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The role of lactic acid in autocrine B-cell growth stimulation

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ABSTRACT **Growth and survival of Epstein–Barr virus (EBV)-immortalized B lymphocytes cultured at low cell densities require autocrine soluble factors. In this study, we have purified a low molecular weight autocrine soluble factor that promotes growth of EBV-immortalized B cells in serum-free conditions and identified it as lactic acid (LA). Synthetic LA stimulated growth in EBV-immortalized B cells at 1–10 mM, a concentration of LA measured in the culture supernatant of EBV-immortalized cell lines. LA alone was found to account for >70% of the autocrine growth factor activity in serum-free supernatants of EBV-immortalized B cells. Aminooxyacetate, a glutamate-oxaloacetate transaminase inhibitor, specifically inhibited B-cell growth induced by LA, suggesting that this process requires mitochondrial–cytosol transfers. Thus, LA is an autocrine stimulatory molecule that in serum-free conditions is essential for the continuous proliferation of EBV-immortalized B cells. This represents an unexpected function for LA.**

Epstein–Barr virus (EBV) can infect human B lymphocytes and induce their immortalization into long-term cell lines (1). The mechanisms responsible for initiation and maintenance of B-cell immortalization by EBV are largely unknown, except that a number of viral genes are believed to play a role in these processes (for a review, see ref. 2).

Over the last several years it has become clear that a critically important event in the process of B-cell immortalization by EBV is the establishment of an autocrine loop, where the virally infected cells secrete growth factors in the culture supernatant and then utilize them to grow (3–5). The existence of such autocrine loop is demonstrated by the observation that, if cultured at critically low cell densities, EBV-immortalized cells stop growing and soon die. This outcome can be prevented by supplementation of the culture medium with supernatants of EBV-immortalized cells cultured at optimal cell densities (3–5).

At present, the molecular nature of the factors responsible for autocrine growth stimulation of EBV-immortalized cells is incompletely defined. Interleukin 6 (IL-6) accounts for a portion (≤30%) of the autocrine growth-stimulating activity in many EBV-immortalized cell lines (5). Other molecules that might contribute to this activity include 3B6 interleukin 1 (IL-1), a structurally unique protein with IL-1 bioactivity (6), and soluble CD23, a B-cell activation antigen (7).

Recently, we found that most of the autocrine growth factor activity (≥70%) in serum-free supernatants of EBV-immortalized cells derives from a low molecular weight (<5000) factor (5). There are many reports of incompletely identified low molecular weight growth factor(s) (8–15). We now report the isolation of one such low molecular weight B-cell-stimulatory factor and its identification as lactic acid (LA).

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MATERIALS AND METHODS

Cells, Cell Lines, and Reagents. Seven lymphoblastoid cell lines (VDS-O, DH, TB, TO, FR, MA, and RY) were used (16). All lines were maintained in RPMI 1640 medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum (Reheis, Armour Pharmaceutical), 2 mM L-glutamine (GIBCO), and 5 μg of gentamicin per ml (Sigma) and were mycoplasma-free. Synthetic LA and oxalic acid were purchased from Aldrich. All other carboxylic acids and all monosaccharides were purchased from Sigma. Recombinant IL-6 was purified from the culture supernatant of COS-7 cells transfected with human IL-6 cDNA (17).

Preparation of Lymphoblastoid Cell Line Supernatants. Exponentially growing lymphoblastoid cells (>90% viable) were first washed extensively in RPMI 1640 medium to remove serum and then incubated for 24 hr at 37°C in RPMI 1640 medium supplemented with 1 mg of bovine serum albumin (BSA) per ml (Miles) and 2.5 μg of transferrin per ml (Sigma) at a cell density of 5×10^5 cells per ml. After incubation, cell-free supernatants were filtered (0.45 μm) and stored either at 4°C or at −20°C. Control supernatants consisted of culture medium (RPMI 1640 with 1 mg of BSA per ml and 2.5 μg of transferrin per ml) incubated at 37°C for 24 hr without cells.

Assay for B-Cell Growth Factor Activity. Exponentially growing lymphoblastoid cells (>90% viable) were washed extensively in RPMI 1640 medium and then cultured ($1.5–2.0 \times 10^4$ cells per ml in flat-bottom microtiter wells, 0.2-ml total volume) in either medium alone (RPMI 1640 supplemented with 1 mg of BSA per ml and 2.5 μg of transferrin per ml) or medium with additives. Cultures were pulsed with [^{3}H]thymidine (0.5 μCi per well, 6.7 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) during the last 18 hr of a 3-day culture. The results are expressed as cpm per culture. Each experiment included a laboratory standard consisting of culture supernatant of the VDS-O cell line prepared as described above.

Determination of IL-6 Activity and LA Concentration. IL-6 bioactivity was determined in a standard proliferation assay using B9 cells as described (5). LA was measured in a lactate dehydrogenase-based enzymatic assay (Sigma, diagnostic procedure no. 720-VV/826 VV).

Purification Procedures. Culture supernatant was filtered through an Amicon ultrafiltration unit with a YM-5 membrane, lyophilized, suspended in water containing 20% ethanol (vol/vol), and then applied to a Bio-Gel P2 (Bio-Rad) column (3 × 100 cm) previously equilibrated in water containing 20% ethanol; elution was done at a flow rate of 10 ml/hr. Active fractions from the Bio-Gel P2 column were pooled, diluted in water to achieve a conductivity of 1.0, adjusted to pH 8.3 with ammonium hydroxide, then applied to a QAE Sephadex A-25 (Pharmacia) column (2.6 × 22 cm) previously equilibrated in water containing ammonium hydroxide (pH 8.3), and then eluted in water with the addition of 100 mM sodium chloride. Active fractions from the QAE

Abbreviations: EBV, Epstein–Barr virus; LA, lactic acid; IL-6, interleukin 6; BSA, bovine serum albumin.

column were pooled, concentrated by centrifugation *in vacuo*, adjusted to pH 6.5 by addition of hydrochloric acid, and then passed in sequence over a Bio-Sil A (Bio-Rad) column (1.5 × 13 cm) and a Bio-Beads SM-2 (Bio-Rad) column (1.5 × 13 cm) equilibrated in water. The unbound material that was biologically active was dialyzed extensively against water using dialysis tubing with a 100 molecular weight cutoff (Spectra/Por, Spectrum, Los Angeles) and then concentrated by centrifugation *in vacuo*. An aliquot (25 μl) of the concentrate was subjected to reversed-phase HPLC over a Hewlett–Packard C_{18} column (5 μm, 100 × 2.1 mm) equilibrated in water and eluted in water at a flow rate of 0.15 ml/min. Active fractions from reverse-phase HPLC were pooled and an aliquot (25 μl) was subjected to hydrophylic interaction HPLC (18) over a Poly LC (Columbia, MD) poly(hydroxyethyl aspartamide) column (100 × 4.6 mm) equilibrated with a solution of 90% acetonitrile in water containing 15 mM triethylamine phosphate (pH 3.8) and eluted for 10 min with the equilibrating solution followed by a linear descending gradient of acetonitrile containing 15 mM triethylamine phosphate (pH 3.8) (from 90% to 30% acetonitrile over 35 min) at a flow rate of 0.5 ml/min.

Mass Spectrometry. Purified fractions from cell culture supernatants that were known to be biologically active as well as corresponding fractions from control medium that were known to be biologically inactive were analyzed first by laser desorption ionization-Fourier transform mass spectrometry (19). Subsequently, equal aliquots of active and inactive fractions were dried *in vacuo* and allowed to react with 50 μl of equal volumes of bis(trimethylsilyl)trifluoroacetamide and acetonitrile at 70°C for 15 min in Teflon-capped tubes. The derivatized extracts were analyzed on a Hewlett–Packard 5970 Mass Selective Detector with a 12 m × 0.25 mm i.d. HP-1 column programed from 90°C to 250°C at 10°C/min while scanning from m/z 50 to 500.

RESULTS

Purification of a Low Molecular Weight Autocrine Growth Factor. The starting material for this isolation was the culture supernatant of the EBV-induced lymphoblastoid cell line VDS-O incubated in serum-free medium at high density (0.5 × 10^6 cells per ml for 24 hr). Recovery of activity at each purification step was monitored by testing the proliferation of the indicator lymphoblastoid cell line VDS-O cultured in serum-free medium at low cell density (1.5 × 10^4 cells per ml) for 3 days. Under these low-density and serum-free conditions the indicator VDS-O cells stop proliferating and die, outcomes that can be prevented by supplementation of the culture medium with supernatant (or its active components) of lymphoblastoid cells cultured at high density (≥2.5 × 10^5 cells per ml). Purification procedures are detailed in Fig. 1. In a representative purification, we recovered ≈15% of the original biological activity. The purified material was analyzed by mass spectrometry (19). Using laser desorption ionization-Fourier transform mass spectrometry, a negative ion series consistent with sodiated LA and oxalic acid [m/z $(M + Na - H)_n(M - H)$] was prominent in the active fractions and absent in corresponding fractions derived from control medium. Subsequently, equal aliquots of active and inactive fractions were allowed to react with bis(trimethylsilyl)trifluoroacetamide and acetonitrile and analyzed on a Hewlett-Packard 5970 MSD with a 12 × 0.25 mm i.d. HP-1 column programed from 90°C to 250°C at 10°C/min while scanning from m/z 50 to 500. The total ion chromatogram indicated a major component with the retention time and mass spectrum of di(trimethylsilyl)lactic acid and a minor component identical to di(trimethylsilyl)oxalic acid in the active fraction that were not detectable in the procedural control. Thus, the purified material that was biologically active contained a major component identified as LA and a minor component identified as oxalic acid.

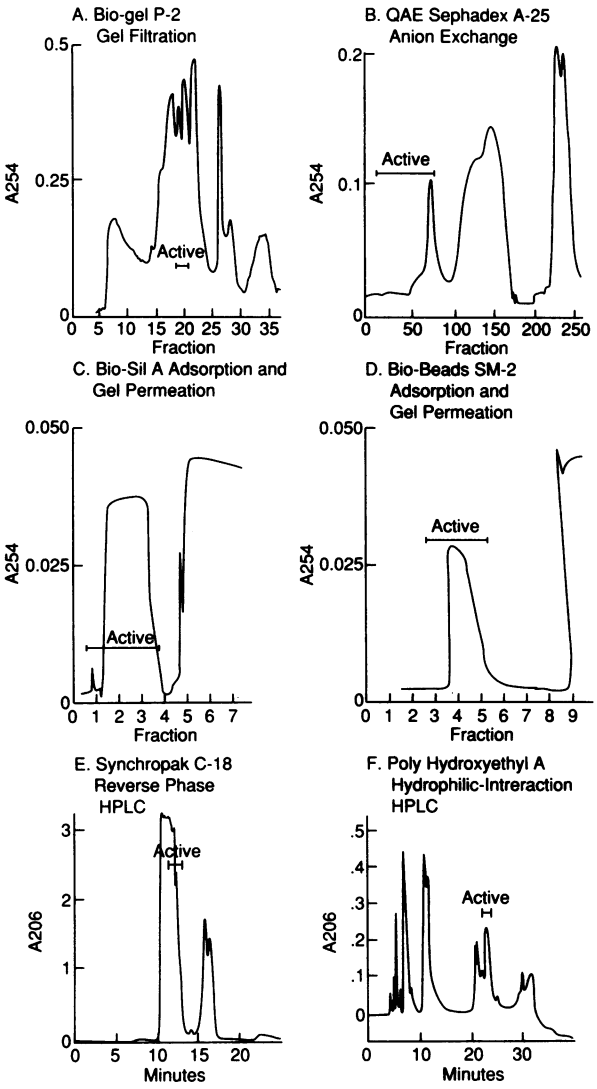

FIG. 1. Purification of a low molecular weight autocrine growth factor. (*A*) Serum-free culture supernatant of the EBV-immortalized cell line VDS-O (700 ml) was filtered through a membrane with a 5000 molecular weight cutoff, lyophilized, suspended in 22 ml of water containing 20% ethanol (vol/vol), applied to a Bio-Gel P2 column, and eluted at a flow rate of 10 ml/hr. (*B*) Active fractions from the gel filtration column were pooled, diluted in water to achieve a conductivity of 1.0 (300 ml), adjusted to pH 8.3, and applied to a QAE Sephadex A-25 column; the material was eluted with water containing 100 mM sodium chloride. (*C* and *D*) Active fractions from anion-exchange chromatography were concentrated to 10 ml, adjusted to pH 6.5, and passed over, in sequence, a Bio-Sil A column and Bio-Beads SM-2 column equilibrated in water. (*E*) The unbound material from the adsorption and gel permeation columns was dialyzed against water, concentrated to 2 ml, and applied (25 μl) to a C_{18} reverse-phase HPLC column; elution was done in water. (*F*) Active fractions from the C_{18} column were pooled and applied (25 μl) to a poly(hydroxyethyl aspartamide) column; elution was in 90% acetonitrile containing 15 mM triethylamine phosphate over 10 min followed by a descending gradient of acetonitrile over 35 min.

Identification of a Low Molecular Weight B-Cell Growth Factor as LA. We tested whether LA and oxalic acid promote growth in EBV-immortalized cells incubated in serum-free medium at low cell densities. Synthetic LA enhanced the proliferation of the indicator VDS-O cells, but synthetic oxalic acid did not (Fig. 2). Maximal stimulation was achieved at LA concentrations ranging between 2.4 and 4.8

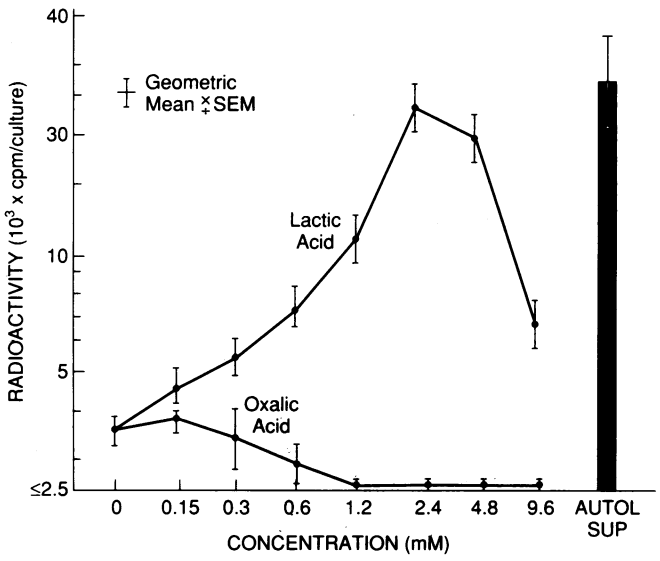

FIG. 2. Induction of proliferation by synthetic LA. EBV-immortalized B cells (VDS-O line) in exponential growth phase (4×10^3 cells per 0.2-ml flat-bottom microwell) were cultured for 3 days in RPMI 1640 medium supplemented with 1 mg of BSA per ml and 2.5 μg of transferrin per ml alone or with the addition of synthetic LA, oxalic acid, or autologous culture supernatant (AUTOL SUP; filtered through a membrane with a 5000 molecular weight cutoff and diluted 1:2). [^{3}H]Thymidine (0.5 μCi per well) was added during the final 18 hr of culture. The results represent the geometric mean radioactivity ×/÷ SEM of five experiments.

mM. This was comparable in magnitude to that achieved by addition of serum-free supernatants of the VDS-O line that had been fractionated to contain predominantly molecules with a molecular weight <5000 and were diluted 1:2. The mean LA concentration in these undiluted VDS-O line supernatants was determined to be 6.7 ×/÷ 1.1 mM (geometric mean ×/÷ SEM). A lactate dehydrogenase-based enzymatic assay was used to measure LA in all cases.

When cultured for 3 days at low cell densities (2.0×10^4 cells per ml) in serum-free medium each of six EBV-induced lymphoblastoid cell lines showed low-level proliferation (Table 1). LA, added at 4.8 mM to these cultures, induced 4.0- to 12.5-fold increases in cell proliferation. This level of growth stimulation in the lymphoblastoid cells was comparable in magnitude to that induced by autologous culture

Table 1. EBV-immortalized cell lines proliferate in response to LA and secrete LA in the culture supernatant

Cell line	Proliferation, cpm per culture			LA content,* mM
	Medium	LA	Autologous supernatant	
DH	541	3874	4279	10.1
TB	924	4693	5835	14.8
TO	290	2253	2151	13.7
FR	1080	4384	5835	11.4
MA	242	3032	3129	10.7
RY	807	6025	6966	7.1

Six EBV-immortalized cell lines were cultured (4×10^3 per 0.2 ml flat-bottom microwell) either in medium alone (RPMI 1640 medium supplemented with 1 mg of BSA per ml and 2.5 μg of transferrin per ml) or with the addition of either synthetic LA (4.8 mM) or 1:2 diluted autologous serum-free culture supernatant prepared by 24-hr culture of the cells (5×10^5 per ml) and filtered through a membrane with a 5000 molecular weight cutoff. Proliferation was measured by [^{3}H]thymidine incorporation during the final 18 hr of culture. Results are expressed as mean radioactivity (cpm) of triplicate cultures. Standard deviations were within 12% of the mean.
*In undiluted cell line supernatant.

supernatants produced in serum-free medium at high cell density (0.5×10^6 cells per ml for 24 hr), filtered through a 5000 molecular weight membrane, and diluted 1:2 (Table 1). The LA concentration in these undiluted supernatants was found to range between 7.1 and 14.8 mM. When crowded, lymphoblastoid cells stop proliferating and LA accumulates in the culture supernatant (7–15 mM). When incubated at lower cell densities, lymphoblastoid cells proliferate and LA concentrations are lower. The mean LA concentration in supernatants of lymphoblastoid cells that proliferated from 5×10^4 to $23–32 \times 10^4$ cells per ml was found to be 3.6 mM (mean of three experiments). Thus, EBV-immortalized cell lines secrete LA in the culture supernatants and the levels achieved are stimulatory for EBV-immortalized cells.

Because IL-6 has been previously identified as an autocrine growth factor for EBV-immortalized B cells (5, 20), we examined the combined growth-promoting effects of LA and IL-6. When added to EBV-immortalized cells from three cell lines (VDS-O, FR, and MA) at the physiologic concentrations measured in the test serum-free culture supernatants of these lines, synthetic LA together with recombinant IL-6 reconstituted >95% of the autocrine growth-stimulatory activity of the autologous supernatants (Fig. 3). Thus, LA and IL-6, together, account for most, if not all, of the autocrine growth factor activity in serum-free supernatants of EBV-immortalized B cells.

Functional Properties of LA. Growth stimulation by LA requires the presence of D-glucose and L-glutamine (Table 2). A number of other monosaccharides, including fructose (Table 2), D-mannose, and *N*-acetyl-D-glucosamine (not shown), failed to substitute for D-glucose over a wide range of concentrations (0.6–40 mg/ml). Fetal calf serum added in culture at levels ≥10% masked the growth-stimulatory effects of LA. After dialysis that removed LA (<0.02 mM residual LA), B-cell growth was still maximal (Table 2). This

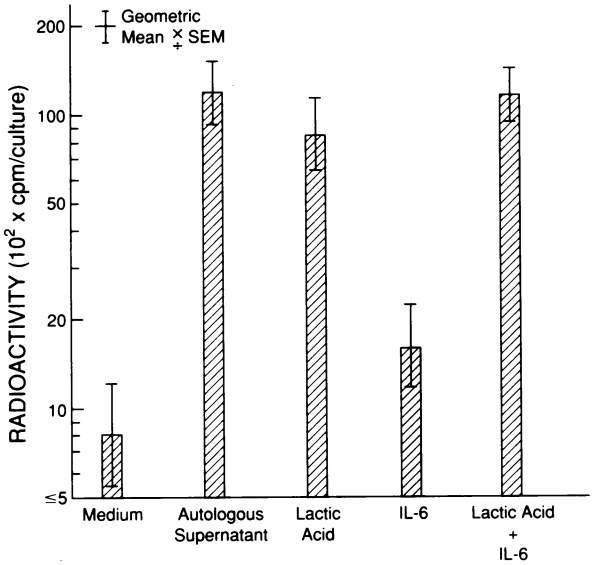

FIG. 3. LA and IL-6 contribution to autocrine growth factor activity of EBV-immortalized B cells. EBV-immortalized B cells from three cell lines (VDS-O, MA, and FR) in exponential growth phase (5×10^3 cells per 0.2-ml flat-bottom microwell) were cultured for 3 days in RPMI 1640 medium containing 1 mg of BSA per ml and 2.5 μg of transferrin per ml. Autologous line supernatant, prepared as described in the text and added unfractionated at 1:2 dilution, was found to contain 10.7–13.5 mM LA and 0.83–3.35 units of IL-6 per ml. Synthetic LA and recombinant IL-6 were added, each at one-half the concentrations measured in the individual supernatants. [^{3}H]Thymidine (0.5 mCi per well) was added during the final 18 hr of culture. The results reflect the geometric mean radioactivity ×/÷ SEM of five determinations.

Table 2. Culture requirements for LA induction of B-cell proliferation

Exp. no.	Culture conditions	Proliferation, cpm per culture	
		Without LA	With LA
1	D-Glucose-deficient RPMI medium	52	70
	+ D-glucose (2 mg/ml)	1,308	10,384
	+ fructose (1 mg/ml)	50	308
2	L-Glutamine-deficient RPMI medium	261	174
	+ L-glutamine (0.3 mg/ml)	8,849	37,911
3	RPMI medium	714	11,292
	+ 1% fetal calf serum	2,564	12,191
	+ 10% fetal calf serum	14,897	13,080
	+ 10% dialyzed fetal calf serum	15,980	15,375
4	RPMI medium	4,405	23,651
	+ pyruvic acid (2.5 mM)	29,197	19,977

Exponentially growing EBV-immortalized cells from the VDS-O cell line were cultured for 3 days (5000 cells per 0.2-ml microwell in experiments 1, 2, and 4 and 1000 cells per 0.2-ml microwell in experiment 3) in RPMI 1640 medium containing BSA (1 mg/ml) and transferrin (2.5 μg/ml) with or without LA (4.8 mM) under the various conditions listed. Cell proliferation was measured by [^{3}H]thymidine uptake during the last 18 hr of culture. Results are expressed as mean radioactivity (cpm) of triplicate cultures. Standard deviations were within 15% of the mean. Experiments 1 and 4 are representative of seven performed; experiments 2 and 3 are representative of five performed.

is likely due to other growth factors present in serum. Pyruvic acid as well as a number of other carboxylic acids (oxaloacetic acid, ketomalonic acid, 3-hydroxybutyric acid, 2-oxopentanoic acid, isobutyric acid, and 2-ketoglutaric acid) stimulated the proliferation of EBV-immortalized cells similarly to LA and, at optimal concentrations, masked stimulation by LA (Table 2). At suboptimal concentrations LA and pyruvic acid had additive growth-promoting effects (not shown).

Aminooxyacetate, an inhibitor of several transaminases, including glutamate-oxaloacetate transaminase, has been shown to selectively inhibit gluconeogenesis from LA while affecting that from pyruvate much less (21, 22). As shown (Fig. 4), over a wide range of concentrations (0.015–1.0 mM), aminooxyacetate inhibited B-cell growth induced by LA by >80% and that from pyruvic acid by <35%. Thus, the autocrine growth-stimulatory effect of LA may require mitochondrial–cytosol transfers, analogous to those for hepatic gluconeogenesis from LA.

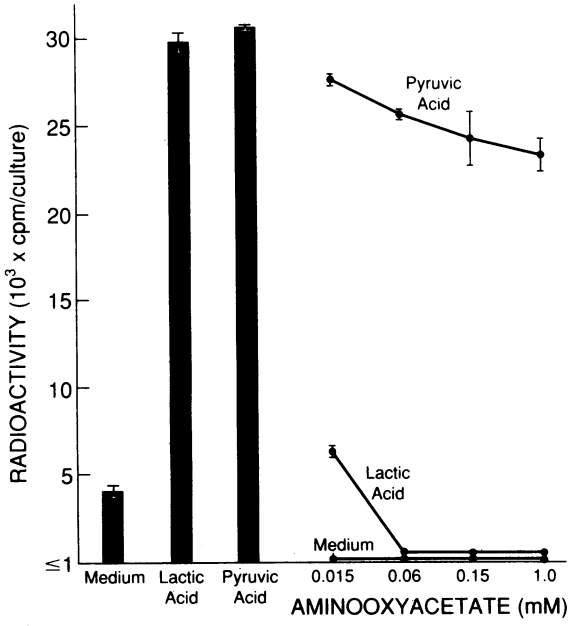

FIG. 4. Specific inhibition of LA-induced B-cell proliferation by the transaminase inhibitor aminooxyacetate. EBV-immortalized B cells (VDS-O line) were cultured (4000 cells per 0.2-ml microwell) in RPMI 1640 medium containing 1 mg of BSA per ml and 2.5 μg of transferrin per ml with or without LA (2.5 mM) or pyruvic acid (2.5 mM). The glutamate-oxaloacetate inhibitor aminooxyacetate was added at the indicated concentrations (0.016–1.0 mM) to cell cultures containing either medium alone or medium supplemented with LA or pyruvic acid.

DISCUSSION

In this study, we have demonstrated that LA is the compound principally responsible for the survival and proliferation of EBV-immortalized B cells grown in serum-free conditions. When incubated at low cell densities in serum-free conditions, EBV-immortalized cells stop proliferating and die, outcomes that can be prevented by the addition of culture supernatants of EBV-immortalized cells grown at higher cell densities in the same medium. We have now found that this growth-promoting effect of culture supernatants is due, for the most part, to the LA they contain. This conclusion is supported by two sets of observations. (*i*) LA was found to be present in the serum-free culture supernatants of EBV-immortalized cells grown at high cell densities at concentrations ranging between 7 and 15 mM and copurified with the autocrine growth-stimulating activity over several steps of purification. (*ii*) Synthetic LA, added at concentrations of 1–10 mM, was found to promote growth in EBV-immortalized cells incubated at low cell densities. The levels of stimulation achieved were comparable to those induced by culture supernatants of EBV-immortalized cells incubated at high cell densities.

The observation that LA plays a major role in the maintenance of an immortalized state by EBV is surprising. Cell growth factors are dominated by complex molecules such as proteins, and we would not have predicted that a simple and ubiquitous molecule like LA could cause B-cell growth stimulation. It is not unique, however, for a simple molecule to exert complex biological effects. For example, nitric acid is turning out to be a pervasive neurotransmitter (23).

Until now, LA was generally considered a biologically inert metabolic product of glycolysis. *In vivo*, it is produced mainly by muscle cells and neoplastic tissues when oxygen becomes limiting (24, 25). *In vitro*, it is produced by a various normal and neoplastic cells even when they are incubated aerobically (26–28). It is known that utilization of LA is mainly limited to liver and kidney cells that are capable of gluconeogenesis from lactate (24). Other cells that have been reported to utilize LA include the retina, which can recycle lactate (29), spermatocytes and spermatides, which can uti-

lize lactate for RNA and protein synthesis (30), splenic T lymphocytes, which are induced to secrete T-cell growth factor (31), and pancreatic islet cells, which are stimulated to secrete insulin (32). However, LA has not been previously found to either be utilized by or promote growth in B lymphocytes.

There is considerable evidence for the existence of specialized transport systems for LA on the plasma membranes of various mammalian cells (33), but the mechanism by which LA promotes growth in EBV-immortalized cells is incompletely understood. LA does not promote glucose transport (not shown) similar to what has been reported for certain growth factors (34) and oncogenes (35). The observation that the glutamate-oxaloacetate transaminase inhibitor aminooxyacetate specifically inhibits growth induced by LA suggests that it may serve to augment cytosol–mitochondrial transfers and thereby increase ATP generation. Increased levels of ATP in the cell may trigger initiation of DNA replication, committing the cell to switch from quiescence to proliferation (36).

In preliminary experiments, LA stimulated the proliferation of EBV-negative cell lines of various lineage, including human T-lymphotropic virus type I-transformed T lines, Burkitt lines, and monocytoid cell lines. Thus, growth stimulation by LA may not be limited to EBV-immortalized cells and may be common among immortalized/transformed cells. We would speculate that LA may have a role in tumorigenesis. Malignant tumors are often poorly perfused as they increase in size. As a result, they become hypoxic and accumulate a number of metabolic products, particularly LA (25, 37). If tumor cells can proliferate *in vivo* in response to LA, as shown here for EBV-immortalized cells *in vitro*, this would allow cell growth to continue in spite of oxygen and nutrient restrictions.

We report here that LA is a potent autostimulatory factor for human B cells immortalized by EBV. This property of LA provides a mean for understanding the molecular basis for autocrine growth stimulation.

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