Proliferative Responses of a Bovine Leukemia Virus-Infected Lymphoblastoid B-Cell Line by Its Culture Supernatant and Cytokines

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ABSTRACT. The growth-promoting activity in the culture supernatant of bovine lymphoblastoid B-cell lines (BL2M3 and BL312) were examined. BL2M3 cells proliferated well in response to conditioned medium (CM) obtained from BL2M3 and BL312 cell cultures. These BL2M3 and BL312 CM were used as sources of BL2M3 cell growth-promoting factor (BL2M3-GPF). BL2M3-GPF was sensitive to acid (pH 2) and alkali (pH 10) and was heat-labile. Proliferative responses of BL2M3 cells were not induced by human recombinant (r)IL 1, rIL 2, rIL 6, granulocyte-colony stimulating factor (rG-CSF) or tumor necrosis factor (rTNF)-α. Human low molecular weight B cell-growth factor (LMW-BCGF) was, however, capable of augmenting the proliferation of BL2M3 cells. BL2M3 cells formed clusters in response to LMW-BCGF, whereas they showed single and discete appearance in the presence of BL2M3-GPF. These results suggested that bovine lymphoblastoid B-cell lines might release and respond to the growth-promoting factor for *in vitro* proliferation of its own cell line, BL2M3.—KEY WORDS: BLV-infected lymphoblastoid B-cell line, cytokine, proliferation.

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The control of normal B-cell growth is dictated by the interplay of several cytokines. These cytokines, such as interleukin (IL) 4 [4], IL 5 [14], IL 6 [9] and low molecular weight B-cell growth factor (LMW-BCGF) [11], are involved in the triggering of B-cell activation, proliferation and differentiation. However, if normal B cells are infected with some viruses such as Epstein-Barr virus (EBV), they become to lymphoblast at a particular stage of differentiation [5, 16], allowing indefinite proliferation. These transformed cells may become malignant by endogenous production of growth factors acting on themselves [3, 7]. It is also known that virally immortalized cell lines synthesize tumor growth factors which act in an autocrine manner *in vitro* [13, 17]. Therefore, transformation of bovine B cells with bovine leukemia virus (BLV) was hypothesized that the early events of antigen-driven activation of BLV-infected B cells were followed by the release of the intrinsic factors involved in the proliferation of transformed cells. The aim of the current study is to investigate the existence of cell growth-promoting factors in the culture supernatant of BLV-infected lymphoblastoid B-cell lines in association with the proliferative responses of these B-cell lines to some human cytokines.

MATERIALS AND METHODS

Cell lines and conditioned medium: Bovine lymphoblastoid cell lines, BL2M3 and BL312, which exhibited B-cell markers, were originally established from cattle with enzootic bovine leukosis, and the expression of BLV was negative in BL2M3 cells and positive in BL312 cells [10]. These cell lines were kindly donated by Dr. Kenji Sekikawa, National Institute of Animal Health, Ibaraki, Japan. These were maintained in RPMI 1640 (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 0.02 mg/m*l* of gentamycin, and 15% heat-inactivated fetal bovine serum (FBS), referred hereafter to as the medium. For the preparation of conditioned medium (CM), BL2M3 and BL312 cells were washed in RPMI 1640, centrifuged (800 ×g for 5 min), suspended at a concentration of 3×10^6 cells/m*l* in medium, and incubated for 5 days at 37°C under 5% CO_2-humidified atmosphere. These CM were collected by centrifugation at 10,000×g for 30 min and stored at −70°C until use.

Cytokines: Human recombinant (r)IL 1-α and -β and human tumor necrosis factor (rTNF)-α were purchased from Genzyme (Boston, MA.) and human LMW-BCGF from Cellular Products Inc. (Fubbalo, N. Y.). Human rIL 2 was generously provided by Shionogi Pharmaceutical Co. (Tokyo, Japan), human rIL 6 by the courtesy of Dr. K. Yasukawa (Biotechnology Research Laboratory, Tosho, Co., Kanagawa, Japan) and human granulocyte-colony stimulating factor (rG-CSF) by Kirin Brewery Co., Ltd. (Maebashi, Japan).

Assay for BL2M3 proliferation: BL2M3 cells from

cultures were harvested and washed twice. Cells (5×10^5/m*l*) were maintained for 15 hr in medium without FBS to make them starved, as described by Sendeman and Thorley-Lawson [15] with minor modifications. These starved cells were seeded at a density of 2.5×10^4 cells/well in 96-well microplates (Falcon 3072; Becton Dickinson Labware, Oxnard, CA.) and incubated with either different concentrations of BL2M3 and BL312 CM or various cytokines for 5 days at 37°C under 5% CO_2 humidified atmosphere. Medium alone was added to wells as a control. The culture of starved BL2M3 cells was performed at a cell viability of more than 95%. The cultures were pulsed with 0.4 μCi/well (1 μCi=37 kBq) of tritiated thymidine (^{3}H-TdR; New England Nuclear, Boston, MA.) for the last 18 hr and then harvested onto glass fiber papers. The radioactivity was measured in a liquid scintillation counter and data expressd as the mean cpm of triplicate cultures ± SEM.

Physicochemical analyses: For testing the temperature stability, BL2M3 and BL312 CM were treated at 4, 20, 37, 60, and 100°C for 30 min, respectively. Some CM were also dialysed against PBS at pH 2, 7, and 10 at 4°C for 4 hr and thereafter, the CM were re-dialysed against PBS at pH 7 at 4°C for 4 hr. After each treatment, the proliferative activities of the treated CM were determined by using BL2M3 cells.

RESULTS

Effect of BL2M3 CM, BL312 CM and cytokines on BL2M3 cell proliferation: As shown in Fig. 1, BL2M3 and BL312 CM were assayed for their abilities to proliferate BL2M3 cells. BL2M3 and BL312 CM added to the culture for 5 days augmented DNA synthesis of BL2M3 cells in a dose-dependent manner. Further, the proliferation of BL2M3 cells was tested for the effect of human cytokines over a wide range of concentrations. IL 1, IL 2, IL 6, G-CSF, and TNF-α did not support BL2M3 cell proliferation at any concentration when compared to that of medium alone control. LMW-BCGF was able to augment a dose-dependent proliferation of BL2M3 cells.

Morphologic differences of BL2M3 cell proliferation in response to BL2M3 CM and LMW-BCGF: As can be seen in Fig. 2, BL2M3 cells proliferated discretely and individually in response to BL2M3 CM (40%). In contrary, BL2M3 cells proliferated in aggregates and clumps in response to LMW-BCGF

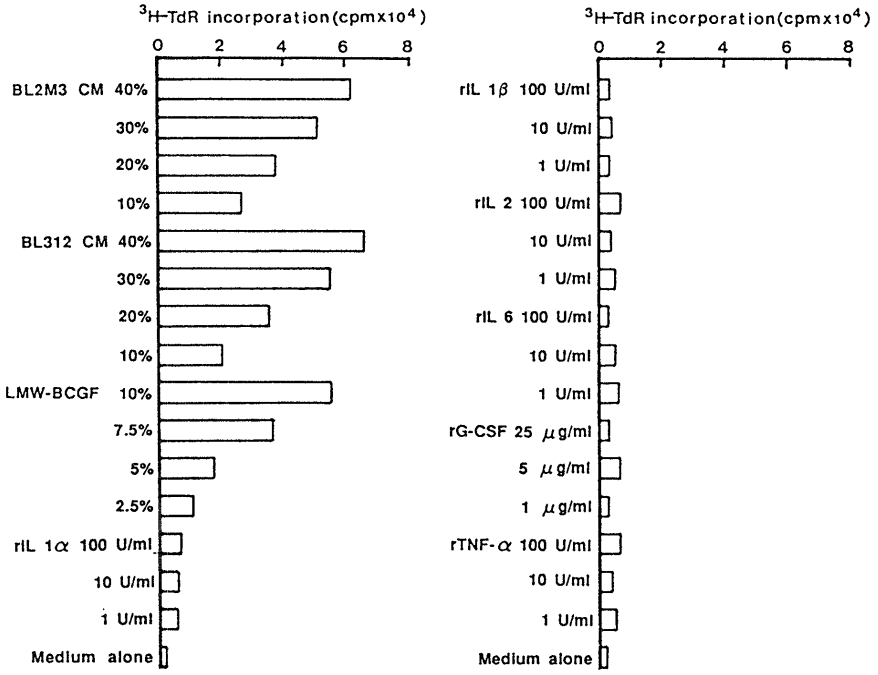

Fig. 1. Proliferative responses of BL2M3 cells to BL2M3 and BL312 CM or various cytokines. BL2M3 cells were cultured for 5 days in the presence of different concentrations of BL2M3 and BL312 CM or various human cytokines and pulsed with 0.4 μCi of ^{3}H-TdR for the last 18 hr of culture. Data expressed as cpm of ^{3}H-TdR incorporation.

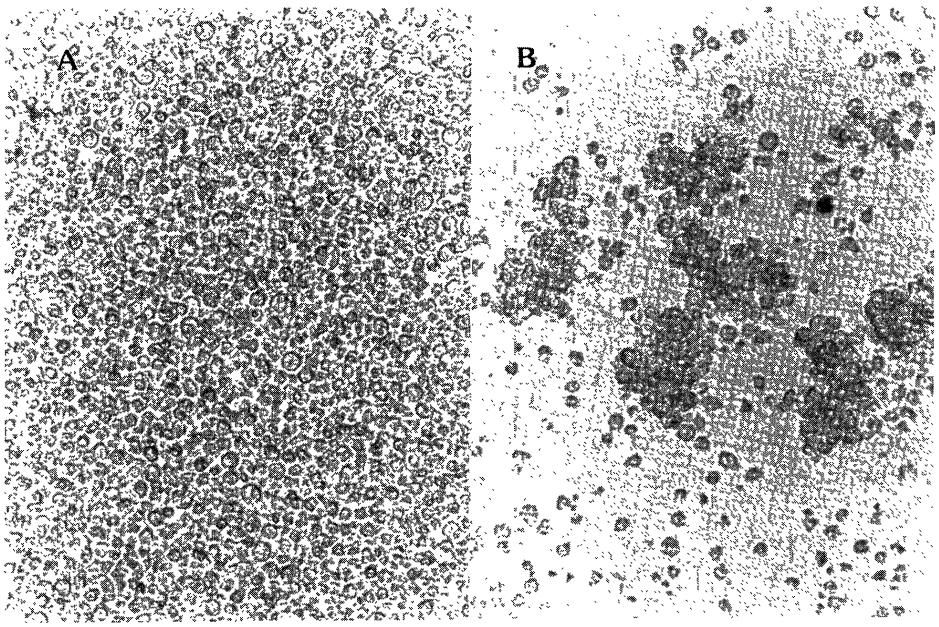

Fig. 2. Phase contrast micrographs (× 20) of BL2M3 cells cultured for 5 days with 40% of BL2M3 CM (A) and 10% of human LMW-BCGF (B).

Table 1. Effect of heat and pH treatment for BL2M3 and BL312 CM on BL2M3 cell proliferative response[a)]

CM (40%)	Temp (°C)[b)]		pH[c)]	
Medium alone	4	922±203[d)]	7	437±109
BL2M3 CM	4	5,380±776	2	309± 87
	20	4,686±518	7	4,044± 52
	37	3,783±384	10	1,707±114
	60	670±197		
	100	695±263		
BL312 CM	4	3,923±597	2	456±195
	20	4,593±231	7	4,214± 87
	37	4,538±239	10	3,529±269
	60	1,128± 58		
	100	1,220±196		

a) Cells were grown for 5 days in the presence of BL2M3 and BL312 CM which were previously treated with either heat or acid and alkali buffers.
b) CM were heated to indicated temperatures for 30 min.
c) CM were dialysed against indicated buffers with different pH for 4 hr.
d) Data were expressed as ^{3}H-TdR incorporation (cpm of mean±SEM, n=3).

(10%).

Physicochemical properties of BL2M3 and BL312 CM: The physicochemical characteristics of BL2M3 and BL312 CM to the proliferation of BL2M3 cells were tested by their temperature stability and their resistance to acid and alkaline treatments. The activity of CM to promote BL2M3 cell proliferation was unaffected by treatment of temperatures between 4 and 37°C for 30 min. In contrast, when heated at 60 or 100°C for 30 min, the activity was completely depleted. The growth activity of BL2M3 and BL312 CM in BL2M3 cells was markedly reduced after treatment with a pH 2 buffer for 4 hr but it was somewhat reduced after treatment with a pH 10 buffer for 4 hr when compared to that of a pH 7 (Table 1).

DISCUSSION

The results suggested that BL2M3 cells could release and respond to specific growth factor(s) necessary for their own growth. BL312 cells were also confirmed to release the soluble factor that supports the continuous proliferation of BL2M3 cells. This activity was designated as "BL2M3 cell growth-promoting factor (BL2M3-GPF)", which was heat-labile and sensitive to acid (pH 2) and slightly sensitive to alkali (pH 10). These findings also indicated the possible presence of receptors for BL2M3-GPF on the surface of BL2M3 cells. Thus, an autocrine mechanism is involved in the *in vitro* growth of the bovine lymphoblastoid B-cell line, BL2M3.

In human and murine cells associated with these results, the surface soluble factors shedded from EBV-transformed cell lines appeared to be implicated in various forms on their cell growth [2, 12]. Furthermore, EBV-transformed lymphoblastoid B-cell lines as well as B-cell neoplasm were shown to release factors which could act in an autocrine manner for their own cells or co-mitogen for normal B cells [6, 8].

Certain cytokines are capable of crossing the species barrier, e.g., human IL 2 shows cross-reactive effect on mouse cells. Human IL 1 and IL 2 actually promoted the proliferation of bovine peripheral blood mononuclear cells [1], indicating that bovine cells may react to these human cytokines. Thus, we determined if various human cytokines exert any effect on the proliferation of BLV-infected B-cell line. In conclusion, human rIL 1, IL 2, IL 6, G-CSF and TNF-α failed to promote the proliferation of BL2M3 cells. This fact suggested that IL 1, IL 2, IL 6, G-CSF and TNF-α were not involved in the continuous growth of bovine lymphoblastoid B-cell line. Furthermore, though both BL2M3-GPF and human LMW-BCGF augmented DNA synthesis of BL2M3 cells, the cells grew as single, nonaggregated cells in response to BL2M3-GPF whereas they tended to aggregate and form clusters in the presence of LMW-BCGF. This observation also suggested that BL2M3-GPF activity was different from LMW-BCGF activity.

Although the B-cell growth factors are often pleiotropic, BL2M3-GPF would be a possible candidate of growth factor for the growth of bovine BLV-induced B-cell lines. The definite conclusion for characterization of BL2M3-GPF will be obtained by further purification and isolation of this factor.

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