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Effect of Mitogens and Anti-bovine Leukosis Virus Serums on DNA Synthesis of Lymphocytes from Cattle*

IKUO TAKASHIMA and CARL OLSON

Department of Veterinary Science, University of Wisconsin, Madison, Wisconsin 53706, *U.S.A.*

Abstract—*Peripheral blood lymphocytes (PBL) from normal cattle and cattle infected with bovine leukosis virus (BLV) were treated with three different mitogens (phytohemagglutinin, concanavalin A and pokeweed mitogens). Lymphocytes from four cattle infected with BLV had increased spontaneous DNA synthesis. Suppressed mitogen responses were observed in lymphocytes from one leukemic steer with enlarged tumorous lymph nodes. Mitogen responses characterized by low stimulation indexes were also observed in lymphocytes from three other infected animals. The spontaneous DNA synthesis in lymphocytes from BLV infected animals was markedly reduced with BLV antiserum in the culture fluid. BLV production in lymphocyte culture, as indicated by syncytium assays was also inhibited by the antiserum. These inhibitory effects were abolished by absorbing the antiserum with BLV. The results suggest that extracellular BLV as well as BLV antigen on cell surface may be responsible for the increased spontaneous DNA synthesis and the altered mitogen responses of PBL from BLV infected cattle.*

INTRODUCTION

THE FOUR clinical forms of bovine lymphosarcoma are the common adult type and the rare calf, thymic, and skin types [1]. Only the adult type of lymphosarcoma appears to be associated with BLV [1]. B lymphocytes are involved in BLV infection, persistent lymphocytosis, and adult type of lymphosarcoma [2, 3]. Infected animals develop antibodies against BLV related antigens (protein 24,000 and glycoprotein antigens) [4]. The antibody against glycoprotein antigens (gp) of BLV inhibits BLV release in the culture of BLV infected lymphocytes [5] and neutralizes cell free BLV [6].

Depression of mitogen responses of lymphocytes from chickens infected with Marek's disease virus and from mice infected with some of the murine leukemia viruses has been demonstrated [7, 8]. High spontaneous uptake of ^{3}H-thymidine and/or depression of mitogen response has been reported in lymphocytes from cattle with leukosis and BLV infection [9, 10]. This study examines the effect of antiviral antiserum on the high spontaneous DNA synthesis, altered mitogen responses, and BLV in the lymphocyte cultures from BLV infected cattle to clarify the involvement of BLV in altered mitogen responses.

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MATERIALS AND METHODS

Animals

Peripheral blood was obtained from 8 clinically normal cattle. These cattle were negative for BLV both by culture and BLV antibody. Four cattle experimentally infected with BLV, confirmed by detection of both BLV and BLV antibody provided BLV infected lymphocytes.

Serums

Serums from three BLV-infected animals (sheep V34 and cow 7 and steer 508) were used. Two animals (sheep V34 and cow 7) had BLV antibodies against both protein 24,000 (P24), the internal BLV antigen, and glycoprotein (gp) antigens as determined by

immunodiffusion (ID) test [5]. Steer 508 had BLV detectable antibodies only against gp antigen by ID test, therefore anti-P24 titer of the serum was considered to be lower than anti-gp titer. Serums from uninfected cattle and a sheep were used as controls. All test serums were sterilized by filtration and inactivated for 30 min at 56°C before use.

Absorption of serum

To characterize inhibiting factors in the serum, serum V34 was absorbed with the BLV pellet derived from the culture fluid of BLV infected fetal lamb kidney (FLK) monolayer culture [11]. Seven hundred millilitres of culture fluid of FLK was centrifuged at 100,000 ***g*** for 90 min. The BLV pellet was mixed with 2 ml of V34 serum diluted to 1:100 in RPMI 1640 medium and incubated for 60 min at 37°C. After incubation, the mixture was centrifuged at 100,000 ***g*** for 90 min, and the supernatant was used as absorbed serum.

Syncytia forming assay

BLV in lymphocyte cultures was determined by the syncytia forming assay using bovine embryo spleen (BESP) monolayer culture [12]. Briefly, 3×10^5 cells of BESP were seeded into 60 mm Falcon integrid tissue culture dishes (Falcon, Oxnard, CA, U.S.A.). Twenty-four hours later, 4 ml of 25 μg/ml DEAE dextran in Eagle's minimum essential media (MEM) with 10% fetal calf serum (FCS) was added to the monolayer culture. After 30 min incubation at 37°C, the cultures were washed and inoculated with 1×10^6 lymphocytes suspended in 4 ml MEM with or without antiserum. After incubation for 48 hr, the monolayer cultures were washed, 4 ml of complete medium was added and the culture was reincubated. When BESP cultures reached confluency, usually 3 days later, the cultures were washed, fixed with methanol, stained with Giemsa stain, and examined for syncytia. All assays were carried out in duplicate. Cells with 5 or more nuclei were counted as positive. Results were expressed as the average of duplicate preparations.

Mitogens

Three mitogens, phytohemagglutinin (PHA) (Bacto PHA-M) (Difco Laboratories, Detroit, MI, U.S.A.), concanavalin A (Con A) (Miles-Yeda Ltd., Kankakee, IL, U.S.A.), pokeweed mitogen (PWM) (Grand Island Biological Company, Grand Island, NY, U.S.A.), were used.

Cultures and stimulation of lymphocytes with antiserum

Lymphocytes from blood collected with heparin (20 units/ml) were isolated with Ficoll-Hypaque (density, 1.077 g/ml) as previously described [3]. Lymphocytes were suspended in RPMI 1640 medium with 10% FCS, 2 mM fresh glutamine, 100 i.u./ml of penicillin, 100 μg/ml of streptomycin and 20 mM HEPES buffer, at a cell concentration of 4×10^6/ml. The lymphocyte preparations, usually 1 ml, were added to an equal volume of complete RPMI 1640 medium or antiserum serially diluted in medium prior to culture. Triplicate samples of 200 μl of cell suspension were incubated in flat bottom microtiter plates with 20 μl of mitogen in media or media alone for 48 hr at 37°C in humidified 5% CO_2/air. To examine the effect of BLV on spontaneous uptake of ^{3}H-thymidine (^{3}H-TdR), 0.2 ml of BLV preparation derived from FLK culture fluid and 0.8 ml of PBL at a cell concentration of 4×10^6/ml were mixed together. BLV was prepared by centrifugation of the culture fluid at 100,000 ***g*** for 90 min. Two hundred microliters of cells and BLV mixture was cultured as described above. The titers of BLV used were 2.0×10^2, 2.0×10^3 and 2.0×10^4 syncytia forming units/6×10^5 lymphocytes. Cultures were pulsed with 1 μCi of methyl-[^{3}H]-thymidine (thymidine, [methyl-^{3}H]-, 6.7 Ci/mM, New England Nuclear, Boston, MA, U.S.A.) in 20 μl RPMI 1640 medium for an additional 18 hr before harvesting. Lymphocyte cultures were harvested with a multiple automatic sample harvester on to glass fiber filter paper (grade 934AH, Reeve Angel, Clifton, NJ, U.S.A.), and ^{3}H-TdR incorporation was determined by counting in a Tri-Carb liquid scintillation spectrometer- (Packard Instrument Company, Downers Grove, IL, U.S.A.). Results are expressed as average of counts per minutes (counts/min) of triplicate samples, and calculated standard errors in triplicate samples were less than 20% in all instances and usually less than 10%. Stimulation index (SI) was calculated by dividing mean counts/min of stimulated cultures with mean counts/min of cultures without mitogen. The significance of the differences observed between the different groups, BLV infected and uninfected animals, was determined by the Wilcoxon two-sample rank test.

RESULTS

Mitogen response of PBL from BLV infected and uninfected cattle

Mitogen stimulation of DNA synthesis was examined for PBL from uninfected and BLV infected cattle (Table 1). The maximal stimulation with mitogens was determined by testing several concentrations of each mitogen: 20, 50 and 100 μg/ml for PHA, 2.5, 5 and 10 μg/ml for Con A and 10, 20 and 50 μg/ml for PWM. Concentrations of each mitogen, 50 μg/ml for PHA, 5 μg/ml for Con A, and 20 μg/ml for PWM were found optimum for the maximal stimulation in the most of tests and used in all the tests. In uninfected animals, incorporation of ^{3}H-TdR into lymphocytes without mitogen ranged from 1.9 to 11.8 (average 4.1) $\times 10^3$ counts/min (Table 1). Responses to mitogens of PBL from uninfected animals were 106.3–402.3 (average 269.5) $\times 10^3$ counts/min with PHA, 67.5–372.6 (average 270.2) $\times 10^3$ counts/min with Con A (Table 1), and 147.3–362.5 (average 248.0) $\times 10^3$ counts/min with PWM (not shown in Table 1). To compare the results, stimulation indices (SI) were determined by dividing mean counts/min value of control cultures (without mitogens) into mean counts/min mitogen stimulated cultures. The SI of mitogen responses of PBL from uninfected animals was 24.8–208.3 (average 117.9) with PHA, 17.3–192.9 (average 115.4) with Con A (Table 1), and 24.3–187.7 (average 100.4) with PWM (not shown in Table 1).

In infected animals, spontaneous incorporation of ^{3}H-TdR without mitogen was high, compared to that of uninfected animals (Table 1) ($P<0.01$). The SI of mitogen responses of PBL from infected animals with PHA and Con A was lower than those of uninfected animals (Table 1) ($P<0.01$). The lymphocytes from steer 9 which had extremely high PBL counts responded poorly with PHA and Con A. Surface Ig bearing lymphocytes (B cells) in blood of steer 9 was 82% by the method previously described [3]. Mitogen responses of PBL from infected animals with PWM gave results similar to those with PHA and Con A.

Effect of anti-BLV serum on mitogen response of PBL from BLV infected and uninfected cattle

The effect of anti-BLV serum on mitogen response of PBL was examined to evaluate the involvement of BLV and BLV related antigens. When anti-BLV serum (10% of sheep V34 serum) was added to the cultures of lymphocytes from infected cattle, total incorporation of ^{3}H-TdR into cells in the presence of mitogens did not change. However, there was a marked reduction of spontaneous incorporation of ^{3}H-TdR into cells without mitogen and an increase of SI when PHA and Con A were added (Table 2). On the other hand, mitogen responses of PBL from an uninfected animal were not changed significantly with anti-BLV serum. PWM also gave results similar to PHA and Con A.

Table 1. Synthesis of DNA in response to PHA and Con A by cultures of PBL from uninfected cattle and BLV infected cattle

Cells from animal	BLV infection*	PBL counts†	Without mitogen counts/min‡	PHA		Con A	
				counts/min	SI§	counts/min	SI
1	−	3.7	1.9	402.3	208.3	372.6	192.9
2	−	8.3	3.8	106.3	27.2	67.5	17.3
3	−	2.7	1.5	262.2	167.3	263.2	168.1
4	−	2.4	1.7	283.0	161.7	295.2	168.7
5	−	3.9	11.8	293.8	24.8	352.6	29.8
6	+	4.2	54.2	305.8	5.6	327.2	6.0
7	+	8.0	63.0	192.6	3.0	255.1	4.0
8	+	4.5	28.5	297.3	10.4	355.5	12.5
9	+	303.8	63.3	56.1	<1	29.2	<1

*BLV infection was tested by the presence of antibodies against BLV and BLV antigens in PBL short term culture by ID.

†Indicated as ($\times 10^3$/mm^3).

‡Indicated as ($\times 10^3$ counts/min), mean counts/min of ^{3}H-TdR in insoluble material for triplicate cultures.

§Stimulation index (SI) = mean counts/min of stimulated cultures/mean counts/min of cultures without mitogen.

Table 2. Effect of anti-BLV serum on mitogen response of PBL from BLV infected and uninfected cattle

Cells from animal	BLV infection*	Serum added	Without mitogen counts/min†	PHA		Con A	
				counts/min	SI‡	counts/min	SI
7	+	No serum	47.9	196.2	4.0	305.4	6.3
		10% sheep V34	5.5	209.4	37.6	275.3	49.5
6	+	No serum	54.2	305.8	5.6	327.2	6.0
		10% sheep V34	5.7	198.5	34.6	355.2	62.0
4	−	No serum	1.7	283.0	161.7	295.2	168.7
		10% sheep V34	0.5	157.3	310.9	174.6	345.1

*BLV infection was tested by presence of antibodies against BLV and BLV antigens in PBL short term culture by ID.
†indicated as ($\times 10^3$ counts/min), mean counts/min of ^{3}H-TdR in insoluble material for triplicate cultures.
‡Stimulation index (SI) = mean counts/min of stimulated cultures/mean counts/min of cultures without mitogen.

Table 3. Effect of anti-BLV serum on BLV-induced syncytia formation and spontaneous ^{3}H-TdR incorporation of PBL from BLV infected cattle

Cells from animal	BLV infection*	Serum added	Antibody† P24	Antibody† gp	^{3}H-TdR uptake‡ counts/min	Virus titer§ SFU	Viability¶
9	+	No serum	—	—	49.5	3410	50.0
		10% sheep V34	320	640	3.0	24	58.0
		10% normal sheep	0	0	27.0	3576	52.0
		10% cow 7	40	160	4.9	48	54.0
		10% normal cattle	0	0	88.6	2736	56.5
7	+	No serum	—	—	176.7	1839	48.2
		10% sheep V34	320	640	2.2	12	51.9
		10% normal sheep	0	0	108.4	2213	49.3
		10% cow 7	40	160	2.0	0	54.0
		10% normal cattle	0	0	228.6	1690	49.0
4	−	No serum	—	—	3.8	0	55.1

*BLV infection was tested by presence of antibodies against BLV and BLV antigens in PBL short term culture by ID.
†Reciprocal of antibody titer of antiserum which was determined by ID.
‡Indicated as ($\times 10^3$ counts/min), mean counts/min of ^{3}H-TdR in insoluble material for triplicate cultures without mitogen.
§Virus titer was expressed as means of syncytia forming unit (SFU)/1×10^6 lymphocytes of duplicate cultures.
¶Viability was determined at end of cultures by a trypan blue exclusion test.

Table 4. Effect of antiserum absorbed with BLV on spontaneous incorporation of ^{3}H-TdR and BLV-induced syncytia formation of PBL from BLV infected animal (8)

Antiserum Added	Dilution†	^{3}H-TdR uptake Counts/min‡	^{3}H-TdR uptake % Inhibition§	Syncytia formation SFU¶	Syncytia formation % Inhibition‖
No serum	—	84.5	0	7301	0
Sheep V34 serum unabsorbed	1/300	6.6	92.2	122	98.3
	1/900	16.9	79.9	796	89.1
Sheep V34 serum absorbed* with BLV	1/300	44.9	46.8	6627	9.2
	1/900	91.8	<0	7460	<0

*Absorption was done according to the text.
†Indicated as final dilutions in the media.
‡Indicated as ($\times 10^3$ counts/min), mean counts/min of ^{3}H-TdR in insoluble material for triplicate cultures.
§% inhibition of ^{3}H-TdR uptake = 100 × (counts/min without serum − counts/min with serum)/(counts/min without serum).
¶Virus titer was expressed as means of syncytia forming unit (SFU)/1×10^6 lymphocytes of duplicate cultures.
‖% inhibition of BLV-induced syncytia formation = 100 × (SFU without serum − SFU with serum)/SFU without serum.

Effect of anti-BLV serum on BLV-induced syncytia formation and spontaneous ^{3}H-TdR incorporation of PBL from BLV infected cattle

The relation of the inhibitory effect of BLV antiserum to BLV-induced syncytia formation and ^{3}H-TdR incorporation was examined. In the presence of antiserum, spontaneous ^{3}H-TdR incorporation and formation of syncytia induced by BLV were markedly inhibited at the same time, whereas normal sheep or cattle serum did not have the inhibitory effect (Table 3). The inhibitory effect of anti-BLV serum was not caused by cytotoxic action of antiserum on lymphocytes as judged by viability of cultured lymphocytes (Table 3). Anti-BLV serum was diluted serially and examined for an inhibition of incorporation of ^{3}H-TdR and BLV-induced syncytia formation. The close pattern of two inhibition curves with diluted antiserum indicates that the same factor was responsible for inhibition of syncytia formation and incorporation of ^{3}H-TdR (Fig. 1). In addition, the inhibitory effects of antiserum on both uptake of ^{3}H-TdR and BLV-induced syncytia formation were abolished after absorption of antiserum with BLV (Table 4). Serum from a steer which contained antibody principally against gp antigens of BLV also inhibited the uptake of ^{3}H-TdR (99%) and BLV-induced syncytia formation (91%). The effect of BLV on the incorporation of ^{3}H-TdR of PBL from normal cattle was examined. BLV at various concentrations (2.0×10^2, 2.0×10^3 and 2.0×10^4 syncytia forming units/6×10^5 lymphocytes) did not affect the incorporation of ^{3}H-TdR of PBL from normal cattle (4.2×10^3 counts/min without BLV and 3.1 to 4.4×10^3 counts/min with BLV).

DISCUSSION

There was high spontaneous DNA synthesis and low SI of mitogen responses of BLV infected lymphocytes. This observation agreed with the results previously reported [9, 10]. Mitogen responses of steer 9 with elevated counts, one of the BLV infected animals were minimal. The depressed response of steer 9 might be due to the predominance of leukemic cells which were not responsive to mitogens and had B cell characteristics. BLV antiserum inhibited the spontaneous DNA

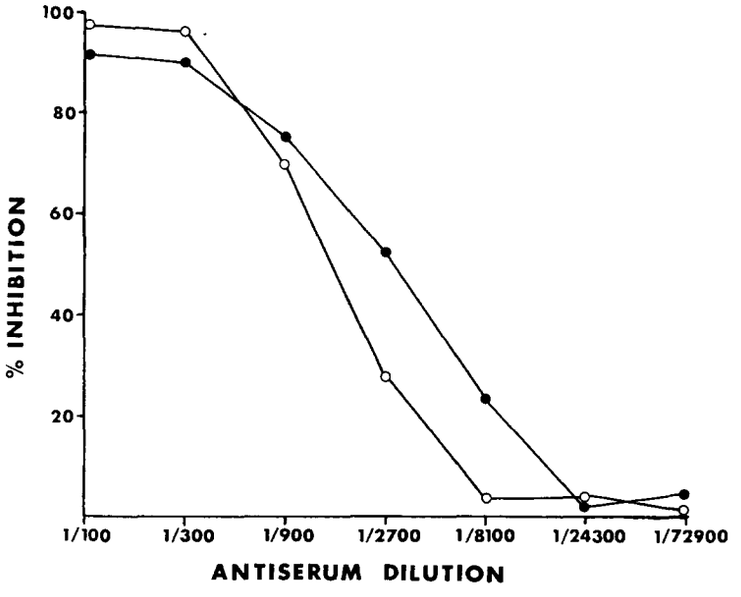

Fig. 1. Inhibition of BLV-induced syncytia formation and DNA synthesis by diluted antiserum. ○——○, *percentage of inhibition of BLV-induced syncytia formation which was calculated from mean of duplicate cultures as* 100 × (*SFU without serum* − *SFU with serum*)/*SFU without serum.* ●——●, *percentage of inhibition of DNA synthesis which was calculated from mean of triplicate cultures as* 100 × (counts/min *without serum*—counts/min *with serum*)/counts/min *without serum PBL studied was obtained from animal* 8. *Dilution of serum was expressed as final dilution in the media.*

synthesis of lymphocytes from BLV infected cattle, which resulted in a higher SI.

The inhibitory effect of antiserum on spontaneous DNA synthesis was not due to cytotoxic effect of the antiserum and/or a complement since lymphocytes at the end of culture were equally viable. The results with diluted antiserum showed a direct correlation of inhibition of DNA synthesis and BLV-induced syncytia formation. Inhibitory effects of antiserum were abolished by absorption with BLV. These results indicated that the responsible factor for the inhibitory effect of the antiserum was the antibody against BLV.

Previous reports from our laboratory demonstrated that BLV antigens were detected in the cytoplasm and on the cell membrane and BLV was released into the culture medium of BLV infected lymphocytes [5, 13, 14]. Antibody to gp of BLV inhibited virus release but antibody to P24 did not inhibit virus release [5]. It was also found that antibody to gp neutralized infectivity of BLV for cells while antibody to P24 did not [6]. BLV gp antigen is located in the virus envelope as well as on the membrane of infected cells [6]. Antigenicity of gp was shown to be ether sensitive [4]. Virus preparations used for absorption of serum in this study were not treated with ether, therefore gp remained intact in the virus envelope. These facts indicate that antibody against BLV gp may be the responsible factor for the inhibitory effect of the antiserum.

Lymphocytes from non-infected cattle had a low rate of spontaneous DNA synthesis which was not significantly altered by BLV antiserum. The high rate of spontaneous DNA synthesis in BLV infected lymphocytes was reduced by BLV antiserum and BLV-induced syncytia formation was also inhibited. With dilution of serum the amount of BLV to induce syncytia in the culture fluid paralleled closely the rate of DNA synthesis of lymphocytes from infected cattle. This might mean that intact extracellular virus in culture fluid played a role in stimulating DNA synthesis or antigen–antibody complexes might be formed and involved in the inhibitory effect of antiserum. Since BLV antigens were not detected in the culture fluid by ID test and BLV antiserum did not interfere with detection of BLV antigens [5], BLV antigen–antibody complexes were absent or present in amounts too small to be detected by ID. BLV did not stimulate DNA synthesis of PBL from uninfected animals. It was reported that only B cells produced BLV in a population of infected lymphocytes [15]. In another report B cells were found to be responsible for the spontaneous DNA synthesis and could be separated from BLV producing cells [16]. Absence of syncytia inducible BLV in cultures with antiserum might be due to inhibition of virus release from the cells and also neutralization of cell free virus, if any escaped from the virus release inhibition effect of the antiserum. The BLV antiserum was shown to react on the cell membrane expressing the BLV antigen [6]. Absence of intact BLV in the culture fluid and masking of BLV antigen on cell surface might cause reduction in DNA synthesis in lymphocytes primed by BLV *in vivo* because of a lack of antigenic stimulation by BLV.

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