Early Syncytium Formation by Bovine Leukemia Virus

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Bovine leukemia virus (BLV) from either persistently infected bat cells or fetal lamb kidney cells induced rapid syncytium formation in F81 indicator cells. Distinct syncytia were seen within 2 h after inoculation of cells with highly concentrated (500-fold) cell-free BLV preparations and within 4 to 8 h when unconcentrated cell-free BLV preparations were used. Indicator cell densities of 1×10^5 to 2×10^5 were optimal for rapid and maximal syncytium formation. Pretreatment of BLV with reference BLV leukemic serum and antiserum prepared against purified BLV significantly inhibited (95%) syncytium formation. Reference bovine viral diarrhea virus serum, foamy-like bovine syncytial virus serum, and control serum had little effect (17% inhibition). Antiserum to BLV gp51 inhibited syncytium formation by greater than 96%, whereas antiserum to BLV p24 reduced syncytium activity to a much lesser extent (38% inhibition). Treatment of BLV with β -propiolactone (0.005 to 0.05%) had little or no effect upon syncytium-forming activity, whereas UV irradiation (15 ergs/mm² per s for 30 min) reduced, but did not completely destroy, the fusion activity. However, both β -propiolactone and UV irradiation drastically reduced the replication potential of BLV, as demonstrated by the lack of p24 expression in the inoculated cells. Concentrations of cycloheximide, cytosine arabinoside, tunicamycin, and 2-deoxy-D-glucose which effectively blocked cellular macromolecular synthesis did not significantly inhibit syncytium formation. These latter results suggested that de novo protein and DNA synthesis as well as protein glycosylation were not required for early syncytium formation. Thus, these experiments demonstrated that replication of BLV by the indicator cells was not essential for cell fusion.

It has been demonstrated that bovine leukemia virus (BLV) can induce cell fusion in several nontransformed indicator cells, such as human embryonic diploid lung, bovine embryonic spleen, and bat lung cells (6), as well as in sarcoma virus-transformed F81 cells (1). Distinct syncytia were seen in nontransformed cells at 4 to 8 days post-inoculation (6) and 16 days after inoculation of the F81 cells (1), suggesting that infection of the indicator cells and replication of BLV in these cells were necessary for syncytium induction. This phenomenon is often referred to as "late" polykaryocytosis or "fusion from within," in contrast to "early" polykaryocytosis or "fusion from without" in which syncytium induction can be seen within a few hours post-inoculation (19). Early polykaryocytosis has been demonstrated with mixed cultures of BLV-producing cells and sarcoma-transformed KC, XC, and CC81 (15, 18) cells. Maximum amounts of syncytia were seen as soon as 24 h after initiation of the mixed cultures. Before the results presented in this paper, induction of early

polykaryocytosis by cell-free BLV had not been reported.

The major objective of this study was to gain a better understanding of the mechanism of syncytium induction by determining whether or not cell-free BLV in the absence of BLV-producing cells could induce early syncytia. To do this, we tested concentrated and unconcentrated cell-free preparations of BLV for the ability to induce syncytia in F81 cells early after inoculation. In addition, the effects of various metabolic inhibitors as well as β -propiolactone (BPL) and UV treatment of BLV were tested on BLV-induced syncytium formation in the F81 indicator cells. If one assumes that early polykaryocytosis is due to a structural component of intact BLV and that infection of the indicator cells is not necessary, metabolic inhibitors, such as cycloheximide, 1- β -D-arabinofuranosylcytosine (ara-C), and tunicamycin, should have no effect upon polykaryocyte formation. In addition, destruction of the infectivity of BLV with BPL and UV irradiation should have little or no effect.

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MATERIALS AND METHODS

Cells and Viruses. Persistently infected bat lung (Tb1Lu) cells, designated BLV-bat₂ (12), and fetal lamb kidney (FLK) cells, designated BLV-FLK (24), were used as the sources of BLV throughout this study. Since BLV preparations from both sources gave similar results in all the experiments presented, the term "BLV preparation" used herein refers to either source unless specified otherwise. The origin and properties of the BLV-bat₂ and BLV-FLK cells have been reported previously (12, 24). The BLV-FLK cells and the F81 feline cell line (8), which contains the murine sarcoma virus genome and was used as indicator for the syncytium assays, were kindly supplied by M. J. Van der Maaten. All cell lines were grown at 37°C in Eagle minimal essential medium supplemented with 10% heat-inactivated fetal calf serum, 100 U of penicillin, and 100 µg of streptomycin sulfate per ml (MEM). They were routinely subcultured every 4 to 5 days by dispersion with 0.05% trypsin and 0.06% EDTA and reseeded into 150-cm² flasks at a concentration of 10⁶ cells per flask. All cells were tested for mycoplasma and found negative.

Cell-free preparations of BLV were obtained by low-speed centrifugation (10,000 × *g* for 10 min) of culture fluids from infected cell lines 72 to 96 h after subculturing, followed by filtration through a 0.45-µm Nalgene filter. Concentrated (500-fold) BLV preparations were prepared initially by concentrating 2,000 ml of cell-free culture fluid to 200 ml with an Amicon concentrator (DC2, hollow fiber H1P100). BLV was pelleted from the concentrated culture fluid by ultracentrifugation (110,000 × *g* for 60 min) and then resuspended in 4 ml of MEM, giving a final 500-fold-concentrated preparation of BLV.

Sera. The bovine sera used in this study were serum 1001, from a normal control animal; serum 4000, from a confirmed case of lymphosarcoma; 3892, from an animal negative for BLV antibodies but positive for antibodies to the widespread foamy-like bovine syncytial virus (BSV); and serum 7767, from an animal positive for antibodies to bovine viral diarrhea virus (BVDV) but negative for antibodies to BLV. Serum 4000 had antibodies to the internal and envelope antigens of BLV but was negative for antibodies to the BSV. These sera were kindly supplied by M. J. Van der Maaten and C. Olson. Antisera to the major internal protein (p24) and the major envelope glycoprotein (gp51) were prepared in rabbits and obtained from A. Burny (11). Radioimmunoassay titers for anti-p24 and anti-gp51, using 1-ng amounts of the respective ¹²⁵I-labeled antigens, were 1:200,000 and 1:20,000, respectively.

Syncytium assays. (i) Direct viral assay. F81 indicator cells were seeded in multiwell cell culture plates (24 wells per plate) at a concentration of 1 × 10⁵ to 2 × 10⁵ cells per well and incubated at 37°C for 20 to 24 h. Growth medium was then removed, and

the cells were treated with 25 µg of DEAE-dextran per ml, using 0.5 ml per well. After 60 min at 37°C, the DEAE-dextran was removed, the cells were inoculated with 0.25 ml of the BLV preparations, and the cultures were incubated at 37°C for 60 min. After adsorption, 0.25 ml of growth medium was added back to each cell culture well. At appropriate times specified herein, the cells were washed with phosphate-buffered saline fixed with absolute methanol, and stained with a 5% Giemsa solution. Multinucleate cells containing five or more nuclei were scored as syncytia. To minimize counting errors, twenty randomly selected fields (1 mm²) were counted in duplicate wells, and the mean number of syncytia per 2.1 cm² was calculated.

The density of cells used for the syncytium assay was found to play a critical role in obtaining maximal syncytium formation. The optimal concentration of cells was found to be between 1 × 10⁵ to 2 × 10⁵ cells per well, when the cell monolayer was nearly confluent. With lower cell densities, fewer syncytia with three to four nuclei were observed. At higher cell densities (4 × 10⁵ or greater), when the monolayers were confluent, a two- to fourfold decrease in the number of syncytia was usually seen.

(ii) Syncytium inhibition assay. Cell-free preparations of BLV were mixed with equal volumes of sera (heat inactivated at 56°C for 30 min) that had been diluted 1:5 or MEM and incubated at 37°C for 30 min followed by incubation at room temperature for 30 min. Samples (0.5 ml) of the mixtures were then added to duplicate wells of indicator cells that had been previously treated with DEAE-dextran. The cells were fixed and stained, and the numbers of multinucleate cells were determined 8 h after the cells were inoculated with the virus-serum mixtures. The percentage of syncytium inhibition for each serum was determined by dividing the number of syncytia seen in virus-serum-inoculated cells by the number of syncytia seen in virus-MEM-inoculated cells.

UV irradiation of BLV. Samples (0.5 ml) of cell-free BLV preparations (concentrated and unconcentrated) were placed into sterile plastic petri dishes and exposed to UV light (Mineralight; Ultra-Violet Products, Inc., South Pasadena, Calif.) for 10, 20, and 30 min at an incident dose rate of 15 ergs/mm² per s. The irradiated BLV preparations and an unirradiated preparation of BLV were then tested on F81 cells for syncytium-forming ability as described above.

BPL treatment. Cell-free preparations of concentrated and unconcentrated BLV were incubated overnight at 4°C with concentrations of BPL ranging from 0.005 to 0.05%, using a method similar to that described by Wainberg et al. (25) for inactivating Sendai virus. Briefly, a freshly prepared 10% solution of BPL was diluted in serum-free MEM to 10 times the desired final BPL concentration. One milliliter of this 10-fold-concentrated BPL solution was then added to 9 ml of cell-free BLV preparation, and the mixture was shaken at 4°C for 10 min and placed onto a shaker in a 37°C incubator and shaken for another 2 h. As a control, a BLV preparation was handled in an identical manner, but without BPL treatment. After overnight incubation of the mixtures at 4°C, they were tested for syncytium-inducing ability as described above.

Immunofluorescence. Our indirect immuno-

fluorescence procedure was a modification of that used by Diglio and Ferrer (6). In brief, inoculated and uninoculated F81 cells were treated with trypsin-EDTA, washed once with phosphate-buffered saline, and adjusted to a concentration of 5×10^5 cells per ml, and 0.05 ml was placed onto a glass slide, air dried, and then fixed with acetone for 10 min. Fixed cells were then incubated with rabbit anti-p24 antiserum (diluted 1:10 in phosphate-buffered saline) for 30 min at 37°C. After this incubation, the cells were washed in phosphate-buffered saline, and fluorescein-conjugated goat anti-rabbit immunoglobulin G diluted 1:10 in phosphate-buffered saline was added. After incubation for a further 30 min at 37°C, the cells were washed with phosphate-buffered saline, a drop of glycerin diluted 1:1 in phosphate-buffered saline was placed on the cells, and the cells were covered with a glass cover slip and immediately examined with a Leitz fluorescence microscope. The fluorescein-conjugated goat anti-rabbit immunoglobulin G was absorbed with feline liver powder and packed F81 cells and showed no reactivity with the F81 indicator cells after absorption. Persistently infected BLV-bat₂ or BLV-FLK cells showed bright cytoplasmic fluorescence, but no nuclear fluorescence.

Evaluation of the effects of metabolic inhibitors on syncytium induction (i) Chemicals. Cycloheximide, ara-C, and 2-deoxy-D-glucose were purchased from Sigma Chemical Co. (St. Louis, Mo.). Tunicamycin was a generous gift of R. L. Hamill, Eli Lilly Laboratories, Indianapolis, Ind. [³H]thymidine (73 Ci/mmol) and D-[1,6-³H]glucosamine hydrochloride (39.6 Ci/mmol) were obtained from New England Nuclear Corp. (Boston, Mass.).

(ii) Treatment with metabolic inhibitors. Each of four wells of the F81 indicator cells was treated with 0.25 ml of cycloheximide (10 µg/ml), ara-C (10 µg/ml), tunicamycin (5 µg/ml), or 2-deoxy-D-glucose (1.64 mg/ml) at -60 min (1 h before virus inoculation) and at zero time (the time at which cells were inoculated with virus). Two of the four wells of cells treated with inhibitors at -60 min and at zero time were inoculated with 0.25 ml of a concentrated virus preparation, which remained on the cells along with the inhibitors throughout the experiment (8 h). These cells, along with duplicate wells of cells inoculated with virus only, were stained in order to determine syncytium formation. To the remaining one-half of the cells that were treated with inhibitors, radioactive precursors were added as described below. In addition, duplicate wells of F81 cells were treated with radioactive precursors only in order to determine the extent of inhibition of macromolecular synthesis.

(iii) Measurement of macromolecular synthesis. Protein and DNA synthesis as well as protein glycosylation were measured by incorporation of radioactive precursors into trichloroacetic acid-insoluble material. In brief, cells were incubated with 10 µCi of ¹⁴C-amino acids, [methyl-³H]thymidine, or D-[1,6-³H]glucosamine hydrochloride per ml in the presence or absence of inhibitors. The radioactive precursors were added at the same time that the cells were inoculated with BLV and remained on the cells until the end of the experiment (8 h). After this time, the cells were washed twice with phosphate-buffered sa-

line, disrupted with 1% sodium dodecyl sulfate, and then precipitated with 10% trichloroacetic acid at 4°C overnight. Precipitates were collected on 0.45-µm membrane filters (Millipore Corp., Bedford, Mass.) and the amount of insoluble radioactivity was determined by placing the filters in a toluene-based scintillation cocktail and counted in a Beckman liquid scintillation spectrometer.

RESULTS

Kinetics of syncytium formation with unconcentrated and concentrated BLV preparations. In our initial studies, inoculation of DEAE-dextran-treated F81 cells with either an unconcentrated or a concentrated (500-fold) BLV preparation from either BLV-bat₂ or BLV-FLK cells resulted in syncytium formation. Discrete syncytia were seen by 4 to 8 h post-inoculation, earlier than had been reported previously for cell-free preparations of BLV (1, 7, 15). To determine the rate of syncytium formation, F81 cells were seeded into multiwell culture plates and treated with DEAE-dextran as described in Materials and Methods and then inoculated with unconcentrated or concentrated BLV preparations. The temporal progression of syncytium formation was followed by staining duplicate samples at 2-h intervals for 24 h. Results from these experiments are shown in Fig. 1. When concentrated BLV preparations were used, the majority of the cells were involved in cell fusion by 2 h post-inoculation and developed into large, discrete syncytia within 24 h. Using unconcentrated cell-free preparations of BLV, fused cells containing two to four nuclei were relatively abundant within 2 h after inoculation (results not shown). The number and size of syncytia increased rapidly with time, and by 4 h a majority of the syncytia contained between five and eight nuclei (Fig. 1). By 8 h, most cells were involved in syncytium formation and the individual syncytia contained between 10 and 20 nuclei. By 24 h post-inoculation, large syncytia could be seen which contained up to 30 to 50 nuclei. No significant increase in the size of the syncytia was observed after 24 h. When normal TblLu cells were inoculated with cell-free preparations of BLV, essentially the same course of syncytium formation was observed. Evidence of cells fusing containing three to four nuclei was seen within 2 to 4 h after inoculation. As with the F81 cells, the number of nuclei per syncytium increased with time until it remained essentially unchanged after 24 h. These results suggested that BLV induced early syncytia by the process of fusion from without, since syncytia were seen as early as 2 to 4 h after inoculation with BLV, earlier than one would expect if BLV infection and replication were necessary for cell fusion.

FIG. 1. BLV-induced syncytium formation in F81 cells. (A) Uninoculated F81 cells (B) F81 cells inoculated with a concentrated preparation of BLV from culture fluid of BLV-FLK cells and incubated for 2 h at 37°C. (C through D) F81 cells inoculated with unconcentrated cell-free preparations of culture fluid from BLV-FLK cells and incubated for 4 (C), 8 (D), and 24 (E) h. All cells were fixed in methanol and stained with 5% Giemsa. Bar = 100 μ m.

Specificity of cell fusion activity by BLV. To confirm that BLV, and not some other adventitious agent, was responsible for syncytium formation, inhibition studies with well-defined reference and control sera were conducted. These experiments were of special importance, since the BLV-FLK cells are also infected with

BVDV (M. J. Van der Maaten, personal communication) and cell-free BLV preparations from this cell line were used for several experiments reported in this study. Reference BLV serum 4000 significantly inhibited (99%) the ability of the preparations from the BLV-FLK cells to form syncytia on the F81 cells, whereas the

control serum 1001, which was negative for antibodies to BLV, BVDV, and BSV, had no significant effect (13% inhibition) (Table 1). Reference sera 7826 and 3892, which had high titers of antibodies to BVDV and BSV, respectively, did not significantly inhibit syncytium formation (17 and 15% respectively).

Sera prepared against purified BLV that had been disrupted by freezing and thawing four times inhibited (95%) syncytium formation quite effectively. These results implied that the syncytium formation in F81 cells by cell-free preparations from BLV-FLK cells was due to BLV, and not BVDV or BSV. Also included in Table 1 are data showing that rabbit antiserum to the major glycoprotein, gp51, showed significant inhibition (96%) of syncytium formation, whereas rabbit antiserum to the major internal protein, p24, inhibited syncytium formation to a much lesser extent (38%). These data suggest that gp51, an external component of BLV, may play a major role in syncytium induction. Experiments with cell-free preparations of BLV from BLV-bat₂ cells were conducted, and similar results were obtained (data not shown).

Effects of BPL and UV irradiation on the cell fusion activity of BLV. To further demonstrate that BLV could induce early syncytia, or cell fusion from without, unconcentrated and concentrated preparations of BLV were treated with various concentrations of BPL or exposed

to UV irradiation for different lengths of time and then tested for syncytium-forming activity. Results from these experiments are shown in Table 2. Treatment of BLV with concentrations of BPL which have been shown to inactivate other syncytium-inducing viruses (20, 25) appeared to have no effect upon syncytium induction by BLV when compared with untreated control cells. UV irradiation reduced the fusion activity of concentrated preparations of BLV, depending upon time of exposure: after 10 min of irradiation, the number of syncytia per 2.1-cm² well was still 93% of the control value; after 20 min, it was 53%; and after 30 min, it was 41%. When unconcentrated cell-free preparations of BLV were treated with UV irradiation, the syncytium-forming activity was decreased quite drastically, and after 30 min of irradiation, over 80% of the activity was lost. However, the syncytium-inducing activity of BLV was not entirely eliminated, even after 60 min of UV treatment, with about 15% of the syncytium-inducing activity retained (data not shown).

To determine whether or not the replicating potential of BLV was destroyed after BPL treatment or UV irradiation, immunofluorescence studies were conducted on F81 cells that had been inoculated with untreated concentrated preparations of BLV and concentrated BLV preparations treated with various amounts of BPL and different times of exposure to UV irradiation. Cells inoculated with BPL-treated BLV showed no cytoplasmic fluorescence at any time period tested (Table 3), indicating that p24

TABLE 1. *Specificity of syncytium formation^a*

Treatment	Avg no. ^b of syncytia/2.1 cm ²	Inhibition (%) ^c
Reference BLV serum 4000	15	99
Reference BSV serum 3892	2,060	15
Reference BVDV serum 7767	1,980	17
Control negative serum 1001	2,080	13
Antiserum to gp 51	84	96
Antiserum to purified BLV	105	95
Antiserum to p 24	1,488	38
None	2,380	0

^a A 2-ml sample of a cell-free preparation of unconcentrated BLV obtained from the culture fluid of 3-day-old BLV-FLK cells was mixed with an equal volume of each serum or antiserum, which had been diluted 1:5. As a control, 2 ml of the virus preparation was added to an equal volume of MEM. The mixtures were incubated, and samples (0.5 ml) of each were inoculated onto duplicate subconfluent monolayers of F81 cells as described in the text. After 8 h, the cells were fixed and stained, and the numbers of syncytia were counted.

^b Twenty randomly selected 1-mm² fields were observed, and the numbers of syncytia per 2.1 cm² were determined.

^c Percent syncytium inhibition compared with the MEM-virus control.

TABLE 2. *Effects of BPL and UV irradiation on syncytium formation*

Treatment	No. of syncytia/2.1-cm ² well ^a	
	Concentrated BLV	Unconcentrated BLV
BPL:		
0.05%	>2,500	>2,500
0.01%	>2,500	>2,500
0.005%	>2,500	>2,500
UV irradiation:		
10 min	2,320 (93)	1,900 (76)
20 min	1,320 (53)	680 (27)
30 min	1,025 (41)	470 (19)
None	>2,500	>2,500

^a Concentrated or unconcentrated preparations of BLV were treated with various concentrations of BPL or exposed to UV irradiation for different time periods as described in the text. F81 cells were inoculated with 0.5 ml of treated virus samples, incubated at 37°C for 8 h, and then fixed and stained, and the numbers of syncytia were counted. Numbers in parentheses are percentages of syncytia compared with nontreated control cells.

TABLE 3. *Effects of BPL and UV irradiation on expression of BLV p24 as detected by immunofluorescence^a*

Treatment	Fluorescence ^b at:	
	8 h post-inoculation	24 h post-inoculation
BPL:		
0.05%	—	—
0.01%	—	—
0.005%	—	—
UV irradiation:		
10 min	—	+
20 min	—	—
30 min	—	—
None	+	+

^a Concentrated preparations of BLV were treated with BPL and UV irradiation and added to indicator cells. These cells were tested for expression of p24 as described in the text.

^b Presence (+) or absence (—) of fluorescence.

expression was not taking place in these cells. Cells inoculated with BLV that had been exposed to UV irradiation (15 ergs/mm² per s) for 20 and 30 min also showed no viral antigen expression at any of the time periods tested. However, cells that were inoculated with BLV that had been exposed to UV irradiation for only 10 min showed weak cytoplasmic fluorescence at 24 h, suggesting that 10-min exposure to UV irradiation was probably not sufficient to destroy all the replicating potential of BLV. No fluorescence was seen in cells inoculated with unconcentrated preparations of BLV that had been treated with either BPL or UV irradiation. Bright fluorescence in both individual and fused cells was seen at 8 and 24 h in F81 cells that had been inoculated with untreated concentrated and unconcentrated BLV preparations, respectively.

Thus, these results suggested that cell fusion and virus replication (expression of BLV p24) were independent events, since BPL treatment and UV irradiation of BLV resulted in a loss of BLV antigen expression, but not a loss of induction of cell fusion.

Effects of metabolic inhibitors on the fusion of F81 cells by BLV. The ability of BLV to induce early syncytium formation in F81 indicator cells was demonstrated. However, to further elucidate the requirements necessary for BLV-induced early cell fusion, experiments were set up with various inhibitors of macromolecular synthesis. F81 cells were treated with ara-C (10 µg/ml), cycloheximide (10 µg/ml), tunicamycin (5 µg/ml), 2-deoxy-D-glucose (1.64 mg/ml), or no inhibitor for 1 h before inoculation or at the same time that cells were inoculated with BLV

and remained on the cells throughout the 8 h of incubation (Table 4). Cycloheximide and ara-C had little or no effect upon syncytium formation by BLV (Table 4). ara-C, an inhibitor of DNA synthesis which has been shown to block the replication of retroviruses by inhibiting the synthesis of proviral DNA (4, 22), significantly inhibited (96% inhibition) cellular DNA synthesis, but inhibited syncytium formation by less than 5%. Furthermore, cycloheximide at a concentration of 10 µg/ml had a drastic effect upon protein synthesis (93% inhibition), but no significant inhibition was observed in syncytium formation (<5%). Thus, early cell fusion by BLV did not appear to require *de novo* protein or DNA synthesis, which can be inhibited by cycloheximide or ara-C (Table 4). Recognizing the fact that specific glycoprotein synthesis or protein glycosylation may have played a role in cell fusion, as has been demonstrated for other systems (10, 21), two inhibitors of protein glycosylation were included in the metabolic inhibitor experiments (Table 4). Both tunicamycin and 2-deoxy-D-glucose reduced glycoprotein synthesis by greater than 68 and 80%, respectively, but had only marginal effects on syncytium formation. These

TABLE 4. *Effects of metabolic inhibitors on syncytium formation and macromolecular synthesis in F81 cells^a*

Treatment	Time of treatment (min)	Inhibition (%)	
		Syncytia ^b	Macromolecular synthesis ^c
Cycloheximide (10 µg/ml)	—60	<5	96
	0	<5	92
ara-C (10 µg/ml)	—60	<5	93
	0	<5	96
Tunicamycin (5 µg/ml)	—60	<5	71
	0	<5	68
2-Deoxy-D-glucose (1.64 mg/ml)	—60	<15	87
	0	<15	80

^a Duplicate samples of F81 cells were inoculated with concentrated preparations of BLV and treated with various inhibitors as described in the text. These experiments were repeated three times, and similar results were obtained.

^b Percentage of syncytia inhibited in treated cells as compared with nontreated control cells.

^c Inhibition by cycloheximide, ara-C, tunicamycin, or 2-deoxy-D-glucose of the incorporation of ¹⁴C-amino acids, [methyl-³H]thymidine, or D-[1,6-³H]glucosamine hydrochloride into trichloroacetic acid-precipitable material as compared with mock-treated control cells.

data, together with the other results presented, suggested that replication of BLV by the indicator cells was not required for early syncytium formation.

DISCUSSION

The results from our studies demonstrate that concentrated cell-free preparations of BLV can induce early syncytium formation in F81 cells. Such rapid virus-induced cell fusion is considered fusion from without, in contrast to fusion from within (19). This conclusion is suggested by: (i) the very rapid appearance of syncytia, within 2 to 4 h after inoculation of F81 cells; (ii) the ability of BPL-treated and UV-irradiated BLV preparations to retain cell-fusing activity; and (iii) the lack of a requirement for protein and DNA synthesis as well as protein glycosylation for syncytium formation to occur. Thus, it seems that the replication of BLV is not essential for cell fusion and that the phenomenon may be considered fusion from without.

The specificity of the cell fusion induced by cell-free preparations of BLV-bat lung and BLV-FLK cell cultures was studied thoroughly. Culture fluids from normal bat₂ cells and FLK cells were assayed for syncytium-inducing ability and found negative. Since the BLV-FLK cells have been shown to be infected with BVDV (Van der Maaten, personal communication), there was concern that this agent or another adventitious agent, such as BSV, may be responsible for syncytium induction. In experiments with specific antisera to these extraneous viruses, no significant inhibition of syncytium formation was observed (Table 1). Even though the BLV-bat₂ cells have been shown to be free of these agents (12), culture fluids from these cells were tested along with the culture fluids from the BLV-FLK cells in the inhibition studies, and similar results were obtained. These results argue against the possibility of BSV and BVDV playing a role in syncytium induction. On the other hand, inhibition of cell fusion by pretreatment of BLV preparations with virus-specific antiserum, either BLV reference serum from a leukemic animal or antiserum prepared against purified BLV, strongly suggests that BLV plays a major role in mediating cell fusion.

Another approach to demonstrate that BLV was the mediator of cell fusion was to use density gradient-purified virus. However, in preliminary studies with sucrose density gradient-purified virus, very little fusion activity was recovered, whereas the original culture fluid and pelletized virus had high levels of early fusion activity (data not shown). Results from these preliminary studies suggest the possibility that a viral

component(s), probably viral spikes or some other external component, is removed during the purification procedure and that this component is necessary for the cell fusion activity. This assumption is further strengthened by the fact that antiserum prepared against the major glycoprotein of BLV significantly inhibits syncytium formation (see below).

The reason why other researchers (1, 7, 15) were unable to obtain early syncytium formation, within 4 to 8 h after inoculation, when using cell-free preparations of BLV is not fully known. However, there are several possibilities that could account for this discrepancy. First, the source of indicator cells used in the syncytium assay probably plays a major role in determining whether or not early syncytium formation is induced. Experiments in our laboratory have shown that CC-81 cells (S+L- feline cells) obtained from P. J. Fischinger (National Cancer Institute, Bethesda, Md.) were quite resistant to syncytium formation, whereas F81 cells (S+L- feline cells) obtained from M. J. Van der Maaten were very susceptible to syncytium induction (unpublished data; a similar observation has been made by Van der Maaten [personal communication]). Ferrer and Cabradilla (7) and Irgens et al. (15) used CC-81 cells, which could be the reason why these researchers did not obtain early syncytium formation with cell-free preparations of BLV. This assumption is further strengthened by the fact that Jerabek et al. (16) noticed that the CC-81 cell cultures contained few cells that were susceptible to BLV infection and thus were capable of becoming syncytium-inducing (effector) cells. Assuming that CC-81 and F81 cells were originally identical, as established by Fischinger et al. (8, 9), the reason for the variation in syncytium-inducing ability is unknown. It is possible that through laboratory manipulations, such as growing and subculturing the cells under different conditions, as in the case of F81 cells, cells susceptible to BLV syncytium induction were cloned, whereas in the case of CC-81 cells, cells resistant to BLV syncytium activity were cloned.

A second possibility is that the density of indicator cells used in the syncytium assays is critical for obtaining optimal early syncytium formation. Results from experiments performed in our laboratory show that a cell density of 1×10^5 to 2×10^5 cells per 2.1 cm^2 is optimal for obtaining a maximum syncytium response. Cell densities lower or higher than 1×10^5 to 2×10^5 cells per 2.1 cm^2 result in a relatively drastic decrease in the number of syncytia observed. In fact, when cell densities of 4×10^5 cells per 2.1 cm^2 or greater are used, discrete syncytia are very difficult to observe even after 24 to 48 h

(unpublished data).

A third possible reason, and maybe a more trivial explanation, why certain researchers did not observe early syncytium formation is the lack of pretreatment of indicator cells with either DEAE-dextran or Polybrene. Pretreatment of cells with either DEAE-dextran (25 $\mu\text{g}/\text{ml}$) or Polybrene (4 $\mu\text{g}/\text{ml}$) enhanced the early syncytium response by at least fivefold. Similar results have been observed for other virus-induced cell fusion systems (4, 14, 23, 26, 27).

Preparations of BLV that were UV irradiated (15 ergs/mm^2 per s) for 20 to 30 min were found to possess significant fusion activity (>19% of the fusion activity of non-UV-irradiated BLV) for F81 cells. These results imply that a functional viral genome is not required for early syncytium formation. Thus, assuming that BLV induces fusion from without, the question arises of why UV irradiation of unconcentrated BLV reduced cell fusion activity by 70 to 80% as compared with non-UV-irradiated BLV. One possible explanation is that UV irradiation had denaturing effects upon some component of BLV, perhaps a protein, necessary for cell fusion (2). More convincing evidence showing that non-infectious and nonreplicating BLV is capable of inducing early syncytia was presented by using metabolic inhibitors. These studies conclusively illustrated that concentrations of cycloheximide (10 $\mu\text{g}/\text{ml}$), ara-C (10 $\mu\text{g}/\text{ml}$), tunicamycin (5 $\mu\text{g}/\text{ml}$), and 2-deoxy-D-glucose (1.64 mg/ml) that significantly inhibited cellular macromolecular synthesis had little or no effect upon BLV-induced cell fusion. These results further support the hypothesis that BLV can induce fusion from without.

The exact nature of the factor or factors responsible for BLV-induced cell fusion in F81 cells is unknown. However, (i) the ability of UV-irradiated and BPL-treated BLV to induce syncytia in F81 cells and (ii) the fact that metabolic inhibitors had little or no effect upon syncytium formation are consistent with the hypothesis that early BLV-induced fusion activity may be associated with a BLV virion component(s). Furthermore, since antiserum prepared against purified BLV particles and against the major glycoprotein of BLV neutralize the syncytium-forming ability of BLV, it is likely that external proteins, possibly the viral spikes, play a major role in cell fusion, as in the case of other lipoprotein-enveloped viruses (3, 20, 27).

In conclusion, the results presented in this paper clearly demonstrate that cell-free preparations of BLV can induce early syncytium formation in F81 cells, a phenomenon referred to as fusion from without (19). On the other hand,

several investigators (1, 6) have reported that BLV induces syncytia by a mechanism that requires infectious virus and corresponds to a phenomenon known as late polykaryocytosis, or fusion from within. It seems feasible, therefore, that BLV, like other enveloped viruses (3, 17, 27), can induce cell fusion by both mechanisms.

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LITERATURE CITED

- Benton, C. V., A. E. Soria, and R. V. Gilden. 1978. Direct syncytial assay for the quantitation of bovine leukemia virus. *Infect. Immun.* **20**:307-309.
- Bister, K., H. E. Varmus, E. Stavneger, E. Hunter, and P. K. Vogt. 1977. Biological and biochemical studies on the inactivation of avian oncoviruses by ultraviolet irradiation. *Virology* **77**:689-704.
- Bratt, M. A., and W. R. Gallaheer. 1969. Preliminary analysis of the requirements for fusion from without by Newcastle disease virus. *Proc. Natl. Acad. Sci. U.S.A.* **64**:536-543.
- Chatterjee, S., and E. Hunter. 1979. The characterization of Mason-Pfizer monkey virus-induced cell fusion. *Virology* **95**:421-433.
- Damsky, C. H., A. Levy-Benshimol, C. A. Buck, and L. Warren. 1979. Effect of tunicamycin on the synthesis, intracellular transport and shedding of membrane glycoproteins in BHK cells. *Exp. Cell Res.* **119**:1-13.
- Diglio, C. A., and J. F. Ferrer. 1976. Induction of syncytia by the bovine C-type leukemia virus. *Cancer Res.* **36**:1056-1067.
- Ferrer, J. F., and C. D. Cabradilla. 1978. The phenomenon of polykaryocytosis induced by BLV in mixed cultures: specificity, mechanisms and application to the diagnosis of BLV infection in cattle. *Ann. Rech. Vet.* **9**:721-728.
- Fischinger, P. J., C. S. Blevins, and S. Nomura. 1974. Simple, quantitative assay for both xenotropic murine leukemia and ecotropic feline leukemia viruses. *J. Virol.* **14**:177-179.
- Fischinger, P. J., P. T. Peebles, S. Nomura, and D. K. Haapala. 1973. Isolation of an RD-114-like oncornavirus from a cat cell line. *J. Virol.* **11**:978-985.
- Gallaheer, W. R., D. B. Levitan, and H. A. Blough. 1973. Effect of 2-deoxy-D-glucose on cell fusion induced by Newcastle disease and herpes simplex viruses. *Virology* **55**:193-201.
- Ghysdael, J., R. Kettmann, and A. Burny. 1979. Translation of bovine leukemia virus virion RNAs in heterologous protein-synthesizing systems. *J. Virol.* **29**:1087-1098.
- Graves, D. C., and J. F. Ferrer. 1976. In vitro transmission and propagation of the bovine leukemia virus in monolayer cell cultures. *Cancer Res.* **36**:4152-4159.
- Guillemain, R., R. Mamoun, D. Levy, T. Aster, K. Igrens, and A. A. Parodi. 1978. Early polykaryocytosis inhibition: a simple quantitative *in vitro* assay for the detection of bovine leukemia virus infection in cattle. *Eur. J. Cancer* **14**:811-827.
- Hirschman, S. Z., and V. Funke. 1975. Effect of diethylaminoethyl-dextran on the replication of a murine sarcoma (Moloney)-leukemia virus complex in mouse embryo cultures. *Proc. Soc. Exp. Biol. Med.* **148**:527-531.
- Irgens, K., C. Pinelli, B. Guillemain, D. Levy, and A.

- L. Parodi. 1977. Early syncytium formation induced by bovine leukemia virus in mixed cultures. *Biomedicine* 27:49-50.
16. Jerabek, L., P. Gupta, and J. F. Ferrer. 1978. An infectivity assay for the bovine leukemia virus based on the induction of the major internal virion antigen on susceptible cell cultures. *Ann. Rech. Vet.* 9:729-734.
17. Ogura, H. 1976. XC cell fusion by murine leukemia viruses: fusion from without. *Med. Microbiol. Immunol.* 162:175-181.
18. Ogura, H., J. Paulsen, and H. Bauer. 1977. Cross neutralization of ovine and bovine C-type leukemia virus-induced syncytia formation. *Cancer Res.* 37:1486-1489.
19. Poste, G. 1972. Mechanisms of virus-induced cell fusion. *Int. Rev. Cytol.* 33:157-252.
20. Scheid, A., and P. W. Choppin. 1974. Identification of biological activities of paramyxovirus glycoprotein. Activation of cell fusion, hemolysis, and infectivity by proteolytic cleavage of an inactive precursor protein of Sendai virus. *Virology* 57:475-490.
21. Takehara, M. 1979. Effect of certain inhibitors of glycoprotein synthesis on cell fusion induced by vesicular stomatitis virus. *Microbiol. Immunol.* 23:167-176.
22. Taylor, J. M., D. E. Garfin, W. E. Levinson, J. M. Bishop, and H. M. Goodman. 1974. Tumor virus ribonucleic acid directed deoxyribonucleic acid synthesis: nucleotide sequence at the 5' terminus of nascent deoxyribonucleic acid. *Biochemistry* 13:3159-3163.
23. Toyoshima, K., and P. K. Vogt. 1969. Enhancement and inhibition of avian sarcoma viruses by polycations and polyanions. *Virology* 38:414-426.
24. Van der Maaten, M. H., and J. M. Miller. 1976. Replication of bovine leukemia virus in monolayer cell cultures. *Bibl. Haematol.* 43:360-362.
25. Wainberg, M. A., R. N. Hjorth, and C. Howe. 1971. Effect of β -propiolactone on Sendai virus. *Appl. Microbiol.* 22:618-621.
26. Wong, P. K. Y., P. H. Yuen, and S. J. Kaufman. 1977. Induction of syncytia by Moloney murine leukemia virus in myoblasts defective in differentiation. *J. Virol.* 21:319-327.
27. Zarling, D. A., and I. Keshet. 1979. Fusion activity of virions of murine leukemia virus. *Virology* 95:185-196.