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Autocrine Growth of Bovine Leukemia Virus Infected-Lymphoblastoid B-Cell Line, BL2M3

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牛白血病 바이러스감염 림프芽球樣 B 세포주(BL2M3)의 autocrine 증식

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요 약

牛白血病 바이러스감염 B 세포주(BL2M3 및 BL312)의 배양상층액에 대한 自己細胞들의 증식반응을 검토하였다. 그 결과 BL2M3 및 BL312세포의 배양상층액을 자기세포인 BL2M3세포에 첨가했을 때 농도에 비례하여 현저한 증식을 유도하였다. 이것은 배양 4-5日次, 배양상층액의 첨가농도는 50-60%, 세포수는 $5x10^4$-$5x10^5$/ml에서 最適의 증식반응을 보였다. 牛胎兒血淸(FBS) 무첨가 BL2M3 및 BL312세포의 배양상층액에 대해서도 BL2M3세포는 동일한 증식반응을 보였다. 한편 BL2M3 및 BL312세포의 배양상층액을 BL312세포에 첨가했을 때는 BL2M3세포의 경우에 비해 현저하지 않았다. 또한 BL2M3 및 BL312세포의 배양상층액은 말초혈액 림프구에 대해서도 pokeweed mitogen(PWM)첨가 有無에 관계없이 증식을 유도하였다. 그러나 PWM刺戟 말초혈액 單核細胞의 배양상층액은 BL2M3 및 BL312세포에 대해서 전혀 증식을 유도하지 못했다.

이상의 결과로부터 우백혈병 바이러스감염 B 세포주 특히 BL2M3세포는 細胞자신이 증식인자를 분비하고 그것과 반응하여 증식하는 소위 autocrine growth 樣相을 보이는 것으로 판명되었다.

Key words : Autocrine growth, BLV-infected lymphoblastoid B-cell line

Introduction

Bovine leukemia virus(BLV) is a retrovirus that mainly infects the B cell lineage in ruminant species[9]. The BLV is sequence homologies with human C-type retrovirus, human T cell leukemia virus(HTLV-I and HTLV-II), possessing an unusual long terminal repeat(LTR) structure[7,8,27]. BLV and HTLV-I contain a *trans* activation protein, which induces transcription of overlapping open reading frame region[28]. Also, one of the sequence pX proteins in HTLV-I, $p40^x$, was shown

not only to activate transcription of proviral gene from LTR in a *trans* acting manner[10,11] but also to transactivate the gene for interleukin(IL) 2 as well as the gene for IL 2 receptor/Tac expressions which are continuously activated in HTLV-I infected cells[19,31]. These comparative observations led to be a hypothesis that B cell growth factors derived from BLV-infected cells and their receptors are involved in the B cell malignances in relation to BLV infection.

Many clonal B cell responsive systems have been studied in human and murine. Especially, several factors are involved in the triggering of B cell activation, proliferation and differentiation, such as IL 4[6], IL 5[29], IL 6[18] and low molecular weight B cell growth factor(LMW-BCGF)[23]. But little was known about the requirement of cytokines for growth and differentiation of bovine B cells.

It is recently found that culture supernatant of a few of enzootic bovine leukosis-derived cell lines contained the specific growth-promoting factors for the autologous B cells[34]. Also, some B cells escape from normal growth control and become malignant by endogenous production of growth factors acting on themselves[5,16]. Similarly, the previous study showed that the tumorous proliferation of bovine B cells expressing tumor associated antigen(TAA) on their cell surfaces appeared to be highly involved in the growth factors released from BLV-infected lymphoblastoid B-cell lines[35]. Thus, this study was to examine the autostimulatory growth for the proliferation of lymphoblastoid B-cell lines associated with BLV infection.

Materials and Methods

Cell lines and conditioned medium : The bovine lymphoblastoid cell lines, BL2M3 and BL 312 which exhibited B cell markers were originally established from cows with enzootic bovine leukosis(EBL). The expression of BLV was confirmed to be negative in BL2M3 cells and positive in BL312 cells[20]. 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/ml of gentamicin, and 15% heat-inactivated fetal bovine serum(FBS), referred hereafter to as medium. For the preparation of conditioned medium(CM), BL2M3 and BL312 cells were washed, centrifuged(880xg for 5 minutes) in RPMI 1640, suspended at a concentration of $3x10^6$/ml in medium, and incubated at 37 ℃ under 5% CO_2-humidified atmosphere. After incubation for 5 days, supernatant as CM was collected and stored at -70 ℃ before use. In some experiments, FBS-free CM was also used. In addition, the cultured supernatant of $1x10^6$/ml of bovine peripheral mononuclear cells(BPMNC) stimulated with 0.5 μg/ml of pokeweed mitogen(PWM) for 3 days was used as a source of bovine cytokines.

Assay of BL2M3 and BL312 cell proliferation : BL2M3 and BL312 cells from cultures were harvested and washed twice in RPMI 1640. These cells were maintained for 15 hours in medium without FBS to make them starved, as described previously[30]. These starved cells were seeded at various densities per 200 μl/well in 96-well microplates(Falcon 3072; Becton Dickinson Labware, Oxnard, CA, USA) and incubated with various dilution of BL2M3 and BL312 CM for different time intervals at 37 ℃ under 5% CO_2-humidified atmosphere. The cultures were pulsed with 0.4 μCi/well(1 μCi=37 kBq) of tritiated thymidine(^{3}H-TdR; New England Nuclear, Boston, Mass., USA) for the last 18 hours and then harvested onto glass fiber papers using an automated cell harvester(Labo Science Co., Tokyo, Japan). The radioactivity was measured in a liquid scintillation counter.

Isolation and proliferation assay for bovine peripheral blood lymphocytes(PBL) : Blood was collected in heparinized tubes by jugular venipuncture from clinically healthy cattle. Buffy coat cells which were obtained by centrifugation for 40 minutes at 800xg, at room temperature, were diluted 1:1 in phosphate saline(PBS, pH 7.4). The diluted cells were layed on the equal volume of Lymphoprep(specific gravity, 1.077; Nycomed As, Oslo, Norway) and centrifuged at 1,000xg for 30 minutes. The cells at the interface between PBS and Lymphoprep were subjected to 0.83% NH_4Cl in Tris-HCl buffer(pH 7.6) and washed 3 times with PBS. The freshly isolated peripheral mononuclear cells were incubated for 1 hour to remove the adherent cells. Resulting PBL was cultured at a cell density of $1x10^6$/ml with or without PWM(0.5 μg/ml) for 20 hours at 37 ℃ under 5% CO_2-humidified atmosphere. The PBL was washed 4 times in PBS, suspended in medium and cultured with various dilution of BL2M3 and BL312 CM for different periods of time. Cultures were pulsed with 0.4 μCi/well of ^{3}H-TdR for the final 6 hours and then harvested onto glass fiber filter papers using an automated cell harvester. The radioactivity was measured in a liquid scintillation counter. All the values in this study were expressed as mean cpm or dpm of triplicate cultures±SEM.

Interleukin assay : BL2M3 and BL312 CM was assayed for several interleukin activities including IL 1[24], IL 2[12], IL 5[32], and IL 6[13,25] by using murine assay systems.

Results

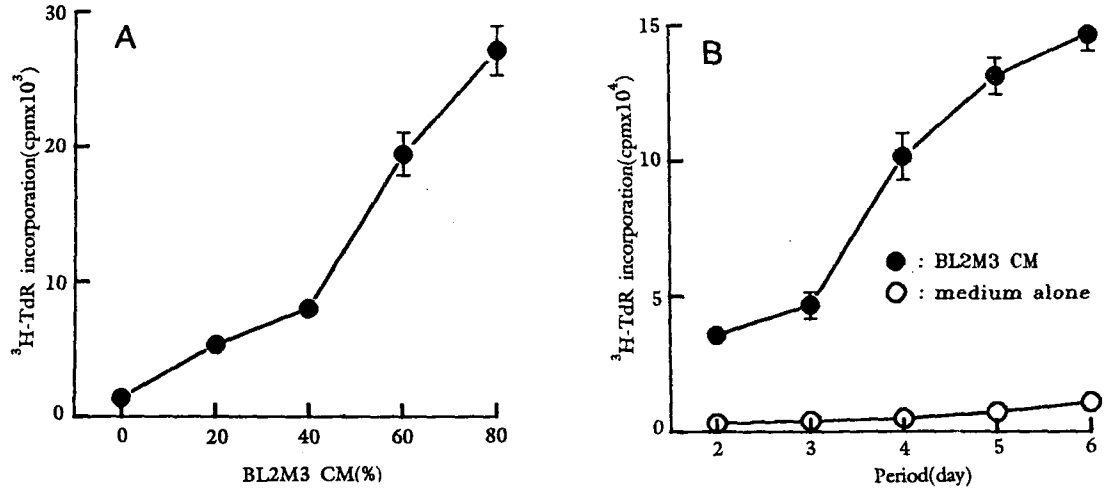
Kinetics for Proliferative responses of BL2M3 and BL312 cells : BL2M3 and BL312 CM was assayed for their abilities to induce the proliferation of autologous cells. BL2M3 CM augmented DNA synthesis of BL2M3 cells in a dose-dependent manner at cell density of $2.5x10^4$/200 μl/well(Fig 1A). This activity started a dramatic augmentation after 3 days of culture and peaked around 4 to 5 days of culture(Fig 1B). However, the BL312 CM showed a weak effect on the proliferation of BL312 cells when compared to those of BL2M3.

As shown in Fig 2, when BL2M3 cells were seeded at cell densities of less than $1x10^5$/200 μl/well, the addition of BL2M3 CM to the culture for 5 days showed proliferative responses in a dose-dependent manner. Whereas little or no activity was seen at higher cell densities(more than $2x10^5$/well). At very low cell densities, less than $1x10^3$/well, BL2M3 cells did not proliferate despite addition of various concentration of BL2M3 CM. BL312 CM had weak activities on BL312 cell proliferation at different cell densities.

To examine whether BL312 CM has the growth-promoting activity in BL2M3 cells or not, BL2M3 cells were cultured with either BL312 CM or BL2M3 CM. DNA synthesis of cultured cells was assessed after 5 days. Not only BL2M3 CM but also BL312 CM induced marked proliferation of BL2M3 cells in dose-dependent manner. Whereas both CM showed weak effect on the induction of BL312 cell proliferation when compared to that of BL2M3 cell proliferation(Fig 3).

To examine whether the growth-promoting activity of BL2M3 and BL312 CM in BL2M3 cells may be due to the presence of FBS in CM, FBS-free CM was prepared by incubating without FBS at a concentration of $4x10^6$/ml for 2 days. Similar growth-promoting activities in FBS-free BL2M3 CM(50%) as well as BL312 CM(40%) were observed in BL2M3 cells but not BL312 cells(Table 1).

BL2M3 cells



BL312 cells

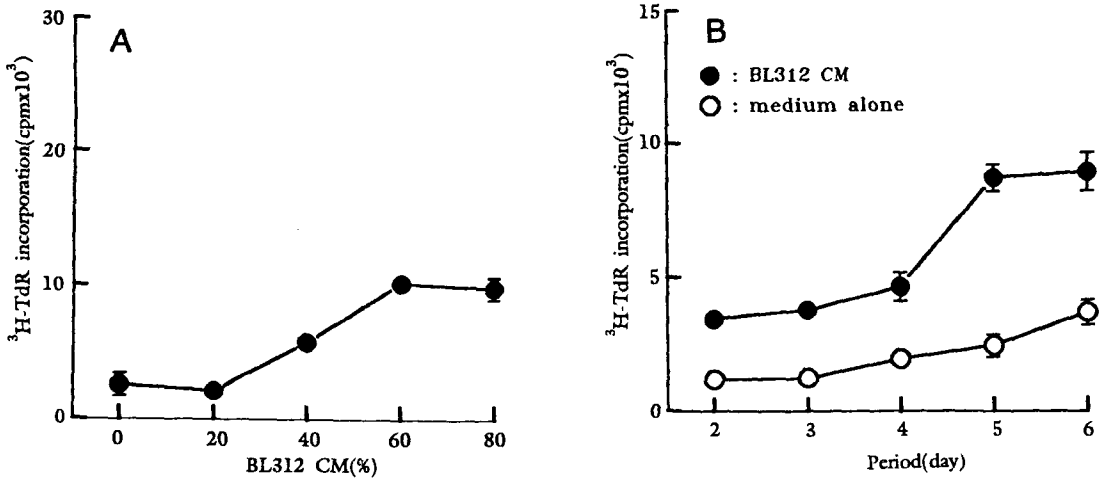


Fig 1. Dose-response(A) and time course(B) on proliferative responses of BL2M3 and BL312 cells in response to BL2M3 and BL312 CM.

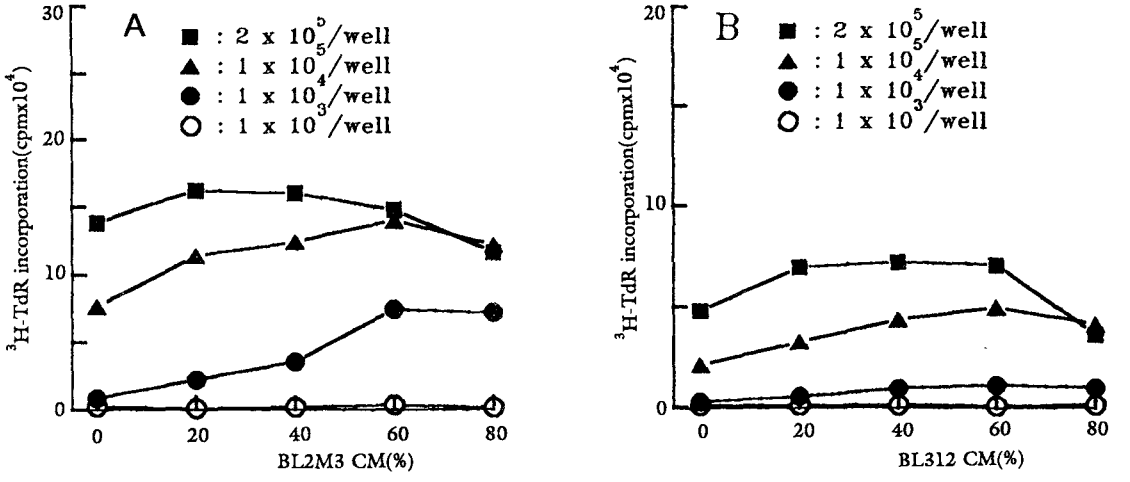


Fig 2. Effect of cell density on proliferative responses of BL2M3(A) and BL312(B) cells in the presence of different doses of BL2M3 and BL312 CM.

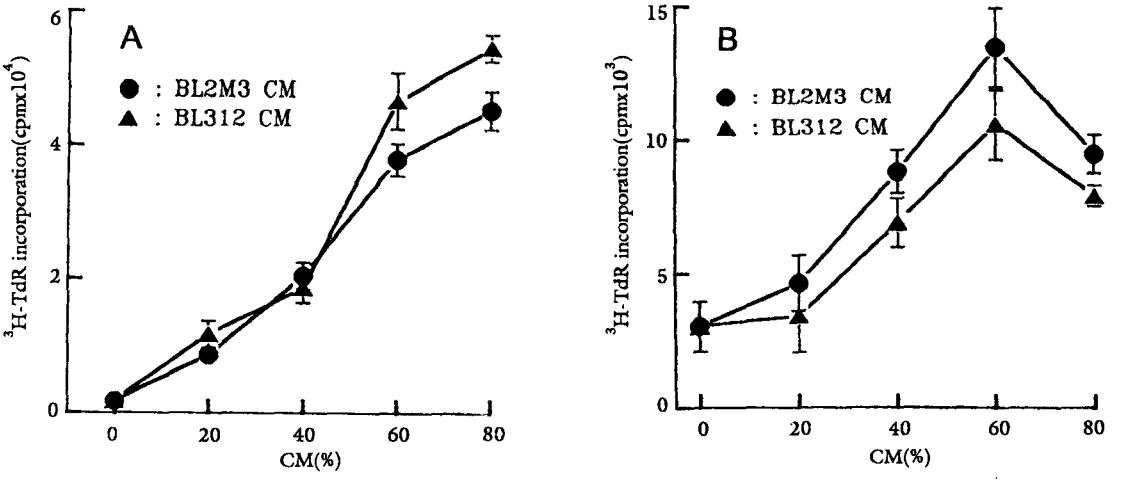


Fig 3. Proliferative responses of BL2M3(A) and BL312(B) cells when cultured with either BL2M3 CM or BL312 CM.

Table 1. Effect of serum-free BL2M3 and BL312 CM in BL2M3 and BL312 cell proliferation

CM[1)]	BL2M3 cells	BL312 cells
Medium alone	219±25[2)]	3,023±383
BL2M3 CM(50%)	14,065±1,588	4,654±456
BL312 CM(40%)	10,217±797	4,685±238

[1)] CM was obtained from supernatant cultured with medium containing no FBS at cell concentration of $4x10^6$/ml for 2 days.

[2)] ^{3}H-TdR incorporation(mean cpm±SEM, n=3)

Biological effect of BL2M3 and BL312 CM on proliferative response of normal bovine PBL : To study the possibility that BL2M3 and BL312 CM is associated with the growth of activated and normal PBL, BL2M3 and BL312 CM was added to either PWM-stimulated bovine PBL blast cells or normal non-stimulated PBL. BL2M3 and BL312 CM induced the proliferation of PWM-stimulated PBL blast cells as well as non-stimulated PBL. Proliferation of PWM- and non-stimulated PBL at cell density of $3x10^4$/200 μl/well to BL2M3 and BL312 CM was dose-dependent and peaked at a concentration of 40% CM(Fig 4A). The time course in proliferation of PWM- and non-stimulated PBL was depicted in Fig 4B. The proliferation of bovine PBL was detected after 2 days of culture and peaked at 4 days of culture regardless of PWM stimulation.

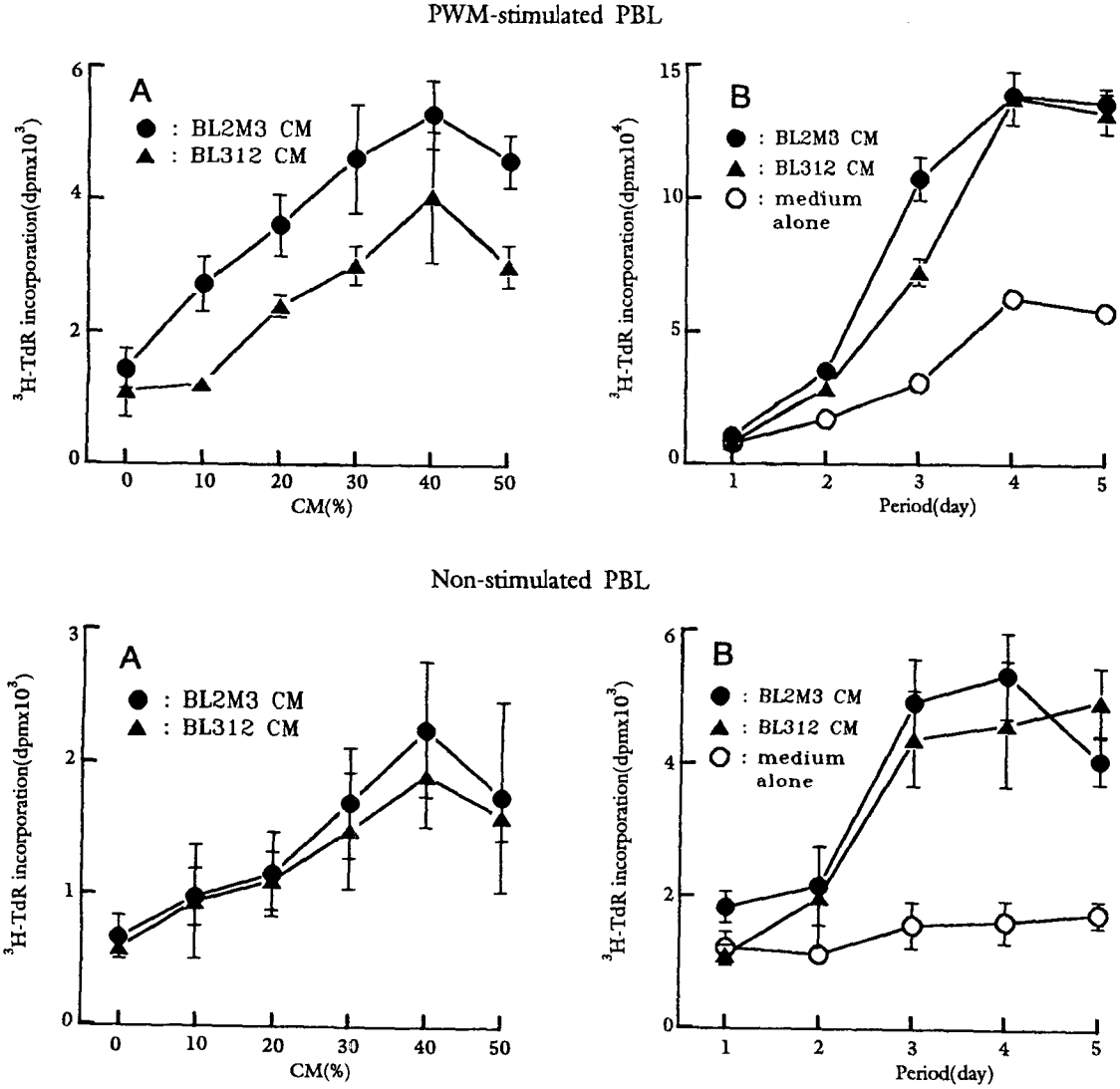


Fig 4. Dose-response(A) and time course(B) on proliferative responses of PWM- and non-stimulated PBL in response to BL2M3 and BL312 CM.

Effect of supernatant of PWM-stimulated BPMNC on BL2M3 cell proliferation : Effects of supernatant of PWM-stimulated BPMNC on the growth of BL2M3 and BL312 cells were tested. Supernatant of PWM-stimulated BPMNC failed to support the growth of BL2M3 and BL312 cells at any concentration(Table 2).

Table 2. BL2M3 and BL312 cell proliferation in response to supernatant of PWM-stimulated BPMNC

CM	BL2M3 cells[1)]	BL312 cells[1)]
Medium alone	5,275±1,248[3)]	8,299± 948
BL2M3 CM(50%)	49,700±2,368	20,977±1,243
BL312 CM(50%)	45,885±1,063	21,410±1,193
PWM-stimulated BPMNC CM(50%)[2)]	3,904± 852	6,676± 514

[1)] Cultured for 5 days in the presence of CM and then pulsed with 0.4 μCi of ^{3}H-TdR for the final 6 hours

[2)] Prepared from culture of 1x10^6 cells/ml with PWM(0.5 μg/ml) for 3 days

[3)] ^{3}H-TdR incorporation(mean cpm±SEM, n=3)

Discussion

The present results demonstrated that BL2M3 CM induce a marked proliferation of BL2M3 cells at optimal cell density. The ability to induce the proliferation of BL2M3 cells also existed in BL312 CM. The CM prepared under serum-free conditions also induced BL2M3 cell proliferation. It was suggested that BL2M3 and BL312 cells release specific growth factor(s) necessary for the BL2M3 and BL312 cell growth. BL2M3 and BL312 CM could also augment the proliferation of autologous cells. It was previously designated this activity as "BL2M3 cell growth-promoting factor(s)(BL2M3-GPF)"[34]. For example, BL2M3 cells release and respond to the soluble growth factor(s) that sustain the continuous proliferation of their own cells, indicating the presence of receptor-like molecules for BL2M3 and BL312 CM on the BL2M3 and BL312 cell surfaces. Thus, these results strongly suggest the probability that an autocrine mechanism appears to be operating in the *in vitro* growth of the bovine B lymphoblastoid cell line, BL2M3.

Several studies have shown the autocrine stimulation of human Epstein-Barr virus(EBV)-transformed B lymphoblastoid cell lines(LCL) that secrete growth factor(s) necessary for their sustained *in vitro* proliferation[3,14,15]. The CD23 molecule which invariably expressed on human EBV-transformed B LCL is released in culture supernatant and reacted again on cell surface[4,17,33]. Namalva Burkitt's lymphoma line produces a 60,000 molecular weight protein, which enhances the growth of normal activated B cells as well as certain B cell lines[1,2]. These surface soluble pro-

teins shed from EBV-transformed cell line or other cell lines appear to be implicated in various forms on its cell growth. It is, therefore, conceivable from autocrine mechanism in BL2M3 and BL312 cell proliferation that the surfaces of BL2M3 and BL312 cells may be transformed or cleaved by BLV or unknown other reasons.

In this study, supernatant of PWM-stimulated crude BPMNC that is thought to include bovine IL 1[21,22], IL 2[26], and other bovine cytokines did not influence the proliferation of BL2M3 cells. Therefore, BL2M3 cells are unable to react with bovine IL 1 and IL 2 on their growth. BL2M3 and BL312 CM showed no IL 1, IL 2, IL 5 or IL 6 activity using murine interleukin assay system(data not shown). These observations indicated that BL2M3 and BL312 CM is a growth factor devoid of, at least, IL 1, IL 2, IL 5 or IL 6 activity. Also, human IL 1, IL 2, IL 6, G-CSF, and TNF-α failed to promote the BL2M 3 cell growth, but LMW-BCGF was capable of augmenting the proliferation of BL2M3 cells[34]. This fact suggested that IL 1, IL 2, IL 6, G-CSF, and TNF-α but not LMW-BCGF were not involved in the continuous growth of BLV-infected lymphoblastoid B cell lines.

BL2M3 and BL312 CM also acted on PWM-stimulated bovine PBL blast cells as well as non-stimulated PBL proliferation. Because PBL used in this study was not separated from B and T lymphocytes, BL2M3 and BL312 CM might stimulate the PBL as a competent factor and then induce the entry of activation and proliferation. This finding indicated that BL2M3 and BL312 CM released from BLV-infected B-cell lines has a paracrine effect for bovine PBL growth.

The B cell growth factors are sometimes multiple and panspecific. It is, nevertheless, assumed that BL2M3 and BL312 CM is a growth factor with regulatory function for the growth of bovine BLV-induced B cell lines. The definite characterization of BL2M3 and BL312 CM will be obtained by further purification and isolation.

Conclusion

The current study was undertaken to investigate the involvement and kinetics of growth factors for continuous growth of bovine leukemia virus(BLV)-infected lymphoblastoid B-cell lines, BL2M3 and BL312. BL2M3 cells proliferated remarkedly to conditioned medium(CM) obtained from either BL2M3 cell culture or BL312 cell culture. They showed a proliferative response even when cultured with serum-free CM. This proliferative activity was dose-dependent at cell concentrations from $5x10^4$/ml to $5x10^5$/ml. Whereas BL312 cells showed a weak proliferative response to either BL2M3 CM or BL312 CM. BL2M3 and BL312 CM also induced proliferation of resting and pokeweed mitogen (PWM)-stimulated bovine peripheral blood lymphocytes (PBL), indicating a paracrine effect that is able to augment the *in vitro* growth for non-transformed PBL. However, proliferative response of BL2M3 and BL312 cells could not substituted by culture supernatant of PWM-stimulated bovine peripheral mononuclear cells instead of BL2M3 and BL312 CM. These results suggested that the *in vitro* proliferation by BLV-infected lymphoblastoid B-cell line, BL2M3, shows an autocrine chain.

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References

1. Ambrus JL, Fauci AS. Human B lymphoma cell line producing B cell growth factor. J Clin Invest 1985; 75: 732-739.
2. Ambrus JL, Jurgensen CH, Brown EJ, et al. Purification to homogeneity of a high molecular weight human B cell growth factor: demonstration of specific binding to activated B cells and development of a monoclonal antibody to the factor. J Exp Med 1985; 162: 1319-1335.
3. Blazar BA, Sutton LM, Strome M. Self-stimulating growth factor production by B-cell lines derived from Burkitt's lymphoma and other lines transformed in vitro by Epstein-Barr virus. Cancer Research 1983; 43: 4562-4568.
4. Cairns J, Flores-Romo L, Millsum MJ, et al. Soluble CD23 is released by B lymphocytes cycling in response to interleukin 4 and anti-Bp50(CDw40). Eur J Immunol 1988; 18: 349-353.
5. Callard RE, Lau YL, Shields JG, et al. The marmoset B-lymphoblastoid cell line(B95-8) produces and responds to B-cell growth and differentiation factors: role of shed CD 23(sCD23). Immunology 1988; 65: 379-384.
6. Defrance T, Vanbervliet B, Aubry JP, et al. B cell growth-promoting activity of recombinant human interleukin 4. J Immunol 1987; 139: 1135-1141.
7. Derse D, Caradonna SJ, Casey JW. Bovine leukemia virus long terminal repeat: a cell type-specific promotor. Science 1985; 227: 317-320.
8. Derse D, Casey JW. Two elements in the bovine leukemia virus long terminal repeat that regulate gene expression. Science 1986; 231: 1437-1440.
9. Fossum C, Burny A, Portetelle D, et al. Detection of B and T cells, with lectins or antibodies, in healthy and bovine leukemia virus-infected cattle. Vet Immunol Immunopathol 1988; 18: 269-278.
10. Fujisawa J, Seiki M, Kiyosawa T, et al. Functional activation of long terminal repeat of human T-cell leukemia virus type I by a *trans*-acting factor. Proc Natl Acad Sci USA 1985; 82: 2277-2281.
11. Fujisawa J, Seiki M, Sato M, et al. A transcriptional enhancer sequence of HTLV-I is responsible for trans-activation mediated by p40x of HTLV-I. EMBO J 1986; 5: 713-718.
12. Gillis S, Ferm MM, Ou W, et al. T cell growth factor: parameters of production and a quantitative microassay for activity. J Immunol 1978; 120: 2027-2032.
13. Goitsuka R, Ohashi T, Ono K, et al. IL-6 activity in feline infectious peritonitis. J Immunol 1990; 144: 2599-2603.
14. Gordon J, Guy G, Walker L. Autocrine models of B-lymphocyte growth: I. role of cell contact and soluble factors in T-independent B-cell responses. Immunology 1985; 56: 329-335.
15. Gordon J, Ley SC, Melamed, et al. Soluble factor requirements for the autostimulatory growth of B lymphoblasts immortalized by Epstein-Barr virus. J Exp Med 1984; 159: 1554-1559.
16. Gordon J, Ley SC, Melamed, et al. Immortalized B lymphocytes produce B-cell growth factor. Nature 1984; 310: 145-147.
17. Guy GR, Gordon J. Coordinated action of IgE and a B-cell stimulatory factor on the CD23 receptor molecular up-regulate B-lymphocyte growth. Proc Natl Acad Sci USA 1987; 84: 6239-6243.
18. Hirano T, Yasukawa K, Harada H, et al. Complementary DNA for a novel human

interleukin(BSF-2) that induces B lymphocytes to produce immunoglobulin. Nature 1986; 324:73-76.

19. Inoue T, Seiki M, Taniguchi T, et al. Induction of interleukin 2 receptor gene expression by p40x encoded by human T-cell leukemia virus type 1. EMBO J 1986; 5: 2883-2888.
20. Itohara S, Sekikawa K, Mizuno Y, et al. Establishment of bovine leukemia virus-producing and -nonproducing B-lymphoid cell lines and their proviral genomes. Leukemia Research 1987; 11: 407-414.
21. Lederer JA, Czuprynski CJ. Production and purification of bovine monocyte-derived interleukin 1. Vet Immunol Immunopathol 1989; 23: 201-211.
22. Lederer JA, Czuprynski CJ. Species preference of bovine thymocytes and fibroblasts for bovine interleukin 1. Vet Immunol Immunopathol 1989; 23: 213-222.
23. Mehta SR, Conrad D, Sandler R, et al. Purification of human B cell growth factor. J Immunol 1985; 135: 3298-3302.
24. Mizel SB, Oppenheim JJ, Rosenstreich DL, et al. Characterization of lymphocyte-activation factor(LAF) produced by the macrophage cell line, P388D1: enhancement of LAF production by activated T lymphocytes. J Immunol 1978; 120: 1497-1503.
25. Ohashi T, Goitsuka R, Ono K, et al. Feline hybridoma growth factor/interleukin-6 activity. J Leukocyte Biology 1989; 46: 501-507.
26. Reeves R, Spies AG, Nissen MS, et al. Molecular cloning of a functional bovine interleukin 2 cDNA. Proc Natl Acad Sci USA 1986; 83: 3228-3232.
27. Rosen CA, Sodroski JG, Kettmann R, et al. *Trans* activation of the bovine leukemia virus long terminal repeat in BLV-infected cells. Science 1985; 227: 320-322.
28. Sagata N, Yasunaga T, Tsuzuku-Kawamura J, et al. Complete nucleotide relationship to other retroviruses. Proc Natl Acad Sci USA 1985; 82: 677-681.
29. Swain SL. Role of BCGF-II in the differentiation to antibody secretion of normal and tumor cells. J Immunol 1985; 134: 3934-3943.
30. Swendeman S, Thorley-Lawson DA. The activation antigen BLAST-2, when shed, is an autocrine BCGF for normal and transformed B cells. EMBO J 1987; 6: 1637-1642.
31. Tagaya Y, Okada M, Sugie K, et al. IL-2 receptor(p55)/Tac-inducing factor: purification and characterization of adult T cell leukemia-derived factor. J Immunol 1988; 140: 2614-2620.
32. Takatsu K, Harada N, Hara Y, et al. Purification and physicochemical characterization of murine T cell replacing factor (TRF). J Immunol 1985; 134: 382-389.
33. Thorley-Lawson DA, Swendeman SL, Edson CM. Biochemical analysis suggests distinct functional roles for the BLAST-1 and BLAST-2 antigens. J Immunol 1986; 136: 1745-1751.
34. Yang MP, Goitsuka R, Tsujimoto H, et al. Proliferative responses of a bovine leukemia virus-infected lymphoblastoid B-cell line by its culture supernatant and cytokines. J Vet Med Sci 1992; 54: 255-259.
35. Yang MP, Goitsuka R, Tsujimoto H, et al. Association of tumor-associated antigen on the proliferation of bovine leukemia virus-infected lymphoblastoid B-cell lines. J Vet Med Sci 1992; 54: 757-761.