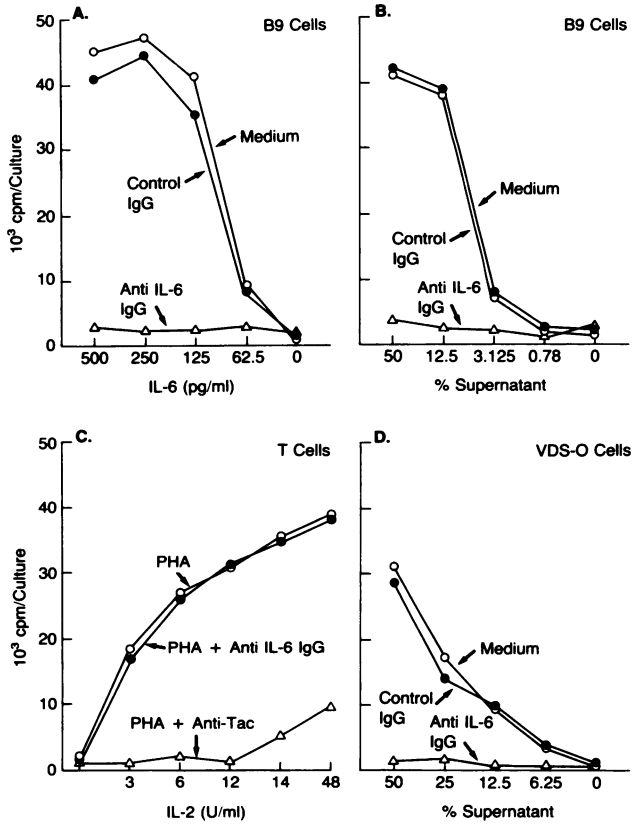

FIG. 5. Autocrine and B9 growth factor activities of a fractionated lymphoblastoid cell line supernatant are neutralized by an anti-human IL-6 rabbit immunoglobulin. (A) Recombinant human IL-6 was incubated for 1 h at 37°C with either medium or control rabbit IgG or anti-human IL-6 rabbit IgG (both at a final concentration of 20 μg/ml). After incubation, B9 cells (2×10^3 in 0.1 ml) were added to each well. Culture medium consisted of RPMI 1640 medium supplemented with 10% FCS and 10^{-4} M 2ME. [^{3}H]thymidine incorporation was measured during the last 4.5 h of a 3-day culture. (B) Twofold-concentrated supernatant of the lymphoblastoid cell line VDS-O, fractionated by ultrafiltration to contain predominantly molecules with molecular weights higher than 5,000, was incubated for 1 h at 37°C under conditions identical to those described in panel A. After incubation, B9 cells (2×10^3 in 0.1 ml) were added to each well. Culture medium and [^{3}H]thymidine labeling were identical to those described in panel A. (C) Recombinant human IL-2 incubated for 1 h at 37°C with either medium or anti-human IL-2 receptor antibody (anti-Tac, 10 μg/ml) or anti-human IL-6 rabbit IgG (20 μg/ml). After incubation, T lymphocytes (10^5 cells in 0.05 ml) and phytohemagglutinin (PHA) (0.04 μg in 0.05 ml) were added to each well. Culture medium consisted of RPMI 1640 medium supplemented with 10% FCS and 2 mM L-glutamine. [^{3}H]thymidine incorporation was measured during the last 4.5 h of a 3-day culture. (D) Twofold-concentrated supernatant of the lymphoblastoid cell line VDS-O, fractionated by ultrafiltration to contain predominantly molecules with molecular weights higher than 5,000 (the same employed in panel B), was incubated for 1 h at 37°C under conditions identical to those described in panel A. After incubation, starved VDS-O cells (3×10^3 in 0.1 ml) were added to each well. Culture medium consisted of RPMI 1640 medium supplemented with 1 mg of BSA plus 2.5 μg of transferrin per ml. [^{3}H]thymidine incorporation was measured during the last 18 h of a 3-day culture.

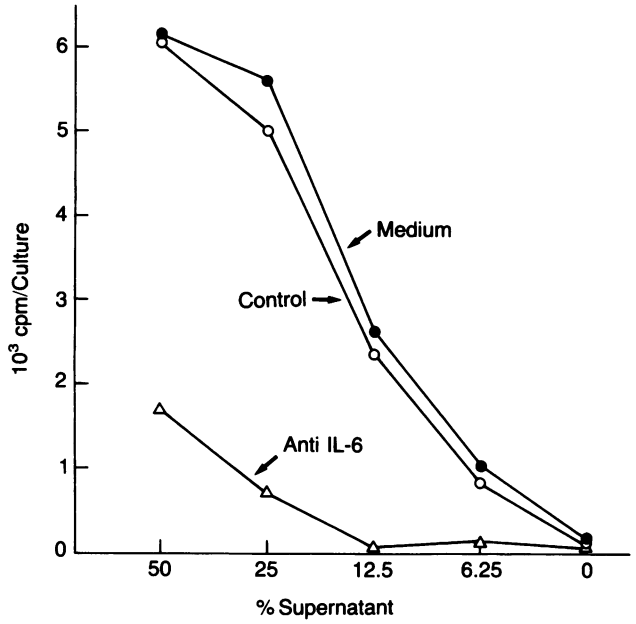

FIG. 6. Autocrine growth factor activity of a lymphoblastoid cell line supernatant is neutralized by a monoclonal antibody to human IL-6. Twofold-concentrated supernatant of the cell line VDS-O, which had been size fractionated to contain mostly molecules with molecular weights higher than 5,000, was incubated (1 h at 37°C) with either a control monoclonal antibody (anti-CD2) or anti-human IL-6 monoclonal antibody (both at a final concentration of 20 μg/ml). After incubation, starved VDS-O cells (3×10^3 in 0.1 ml) were added to each well. Culture medium consisted of RPMI 1640 medium supplemented with BSA (1 mg/ml) and transferrin (2.5 μg/ml). Proliferation was measured by [^{3}H]thymidine incorporation during the final 18 h of a 3-day culture.

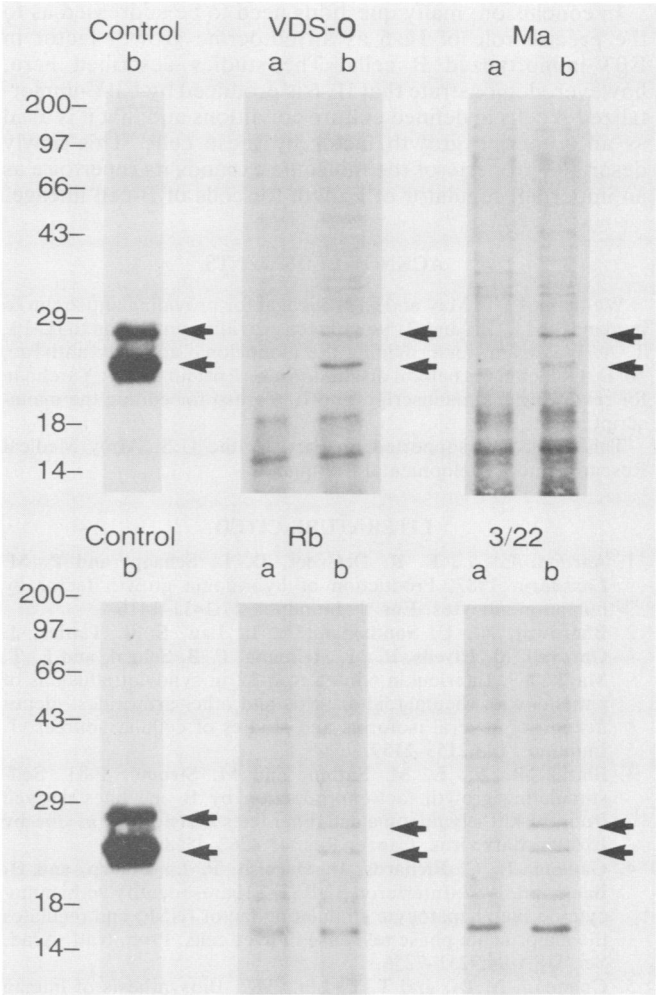

FIG. 7. Immunoprecipitable IL-6 detected in the culture supernatant of EBV-immortalized B cells. ^{35}S-labeled supernatants of either activated peripheral blood monocytes or the indicated EBV-immortalized cell lines (VDS-O, Ma, Rb, and 3/22) were immunoprecipitated with a control rabbit serum (lanes a) or an anti-human IL-6 rabbit serum (lanes b). Immunoprecipitates were boiled and electrophoresed through a 12.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate. Molecular weights (in thousands) are indicated at the left.

factor activity exhibited by the high-molecular-weight (>5,000) factor.

The high-molecular-weight autocrine growth factor eluted from a G-75 gel filtration column with a relative molecular weight of 45,000 to 67,000, a weight higher than that of IL-6 (19,000 to 30,000). This finding initially supported the possibility that molecules other than IL-6 may contribute to autocrine growth stimulation in lymphoblastoid cell lines. However, the same G-75-eluted fractions that contained autocrine growth factor activity also contained IL-6 bioactivity. In addition, when monocyte supernatants were prepared in the same culture medium (RPMI 1640 medium plus BSA plus transferrin), IL-6 bioactivity also eluted from a G-75 gel filtration column with a relative molecular weight of 45,000 to 67,000 (not shown). This early elution of IL-6 from the column could have been due to IL-6 being trapped by BSA and/or transferrin, which are present at high concentrations. Indeed, when both lymphoblastoid cell line supernatants and monocyte supernatants were prepared in a different serum-free culture medium, Opti MEM (GIBCO), autocrine and IL-6 bioactivities eluted from a G-75 gel filtration column with relative molecular weights of 20,000 to 35,000 (not shown). Thus, gel filtration chromatography provides no evidence for the existence of a high-molecular weight autocrine growth factor distinct from IL-6.

As noted above, most of the autocrine growth factor activity produced by lymphoblastoid cells is mediated by molecules with relative molecular weights lower than 5,000. Therefore, it is not surprising that previous experiments (including those in our laboratory) have shown that antibody which neutralized IL-6 failed to inhibit autocrine growth by unfractionated supernatants of lymphoblastoid cell lines (27). In addition to representing only a small proportion (usually less than 30%) of the autocrine growth factor activity, the production of IL-6 by lymphoblastoid cells appears to be dependent on the culture conditions. A variety of studies in our laboratory have consistently failed to show evidence of IL-6 production by immunoprecipitation when the cultures were supplemented with as little as 3% FCS (27). Only when the FCS concentration was reduced to 0.5%, or when BSA was used as a substitute, were we able to see evidence of IL-6 induction by this method. The reason for this effect is not clear. The FCS employed in these experiments does not contain substantial IL-6 activity, as assessed in B9 cells. It is possible, however, that the FCS may contain another IL-6-like factor which makes it unnecessary for the cells to produce autocrine IL-6. Additional studies are under way to address this issue.

In conclusion, many questions need to be addressed as to the precise role of IL-6 as an autocrine growth factor in EBV-immortalized B cells. The studies described here, however, demonstrate that IL-6 is produced by EBV-immortalized B cells in defined culture conditions and that it is used as an autocrine growth factor by these cells. This newly described property of the molecule expands its repertoire as an important regulator of growth for cells of B-cell lineage.

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