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Induction of B cell responsiveness to growth factors by Epstein–Barr virus conversion: comparison of endogenous factors and interleukin-1

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SUMMARY

Immortalized B lymphocytes produce a factor(s) that stimulates growth of B cell lines carrying Epstein–Barr virus (EBV). Stimulatory supernatants derived from B cells also exhibit interleukin-1 (IL-1) activity in costimulator assays with the D10.G4.1 helper T cell line. Experiments with purified macrophage-derived IL-1 and recombinant IL-1β demonstrate that IL-1 stimulates proliferation of the cell lines that respond to the factors from B lymphocyte lines. One B cell line, Ramos, an EBV$^-$ Burkitt's lymphoma, contrasts with other B cell lines in that it is refractory to the growth enhancing effects of B cell conditioned medium and macrophage-derived IL-1. When EBV was introduced into Ramos cells, growth was enhanced by the factor(s) in B cell conditioned medium (six out of seven lines); growth of EBV-converted Ramos lines (six out of seven lines) also was enhanced by IL-1. These findings demonstrate that infection of a non-responsive transformed B lymphocyte by EBV induces cellular responsiveness to factor-mediated growth stimulation.

Keywords Epstein–Barr virus growth factor cell lines

INTRODUCTION

Epstein–Barr virus (EBV) activates the human B lymphocyte, causing it to proliferate, secrete immunoglobulin, and develop into a cell line. The way by which EBV causes B lymphocyte activation is the subject of much current investigation. Earlier studies demonstrate that the continuous proliferation of EBV-immortalized B lymphocytes involves a factor-dependent autocrine cycle (Blazar, Sutton & Strome, 1983; Gordon *et al.*, 1984). Autostimulation exemplifies one pathway through which cells can become immortal, a necessary change in the progression of the cell to oncogenesis. Does this autocrine cycle result from the molecular events following B cell activation or is it induced by EBV infection?

Normal B lymphocytes responding to antigenic stimulation require soluble factors from T cells and accessory cells for proliferation to occur (Kishimoto, 1987). Normal B cells may produce some soluble factors when stimulated by antigen (Jurgensen, Ambrus & Fauci, 1986; Muraguchi *et al.*, 1986). EBV-carrying immortalized B lymphocytes continuously proliferate in the absence of exogenously supplied interleukins or other cell types. Shed stimulatory activity has been identified at various times as interleukin-1 (IL-1) (Blazar *et al.*, 1986; Matsushima *et al.*, 1985; Rimsky *et al.*, 1986), T cell derived B cell growth factor (BCGF) (Ambrus *et al.*, 1985; Gordon *et al*, 1984; Muraguchi *et al.*, 1986), CD23 (Swendeman & Thorley-Lawson, 1987), and no known lymphokine (Buck *et al.*, 1987). Our previous studies have demonstrated that supernatants from B cells function as co-stimulators in T cell assays involving concanavalin A (Con A) stimulated human peripheral blood lymphocytes and mitogen-stimulated murine thymocytes (Blazar *et al.*, 1986).

Here we address the question whether EBV infection induces the capacity to respond to growth factors in the B lymphocyte. The effect of EBV transformation was evaluated using the EBV-negative American Burkitt's lymphoma (BL) line, Ramos (Klein *et al.*, 1976) and Ramos sublines carrying EBV-DNA and the Epstein–Barr nuclear antigen (EBNA), derived by transformation *in vitro* with EBV (Fresen & zur Hansen, 1976; Klein *et al.*, 1976). Differences in cell surface phenotypes and growth properties have been reported between EBV$^-$ BL lines and their virally converted sublines (Steinitz & Klein, 1975, 1977; Spira *et al.*, 1981; Ernberg *et al.*, 1983; Klein, Ehlin-Henriksson & Schlossman, 1985). We describe for the first time the induction of growth factor responsiveness in a nonresponsive B cell lymphoma line, Ramos, by EBV infection. Proliferation of EBV converted Ramos sublines is enhanced in the presence of either B cell supernatants containing autostimulatory activity or macrophage-derived IL-1, whereas neither stimulator augments the proliferation of the uninfected Ramos line.

MATERIALS AND METHODS

Cell Lines

Cell lines were propagated as suspension cultures in RPMI 1640

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with 8% fetal calf serum (FCS) (regular culture medium) and subcultured twice weekly. These lines were found to be free of cultivable mycoplasma by Dr Ruth Kundson, Brigham & Women's Hospital, Boston, MA. The following cell lines were obtained through the courtesy of Dr George Klein, Stockholm, Sweden: Raji (Ernberg *et al.*, 1983), Daudi (Klein *et al.*, 1968), and Rael (Klein, Dombos & Gothoskar, 1972). EBV-carrying BL lines; BJAB (Klein *et al.*, 1974) and Ramos (Klein *et al.*, 1976), EBV-negative BL lines; lymphoblastoid cell lines (LCL) transformed with the B95-8 strain of EBV in Stockholm; NAD-20, -22, -23, from normal human lymphocytes, and AT-A-CL7-111 from a patient with ataxia telangiectasis; EBV-converted Ramos sublines B95-8 Ramon, E95-A-Ramos, E95-D Ramos, E950G Ramos, AW- Ramos, EHR-A-Ramos, and HRIK-Ramos (Fresen & zur Hansen, 1976; Klein *et al.*, 1976). LS and SS were transformed from normal human lymphocytes in our laboratory with the B95-8 strain of EBV.

EBNA staining

EBNA staining was determined by anti-complement immunofluorescence on methanol–acetone-fixed smears (Reedman & Klein, 1973).

Preparation of growth-enhancing medium

Preparation of growth-enhancing supernatants (GES) has been described elsewhere (Blazar *et al.*, 1983). Briefly, cells at a concentration of 5×10^5/ml were seeded into 75-cm^2 tissue culture flasks containing RPMI either with 8% FCS or without FCS. Viable cell number was checked after 24 and 27 h in culture by counting trypan blue excluding cells in a Neubauer haemocytometer. If growth had occurred as manifested by 20–40% increase in cell number, the cells were removed by centrifugation and the media filtered through a 0·20-μm Nalgene filter (model 120-0020; Nalgene Labware Division, Rochester, NY) and stored at 4°C. This medium was prescreened with Raji cells; batches that enhanced growth by 100-fold were pooled and saved as a single source of conditioned medium, GES, for this study.

IL-1 assay

IL-1 activity was measured in assay with D10.G4.1 cells in flat-bottomed tissue culture wells. Serial dilutions of IL-1 or GES were cultured in 200 μl of Click's medium (Irvine Scientific) containing 10% FCS, 2 mM L-glutamine, and 50 μM 2-ME; 2×10^4 D10.G4.1 cells and 2·5 μg Con A (Sigma) were added per well. After 48 h, 0·1 μci of ^{3}H-thymidine was added to each well for the following 18–24 h of culture. All tests were performed in triplicate. The cells were harvested by filtration and radioactivity was determined in a scintillation counter (Kurt-Jones *et al.*, 1985).

Cell growth experiments

To determine the effect of conditioned media, GES, on cell growth, 20–30 ml volumes of cells were seeded at 5×10^3 or 1×10^4/ml into 75-cm^2 tissue culture flasks. Twenty four hours later, the cells were centrifuged, the medium was decanted, and triplicate samples were resuspended into normal medium, conditioned medium, or IL-1-containing medium. Purified macrophage-derived (Genzyme, Boston, MA) or recombinant (Cistron, Pine Brook, NJ) IL-1, 0·5 U/ml and 0·25 U/ml, titered in an earlier growth assay with Raji cells, were added where indicated. Cells were then allowed to grow in flasks at 37°C and aliquots of 0·2 ml were removed from the flasks every 2 days to determine the number of viable cells. All cell counts were performed by an observer who was unaware of the source of cells that were counted. The data were analysed with Student's *t*-test.

RESULTS

B cell supernatants stimulate helper T lymphocyte proliferation

Following stimulation by Con A, the D10.G4.1 cell line is very sensitive to IL-1. The effect of recombinant IL-1β (Cistron Corporation) on the response of Con A-activated D10.G4.1 cells is titered in Table 1. B cell supernatants (GES) as the source of stimulatory factors were tested in assays with D10.G4.1 cells because these endogenous stimulators are not yet completely identified or purified. The results of one representative assay from a series of experiments are illustrated in Table 1. Supernatants from the NAD-20 line greatly augmented the proliferation of D10.G4.1 cells activated by Con A. This supernatant was produced in RPMI without FCS. NAD-20 is an EBV-carrying lymphoblastoid cell line derived from the transformation of normal adult lymphocytes by EBV. Similar stimulation was found in the D10.G4.1 assay with GES-containing supernatants from other B lymphoblastoid cell lines.

Table 1. Supernatants from B cell lines are stimulatory in D10G4.1 cell assay

	^{3}H-thymidine incorporation (mean ct/min ± s.d.)*	
	−Con A	+ Con A
Normal medium	165 ± 4	1047 ± 307
IL-1β (ng)†		
25·00	440 ± 20	10324 ± 526
12·50	356 ± 39	6826 ± 32
6·25	256 ± 24	3655 ± 292
3·13	284 ± 31	2384 ± 206
1·57	246 ± 19	1223 ± 28
0·78	258 ± 6	1180 ± 119
0·39	156 ± 8	1033 ± 65
Growth-enhancing supernatant (NAD-20 (%)‡		
25·0	515 ± 95	6314 ± 374
12·5	471 ± 31	3068 ± 194
6·25	459 ± 113	2375 ± 227
3·13	449 ± 51	1928 ± 341
1·56	279 ± 102	1916 ± 12
0·78	310 ± 77	1795 ± 249
0·39	251 ± 104	1108 ± 153

* Triplicate cultures incubated at 37°C for 48 h and pulsed subsequently for 18 h.

† 10 μl recombinant interleukin-1β (IL-1β) from a beginning stock of 2·5 μg/ml.

‡ Prepared in RPMI without fetal calf serum.

Con A, concanavalin A.

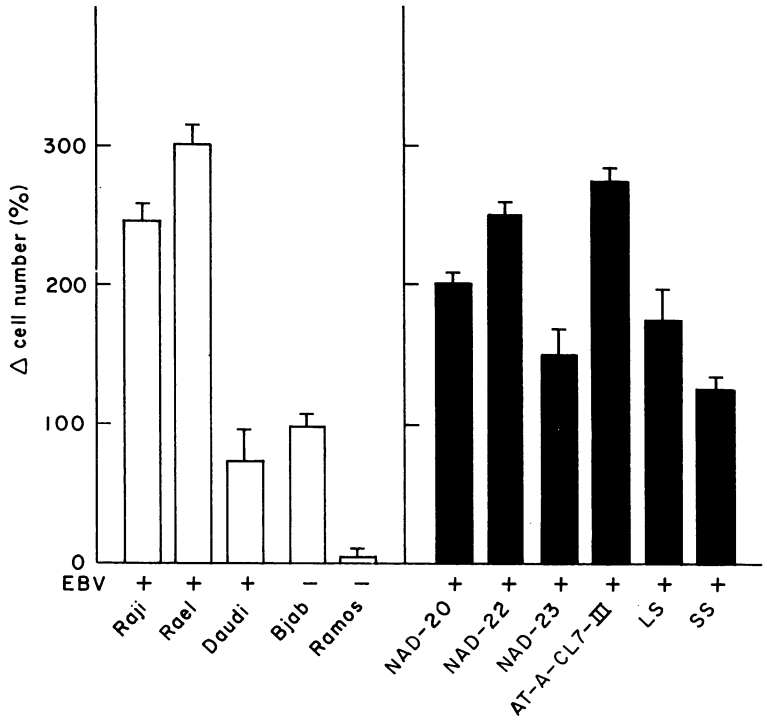

Fig. 1. Burkitt's lymphoma (□) and lymphoblastoid cell lines (■) from growing cultures were density-arrested at 5×10^3/ml. They were then transferred into regular medium or filtered (0·2 μm) growth-enhancing supernatant produced by a 24-h incubation of logarithmically growing NAD-22 cells seeded at a concentration of 5×10^5/ml; 0·2-ml aliquots were removed daily and counted. Results presented here for day 4 following transfer are mean ± s.e.m. of a minimum of three experiments and represent change in cell number compared with cell number grown in regular culture medium. EBV, Epstein–Barr virus.

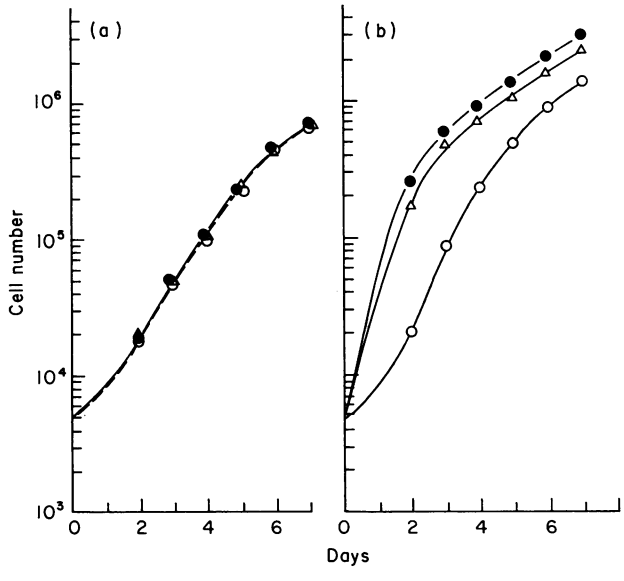

Fig. 2. Kinetics of cell growth for the Ramos line (a) and one of its Epstein–Barr virus (EBV) converted sublines B95-8 Ramos (b) is presented following transfer from 24-h cultures at low cell density (5×10^3/ml). Cells were cultured in either control medium (○), 100% growth-enhancing supernatant (GES) (△), or 10% GES containing control medium (●). Results represent the mean of a minimum of three experiments. Compared with growth in usual culture media differences were greatly significant ($P < 0.01$) from day 2 to day 4 and significant ($P < 0.05$) thereafter.

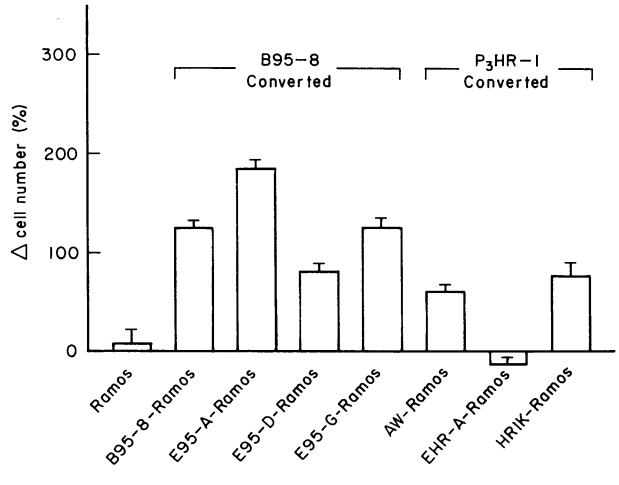

Fig. 3. Density-arrested cells (5×10^3) from cultures of Ramos and seven of its EBV-converted sublines were transferred into either growth-enhancing supernatant or regular medium. Results presented here for day 4 following transfer are the mean ± s.e.m. of a minimum of three experiments and represent change compared with growth in regular culture medium.

B cell response to supernatants and IL-1

Growth of all the B cell lines in this study was enhanced by GES-containing supernatants, with the exception of the Ramos cell line, including EBV$^+$ BL, and EBV$^+$ LCL. Figure 1 presents the average of a minimum of three growth experiments with each

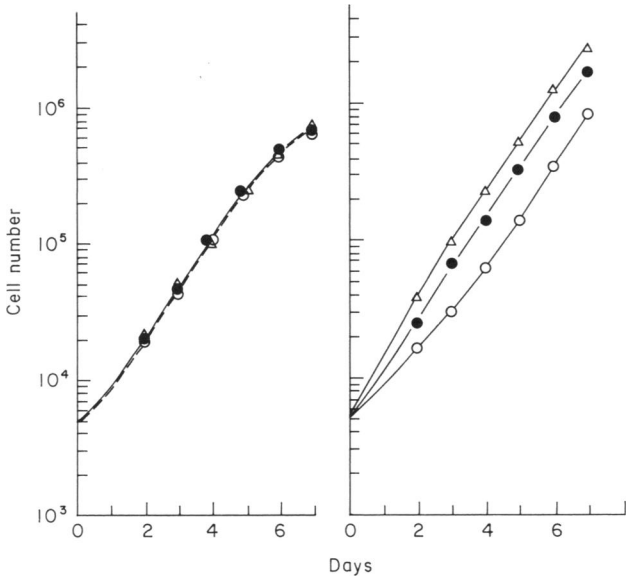

Fig. 4. Kinetics of cell growth for the Ramos line (left) and its Epstein–Barr virus-converted subline, B95-8 (right) is presented following transfer into medium containing interleukin-1 (IL-1) (0·5 U/ml, ●; or 0/25 U/ml, △) (○, control). Results represent the mean of a minimum of three experiments. Significant differences ($P < 0.05$) were found for cells growing in 0·5 U/ml IL-1 from days 2 to 7 and for cells growing in 0·25 U/ml IL-1 for days 3–5 compared with growth in usual culture media.

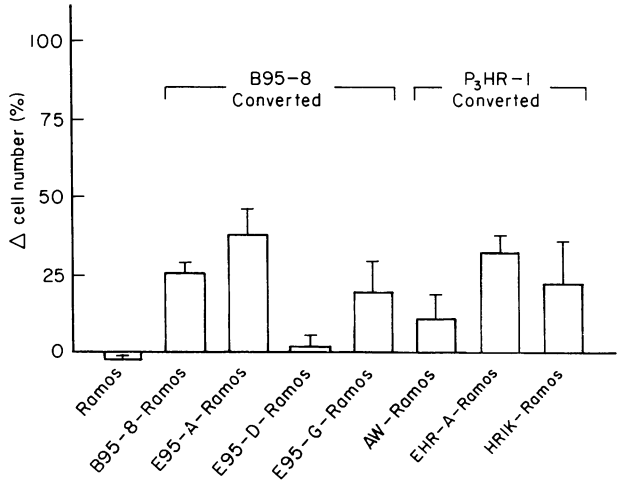

Fig. 5. Density-arrested cells (5×10^3) from cultures of Ramos and seven EBV-converted sublines were transferred into regular medium or medium containing 0·5 U/ml IL-1. Mean ± s.e.m. for a minimum of three experiments is presented for day 4 following transfer for cells grown in IL-1 compared with those grown in regular culture medium.

line. Two EBV$^-$ lines were tested; growth of the EBV$^-$ BJAB line was stimulated whereas growth of the EBV$^-$ Ramos line was not. Although variations occurred in repeat assays of the same line, the pattern of response remained consistent from assay to assay. To insure uniformity, GES-containing B cell supernatants from one cell line, NAD-22, were used. Growth of EBV$^+$ BL and LCL also was enhanced by the addition of purified macrophage-derived or recombinant IL-1β to culture media (data not shown). Again the Ramos line contrasted with the other lines in that its growth was not affected by IL-1.

Converted Ramos sublines respond to B cell supernatants

The effects of B cell supernatants containing GES were evaluated on EBV-converted Ramos sublines carrying EBV-DNA and determined to express EBNA. Cells in these lines were seeded at low concentrations (5×10^3/ml) for 24 h to stop growth and then transferred into either 100% GES, 10% GES and 90% regular culture medium, or 100% regular culture medium. The kinetics of growth in GES for one Ramos subline, B95-8 Ramos, and the parental Ramos lines is presented in Fig. 2. In contrast to the Ramos line, growth of B95-8 Ramos is markedly enhanced in both 100% and 10% GES. In these studies 10% GES was consistently more growth enhancing than 100% GES. This may result from the 24-h growth of cells in GES for the production of conditioned medium. Growth enhancement occurred in six out of seven of the EBV-converted Ramos sublines tested. As shown in Fig. 3, four different Ramos sublines independently converted by the transforming B95-8 strain of EBV, and two of three sublines converted by the nontransforming P3HR1 strain, proliferated better in GES than in regular medium. In contrast to the other sublines, EHR-A Ramos line was not enhanced by GES, but actually grew slower in its presence than in regular culture medium.

Converted Ramos sublines respond to IL-1

In a manner similar to the studies with GES-containing supernatants, proliferation of the EBV$^-$ Ramos lines and its EBV-converted sublines was compared in the presence of IL-1. Cells were seeded at low density for 24 hours and transferred into regular culture medium containing IL-1 (Genzyme, Boston, MA). The doses of IL-1 were determined to maximally enhance growth in another study (Blazar *et al.*, submitted for publication). Kinetics of growth for the converted subline, B98-8 Ramos, and for the parental Ramos line are illustrated in Fig. 4. Growth of the B95-8 Ramos subline was enhanced at both 0·5 and 0·25 U/ml of IL-1. Greater proliferation occurred at the higher dose of IL-1. Kinetic studies published earlier demonstrate that growth rates for cells vary from experiment to experiment (Blazar *et al.*, 1983) but regardless of differences in growth, proliferation of the EBV-converted subline, B95-8 Ramos always was augmented by IL-1. The seven Ramos sublines varied in their responsiveness to IL-1; but six out of seven of the EBV-converted sublines grew better with the addition of IL-1 (Fig. 5). The AW-Ramos line showed minimal growth enhancement by IL-1 at both of the tested concentrations. In addition recombinant IL-1β added to cultures of two sublines, E95-A-Ramos and B95-8 Ramos caused significant growth enhancement.

DISCUSSION

EBV infection *in vitro* activates B lymphocytes and enables them to proliferate without exogenously supplied lymphokines. Stimulated normal B lymphocytes require factors from T lymphocytes and macrophages to maintain even short term growth (Falkoff *et al.*, 1983; Howard *et al.*, 1983; Lipsky *et al.*, 1983).

The experiments reported here are directed firstly towards evaluating the effects of EBV conversion on B lymphocyte responsiveness to factors, and secondly to comparing the effects of endogenously produced B cell factors with macrophage-derived IL-1. EBV-converted Ramos sublines contrast with the parental Ramos line in that they are sensitive to growth stimulation by endogenously produced factors and by macrophage-derived IL-1.

The Ramos line was established from an undifferentiated American lymphoma of the 'Burkitt's' type (Klein *et al.*, 1976). Ramos cells do not have detectable amounts of EBV-DNA or EBNA but they do have an 8:14 chromosomal translocation and are tumourigenic in nude mice. Following EBV conversion, EBV-carrying Ramos sublines exhibit many changes including different patterns of capping, Con A agglutinability, antigen expression, and *in vitro* growth characteristics (Steinitz & Klein, 1975, 1977; Spira *et al.*, 1981; Ernberg *et al.*, 1983; Klein *et al.*, 1985). EBV-converted Ramos sublines remain viable at high cell concentrations for longer periods and have increased expression of insulin receptors compared with the EBV^- parental Ramos line (Spira *et al.*, 1981).

This report is the first of a functional change occurring with EBV conversion of the Ramos line. This change is the induction of responsiveness to growth factors in the Ramos cell following EBV conversion. Responsiveness in the Ramos cell mechanistically might result from the induction of a growth factor receptor or from some intracellular change in a signalling pathway. Responsiveness follows conversion by either the transforming strain B95-8, or the non-transforming P_3HR-1 strain of EBV. Only the transforming B95-8 virus can immortalize normal resting B lymphocytes (Menezes, Leibold & Klein, 1975). The P_3HR-1 strain of EBV contains deletions in its DNA compared with the B95-8 strain and it is thought that the loss of the EBNA-2 gene results in the inability of this viral strain to immortalize normal resting B lymphocytes (Adams, 1980). The induction of growth factor responsiveness in the Ramos sublines by the P_3HR-1 substrain suggests that the EBNA-2 gene is not required for autocrine stimulation to develop. Viral transformation of normal resting B cells involves several critical cellular changes that occur simultaneously and the induction of this growth factor response by the P_3HR-1 strain is not sufficient to cause immortalization of normal lymphocytes. Growth factor responsiveness might be coded for by viral genes, result from indirect viral induction of cellular genes, or be one of the events that follows B lymphocyte stimulation and activation. EBV infection of an established cell line such as Ramos permits expression of virus associated molecular changes even in the absence of one or more EBV functions essential for cell transformation.

The endogenous B lymphocyte derived activity has been reported to be IL-1, T cell derived BCGF (Gordon *et al.*, 1984; Ambrus *et al.*, 1985; Muraguchi *et al.*, 1986), soluble CD23 (Swendeman & Thorley-Lawson, 1987), as well as a new lymphokine (Buck *et al.*, 1987). The possibility of shed CD23 being an autocrine growth factor has been questioned recently (Uchibayashi *et al.*, 1989). Activated normal B lymphocytes express the CD23 differentiation antigen which has been reported to be identical with the Fc receptor molecule for IgE (Yukawa *et al.*, 1987). CD23 antigen expression on B cells follows EBV transformation, and transfection of B lymphocytes by the EBNA-2 or LMP genes from the EBV genome upregulates CD23 expression (Wang *et al.*, 1987). The soluble CD23-Fcε receptor molecule has recently been cloned and the recombinant molecule did not stimulate B cell growth although it did bind IgE (Uchibayashi *et al.*, 1989). The preparation containing native CD23 (Swendeman & Thorley-Lawson, 1987) reported to cause autocrine stimulation of EBV-carrying cells may have contained other molecules that associate with or copurify with CD23 that stimulate B cell growth.

This report extends earlier findings that supernatants containing GES function similarly to members of the IL-1 family, primarily in regard to effects on T lymphocyte responses (Blazar *et al.*, 1986). IL-1-like activity has been reported for EBV-carrying B cells (Gery & Waksman, 1972; Pistoia *et al.*, 1986; Rimsky *et al.*, 1986), fresh cultures of purified B cells from B cell chronic lymphocytic leukaemias (Pistoia *et al.*, 1986) and normal peripheral blood B cells (Matsushima *et al.*, 1985). Our studies indicate that purified IL-1 derived from cultures of stimulated macrophages enhances the growth of B lymphocyte lines (manuscript submitted) and we here demonstrate that recombinant IL-1β also enhances growth of B lymphocyte lines.

Similarly to macrophages, B lymphocyte lines, are able to present antigen to antigen specific T cell clones (Issekutz, Chu & Geha, 1982; Chu *et al.*, 1984; Gerrard & Volkman, 1985). Antigen presentation requires antigen uptake, antigen processing, and the expression of class II MHC molecules on the surface of the antigen presenting cell (Ziegler & Unanue, 1981; Shimonkevitz *et al.*, 1983). Cells in the macrophage lineage which present antigen also secrete IL-1 (Gery & Waksman, 1972). The role of IL-1 in antigen presentation is unclear; it may be required for antigen to induce T lymphocyte responses or may only enhance such responses (Mizel, 1982; Scala & Oppenheim, 1983; Chu *et al.*, 1984; Dinarello, 1984). IL-1 has not been detected thus far in most studies with B cell lines that present antigen to T lymphocytes. The inability to detect IL-1 functionally could result from the production of an IL-1 inhibitor but reports differ as to the presence of such an inhibitor (Scala *et al.*, 1984; Gerrard & Volkman, 1985). A 'membrane' bound form of IL-1 also has been demonstrated on paraformaldehyde fixed murine macrophages and B lymphocytes (Kurt-Jones *et al.*, 1985a, b). Conceivably a cell bound form of IL-1 could be involved in antigen presentation by EBV-carrying B cells. Experiments now in progress indicate that cell surface molecules exist on B cells that costimulate Con A-activated T lymphocytes.

The endogenous B cell stimulatory activity appears to differ with IL-1 as suggested by two findings. Firstly, the consistent observation of greater growth enhancement by the B cell derived GES than the macrophage derived IL-1. Secondly, results with individual virus converted Ramos sublines also suggest that B cells produce a unique molecule in that the B cell supernatant factor, GES, and macrophage derived IL-1 differed in regard to the virally converted Ramos sublines they stimulated, and in regard to the augmentation of proliferation that they caused.

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