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Persistent Infection of Rabbits with Bovine Leukemia Virus Associated with Development of Immune Dysfunction

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Bovine leukemia virus (BLV) infection of rabbits provides a safe and relatively inexpensive in vivo mammalian system for the study of the mechanisms controlling expression of a unique group of lymphotropic retroviruses. This group of viruses, which includes C-type human T-lymphotropic virus types I and II and lentiviruslike human immunodeficiency virus type 1, possesses genes coding for "*trans*-activating" products. Rabbits experimentally inoculated with BLV became persistently infected, as demonstrated by a number of tests. All BLV-inoculated rabbits developed persistent serum antibody to BLV. Furthermore, all BLV-inoculated rabbits had peripheral blood mononuclear cells which, when stimulated, expressed the virus, as demonstrated by viral induction of syncytium formation in a BLV-susceptible fibroblast line. The presence of BLV in circulating cells was confirmed by using peripheral blood mononuclear cells from randomly selected BLV-inoculated rabbits, which showed the presence of viral reverse transcriptase activity, BLV transcriptional activity, or BLV proviral DNA. Additional tests showed that infected lymphocytes maintained in culture with recombinant human interleukin-2 formed multinucleated giant cells and produced virus when incubated in cytokine-containing medium. BLV-infected rabbits also showed alterations in several parameters associated with immunity, beginning 6 months after inoculation. Thirty-eight percent of infected rabbits developed abnormally low T-cell responses, as measured by phytolectin stimulation, and T-cell responses cycled between normal and abnormally low over a period of 20 to 24 months. Forty-four percent of rabbits infected for longer than 12 months suffered from recurrent conjunctivitis and rhinitis. By 24 months postinoculation, 28% of infected rabbits were dead or were killed because of poor clinical condition.

Bovine leukemia virus (BLV) is a type C lymphotropic retrovirus which is associated with a disease complex termed enzootic bovine leukosis, often characterized by persistent lymphocytosis and development of B-cell lymphomas after irregular, long incubation periods (3, 5, 15). The proviral genome structure of BLV is similar to that of human T-lymphotropic virus type I (HTLV-I), the causal agent of adult T-cell leukemia/lymphoma, and human T-lymphotropic virus type II (HTLV-II), the causal agent of a T-cell variant of hairy cell leukemia (41). Although more distantly related to the lymphotropic lentivirus human immunodeficiency virus type 1 (HIV-1), the causal agent of acquired immunodeficiency disease, the BLV genome has an important feature in common with the HTLVs and HIV. That is, BLV, HTLV-I, HTLV-II, and HIV-1 all have a common structural organization in that they each possess a gene sequence that codes for a "*trans*-regulatory" protein that activates viral gene expression (8, 23, 31).

Previously, BLV had been reported to infect a number of small animal species, including cats, dogs, and rabbits. However, infection in these animals had only been defined by the continual presence of anti-BLV antibody (2, 32, 36). Thus, until this report, convincing evidence of persistent BLV infection in terms of the presence of the virus or viral genes in rabbits had not been demonstrated. We used a variety of techniques to demonstrate the persistence of the BLV genome in rabbits infected for up to 18 months. Additionally, we demonstrated that within 6 months of BLV inoculation, about one-third of the rabbits had decreased T-cell responsiveness to phytolectin stimulation, and we observed that two-thirds of BLV-infected rabbits exhibited indications of clinical disease, including conjunctivitis, rhinitis, and severe weight loss, or died unexpectedly.

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MATERIALS AND METHODS

Animals. New Zealand White rabbits (male and female), 2 to 4 months old, were purchased from a U.S. Department of Agriculture-approved rabbitry and maintained at Washington State University, which is fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

Cell separation. Peripheral blood mononuclear cells (PBMC) from BLV-inoculated and control rabbits were separated from heparinized whole blood by Ficoll-Hypaque sedimentation (density, 1.09 g/ml) as described before (40).

Inoculation with BLV. Over a period of a year, groups of two to six young adult rabbits were inoculated with BLV or other antigens. Twelve rabbits received two inoculations of approximately 5×10^6 phytolectin-stimulated PBMC from a BLV-seropositive cow with persistent lymphocytosis. Four rabbits received one inoculation of approximately 5×10^6 cells from a BLV-producing fetal lamb kidney fibroblast line (BLV-FLK), and tested negative for serum antibody to bovine viral diarrhea virus (a potential contaminant of BLV-FLK cultures) 2 to 4 weeks later. Two rabbits received three separate inoculations of cell-free BLV (400 ml of supernatant from BLV-FLK cultures concentrated 100-fold; 0.5 ml given per injection) over an 8-week period. Electron microscopy of both BLV-FLK cells and virus purified from those cells confirmed the presence of type C virus particles. Two

control rabbits received 5×10^6 phytolectin-stimulated PBMC from a BLV-seronegative cow, and 11 additional control rabbits were given antigens other than BLV ($tRNA^{Ala}$ linked to bovine serum albumin [one rabbit], gluteraldehyde-treated STa exotoxin from *Escherichia coli* [two rabbits], barley yellow dwarf virus inclusion bodies [two rabbits], mouse P815 cells [one rabbit], RPMI 1640 medium [one rabbit], or nothing [four rabbits]).

ELISA. An enzyme-linked immunosorbent assay (ELISA) was performed on purified BLV as described previously (4). The color reaction between peroxidase and 5-aminosalicylate was allowed to proceed for 2 h, after which color intensity was evaluated at 570 nm with an ELISA plate reader (Cambridge Technology, Cambridge, Mass.). Antibody titer was defined as the reciprocal of the serum dilution which gave color intensity twice that of either a preinoculation serum sample from the same rabbit or serum from a control rabbit at the same dilution.

Syncytium formation assay. PBMC from BLV-inoculated rabbits were suspended at 4×10^6 cells per ml of RPMI 1640 containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and antibiotics (RPMI) with 5% normal rabbit serum and incubated with 2.5 mg of phytohemagglutinin-L (PHA-L; Sigma Chemical Co., St. Louis, Mo.) per ml for 48 h at 37°C in 5% CO_2. The PBMC were then washed and resuspended in RPMI. The suspension was divided into two equal portions. Each portion was made 10% serum with either anti-BLV bovine serum or normal bovine serum and placed on a monolayer of bat lung fibroblasts (Tb1Lu; Wistar Institute), a cell line that is free of bovine syncytial virus and parainfluenza virus type 3 and is not susceptible to infection by bovine herpesvirus type 1 or bovine viral diarrhea virus (American Type Culture Collection). After 18 h at 37°C and 5% CO_2, the PBMC were removed, and the monolayers were washed in Dulbecco modified Eagle medium containing 10 mM HEPES and antibiotics (DMEM) and incubated overnight in DMEM containing 10% fetal bovine serum (FBS). Monolayers were maintained in growth phase for 4 to 5 days, and then 5×10^5 cells were placed in a 25-cm^2 flask and incubated for an additional 1 to 2 days. The relative numbers of syncytia (defined as cells containing five or more nuclei [9]) were determined by counting the syncytia present in 20 consecutive fields with an inverted microscope at a magnification of 100×. Backgrounds were subtracted, and results are expressed as number of syncytia per square centimeter. Controls consisted of identically treated PBMC from 10 different control rabbits.

RT assay. Clarified supernatants from 1×10^7 to 2×10^7 PBMC stimulated with PHA-L for 48 to 72 h were centrifuged at 90,000 × *g* for 1 h at 4°C, and the resulting pellets were suspended in 160 ml of 0.1 M Tris, pH 7.8. Reverse transcriptase (RT) activity was determined in triplicate 50-μl samples in the presence of magnesium by an assay modified for BLV (16, 19). Activity is expressed as picomoles per hour per 10^8 cells. Negative controls consisted of identically obtained supernatants from 1×10^7 to 2×10^7 control rabbit PBMC. Positive controls were clarified, ultracentrifuged virus pellets from supernatants of BLV-FLK cells.

Northern (RNA) blot analysis. Blood was drawn from three BLV-inoculated rabbits, and PBMC were isolated as described before (40). Because only a limited amount of blood could be drawn from each infected rabbit at a given time, the PBMC were pooled to obtain sufficient RNA to analyze. RNA was isolated from PBMC of BLV-inoculated and control rabbits and a BLV-infected cow by CsCl gradient centrifugation as described before (7). Denaturing formaldehyde gels were run on a total RNA from PBMC which had been stimulated for 24 to 48 h with concanavalin A (ConA; 5 mg/ml). The probe (a gift of A. Burny, Brussels, Belgium) consisted of an 8.3-kilobase (kb) clone of BLV proviral DNA.

PCR. Samples of DNA isolated as described before (7) from PBMC of BLV-inoculated and control rabbits were amplified by the polymerase chain reaction (PCR). Briefly, the reaction mixture contained 1 μg of PBMC DNA or 30 ng of control plasmid DNA, 200 mM each of four deoxyribonucleoside triphosphates, 10 mM Tris chloride (pH 8.31), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 U of thermoresistant *Taq* DNA polymerase, and 100 pmol each of oligonucleotide primers. The primers are complementary to a region of the *env* gene and have the following sequences:

First primer: 5'-TAA AAG CAG GGC CCA TGA TCT GAC AG-3'

Second primer: 5'-TGG ATG ACA GCA TAT AAC CAA GAG GC-3'

A standard amplification procedure was followed by adding primers in large excess to cellular DNA, heat denaturing at 95°C, and then cooling to 37°C. Primer extension was carried out at 72°C with heat-stable *Taq* polymerase (33). This cycle was carried out 35 times, and Southern blot analysis was performed on 1/10 of the reaction mixture with an 8.3-kb BLV cDNA probe (27).

IL-2-dependent lymphocyte cultures. PBMC from BLV-inoculated or control rabbits were suspended at 3×10^6/ml of RPMI containing 10% FBS and stimulated with 2.5 mg of the phytolectin ConA per ml. The cultures were maintained by adjusting the cell count to approximately 3×10^6 cells per ml with fresh RPMI containing 10% FBS and 50 U of human recombinant interleukin-2 (IL-2; Chiron Corp., Emeryville, Calif.) per ml every 3 to 4 days.

Induction of BLV production from IL-2-dependent virus-infected lymphocytes. After 3 months or more in culture, BLV production was induced from IL-2-dependent lymphocytes from BLV-infected rabbits by suspending the lymphocytes in a mixture of RPMI containing 50 U of IL-2 per ml and 5% normal rabbit serum with 33% supernatant from phytolectin-stimulated PBMC from uninfected rabbits or 33% RPMI containing 5% normal rabbit serum. Virus production was monitored by incubating the IL-2-dependent lymphocytes on Tb1Lu monolayers in the presence or absence of bovine anti-BLV serum and counting the relative numbers of syncytia. IL-2-dependent lymphocytes obtained from a control rabbit and treated in the same manner were used as negative controls.

Response to phytolectin stimulation. Two hundred milliliters of 1:10 dilutions of blood leukocytes in RPMI were added to U-bottomed microtiter plates. Triplicate cultures were stimulated with PHA-P (5 and 10 mg/ml) or ConA (5 and 10 mg/ml). After 48 h of incubation at 37°C, 0.5 mCi of [*methyl*-^{3}H]thymidine (6.7 Ci/mmol; Du Pont New England Nuclear Research Products, Boston, Mass.) in 20 μl was added to each well. After a further 22 h, cultures were terminated with a microharvester, and radioactivity was counted in a liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.). Responses were evaluated prior to inoculation with BLV and at intervals thereafter. Controls consisted of identical cell preparations taken from control rabbits. Results are expressed as a percentage of the response of leukocytes from an age-matched control animal run at the same time.

TABLE 1. Summary of results

Inoculum source	Inoculum	No. of rabbits inoculated	No. of rabbits positive/no. tested					
			Anti-BLV antibody	PBMC inducing syncytia	RT activity	Virus genome present[a]	Multinucleated giant cells in culture	Clinical disease[b]
BLV-infected lymphocytes	1×10^7 cells	12	12/12	12/12	1/5	1/5	8/8	6/12
BLV-FLK	5×10^6 cells	4	4/4	4/4	2/3	1/2	2/2	4/4
Cell-free BLV	100 × concentrated virus (2 ml)	2	2/2	2/2	1/2	1/1	N.T.[c]	2/2

[a] Analysis by Northern blot or PCR.
[b] Includes rabbits that died.
[c] N.T., Not tested.

Pathology. Food and water consumption and clinical condition were visually monitored for all the BLV-inoculated and control rabbits. Rabbits were weighed monthly, and rabbits (three female, two male) which exhibited severe decline in clinical condition or died unexpectedly were necropsied. The following structures were grossly examined at necropsy: eyes, integument, teeth, oral cavity, tongue, larynx, heart, lungs, esophagus, trachea, diaphragm, liver, gallbladder, spleen, pancreas, stomach, small intestine, cecum, colon, adrenal glands, kidneys, ureters, urinary bladder, ovaries, uterus, cervices, vagina, vulva, brain, pituitary gland, bone marrow, coxofemoral joints, and stifle joints. Tissues were placed in 10% buffered Formalin, paraffin embedded, sectioned at 4 to 6 μm, and stained with hematoxylin and eosin for histologic examination.

RESULTS

Evaluation of infection. Two major criteria were used to demonstrate that all BLV-inoculated rabbits become persistently infected with the virus. First, sera from all BLV-inoculated rabbits were periodically tested for the presence and persistence of antibody to BLV. The development of persistent antibody to BLV is commonly used to determine BLV infection in cattle (31). Second, PBMC from all BLV-inoculated rabbits were assayed for virus expression by the development of syncytia on BLV-sensitive fibroblasts. Cocultivation of BLV-susceptible fibroblasts with lymphocytes from BLV-infected cattle has been used to detect the production of BLV by lymphoid cells (12). The syncytia assay had two functions. It confirmed that the rabbits were infected with BLV, because Tb1Lu is susceptible to BLV but is not susceptible to several other common bovine pathogens (see description in Materials and Methods). Additionally, it served as a means to demonstrate recovery of BLV from circulating cells of inoculated animals, because BLV-induced cell fusion is the cytopathic effect seen in Tb1Lu (9, 19). Additional confirmation of the presence of BLV in inoculated rabbit cells was achieved through evaluation of PBMC from randomly selected animals for viral RT activity, presence of viral mRNA by Northern blot analysis, or presence of provirus as detected by PCR (33). All BLV-inoculated rabbits were monitored for clinical signs suggestive of suppressed immune function. A summary of the results of the tests is shown in Table 1.

Antibody response to BLV. To confirm and extend earlier reports of persistent anti-BLV antibodies in rabbits (6), 18 New Zealand White rabbits were inoculated with BLV. Sera were collected from these rabbits over a period of 18 months and analyzed by ELISA. For animals which survived longer than 18 months, samples were collected up to 24 months postinoculation. Table 2 shows four separate examples of antibody titer patterns observed in BLV-inoculated rabbits tested repeatedly over 24 months postinoculation. Titers varied considerably with time, in some cases falling to undetectable levels and then becoming detectable again later in the infection. Although no consistent pattern of rising and falling anti-BLV titers was observed, each rabbit produced antibody over an extended period of time. In Table 3, the ranges of anti-BLV antibody titers are shown for all BLV-inoculated rabbits for early (2 to 3 months), middle (6 to 8 months), and late (12 to 18 months) time points after inoculation. All of the BLV-inoculated animals produced anti-BLV antibodies for prolonged periods postinoculation. None of the rabbits which received inocula other than BLV developed detectable levels of anti-BLV antibody.

Syncytium formation in BLV-susceptible indicator fibroblasts and inhibition with anti-BLV antibody. In order to further demonstrate that BLV-inoculated rabbits had become persistently infected and to recover BLV from circulating PBMC, we evaluated the ability of phytolectin-stimulated PBMC from BLV-inoculated rabbits to produce syncytia upon cocultivation with susceptible bat lung fibroblasts (Tb1Lu). Table 4 shows syncytium formation induced by phytolectin-stimulated PBMC from 12 of the BLV-inoculated rabbits randomly tested for virus expression beginning 2 months and continuing at least 12 months postinoculation. In these experiments, two observations were made consistently. First, syncytia always formed (8 to 26 syncytia

TABLE 2. ELISA titers over time for four BLV-inoculated rabbits

Inoculum	ELISA titer[a] at mo postinoculation:											
	2	4	6	8	10	12	14	16	18	20	22	24
Cell-free BLV	10,000	10,000	10,000	1,200	1,200	1,200	NT	1,200	1,200	NT	NT	1,200
BLV-infected lymphocytes	1,000	1,200	1,000	250	250	NT	1,000	250	Dead			
BLV-FLK	250	1,000	100	100	BD	BD	NT	NT	BD	NT	BD	250
BLV-FLK	1,000	250	1,000	NT	1,000	50	NT	250	250	BD	50	50

[a] NT, Not tested; BD, below detectable levels.

TABLE 3. Anti-BLV antibody titers[a]

Inoculum (no. of rabbits)	Titer range (no. of rabbits with indicated titer)		
	2–3 mo	6–8 mo	12–18 mo
BLV-infected lymphocytes (12)	100 (4)–10,000 (1)	100 (7)–10,000 (1)	100 (5)–6,000 (1)[b]
BLV-FLK (4)	100 (1)–1,000 (3)	100 (2)–1,000 (2)	BD[c] (2)–250 (1)
Cell-free BLV (2)	10,000 (2)	10,000 (2)	10,000 (2)
Uninfected lymphocytes (2)	BD (2)	BD (2)	BD (2)

[a] Titers were determined by ELISA; sera from rabbits receiving no BLV gave no detectable titers.
[b] Animal which had titer of 10,000 was dead at 12 months.
[c] BD, Below detection limit.

per cm^2) when PBMC from BLV-inoculated rabbits were incubated in normal bovine serum. Second, syncytium formation was always decreased (2.7- to >25-fold) when PBMC from BLV-inoculated rabbits were incubated with anti-BLV serum. None of 10 age-matched control rabbits tested at the same time had PBMC which induced syncytium formation above background. The formation of syncytia in the presence of BLV-inoculated rabbit cells and the inhibition of syncytium formation in the presence of anti-BLV antiserum provided further evidence that cells from BLV-inoculated rabbits were indeed persistently infected with the virus.

RT activity. The presence of RT activity in virus-infected cells has often been used as an indicator of retrovirus infection. To assess whether RT activity could be detected in BLV-inoculated rabbits, PBMC from a number of animals were tested. Culture supernatants from phytolectin-activated PBMC from control and BLV-inoculated rabbits were assayed for RT activity in an assay mix containing magnesium and modified to measure BLV RT (9, 16). Whereas no RT activity above background was detected in samples from seven uninfected rabbits, samples from 4 of 10 BLV-inoculated rabbits sampled between 6 and 20 months postinoculation exhibited appreciable RT activity (16.4 ± 8.0 pmol/h per 10^8 cells) (data not shown). Detectable RT activity in BLV-inoculated animals strongly suggests that circulating PBMC from such animals are infected with the virus.

Northern blot analysis. Like HTLV-I, BLV is not produced at detectable levels in freshly isolated lymphocytes of the infected host but is often expressed when those cells are cultured in vitro (11, 14, 23, 24, 28). To determine whether BLV transcripts could be detected in infected rabbit PBMC, Northern blot analyses with a BLV cDNA probe were performed on total RNA obtained from phytolectin-stimulated PBMC from several of the BLV-inoculated rabbits. Figure 1 shows an experiment in which RNA was isolated and pooled from a limited amount of blood from three BLV-inoculated rabbits and compared with RNA obtained from PBMC from an uninfected rabbit and from a BLV-infected cow. BLV transcripts were detected in RNA from the BLV-inoculated rabbits and the BLV-infected cow but not in the control rabbit RNA. Different hybridizing mRNA species were observed in the infected-rabbit mRNA population and might be the result of alternative splicing events.

PCR. PCR analysis was also performed to determine whether proviral DNA could be detected in cells from BLV-infected rabbits. In HTLV-I- and HIV-1-infected humans, only a small percentage of lymphocytes can be demonstrated to contain proviral DNA (34). We suspected a similar situation might exist in cells from BLV-infected rabbits because of the difficulty we encountered in demonstrating RT activity and BLV transcripts. In order to detect such rare events, an extremely sensitive method such as the PCR assay (33) is required. As shown in Fig. 2, BLV sequences could be detected in one of two rabbits inoculated with BLV 12 months previously but could not be found in the DNA from the control rabbit. The rabbit with the positive PCR was not one of the three rabbits from which pooled RNA was taken for Northern blot analysis (Fig. 1). These data indicate that BLV provirus can be demonstrated in circulating PBMC from at least some BLV-infected rabbits.

Multinucleated-cell formation in long-term rabbit PBMC cultures. To determine whether lymphocytes from BLV-

TABLE 4. Syncytium formation induced by PBMC from twelve BLV-inoculated rabbits

BLV source	Time post-inoculation (mo)	No. of syncytia/cm^2 [a]	
		Normal serum	Anti-BLV serum
BLV-FLK	2	19	1
	2	24	9
	5	25	0
	8	19	4
Cell-free BLV	10	10	1
	11	18	0
BLV-infected lymphocytes	5	26	8
	6	26	4
	7	8	0
	7	10	0
	7	15	0
	12	9	0

[a] Background level of syncytium formation (five or more nuclei per cell) for Tb1Lu is less than 1 per 1,000 cells. PBMC from control rabbits did not form syncytia above background levels in either normal serum or anti-BLV serum.

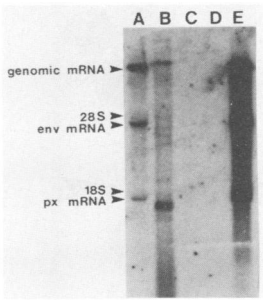

FIG. 1. Expression of BLV mRNA in BLV-infected rabbit PBMC. Northern blot analysis was performed on total RNA isolated from ConA-stimulated bovine and rabbit PBMC. Lanes A and E contain RNA isolated from 24-h-stimulated bovine PBMC; lane B, RNA pooled from 20- to 48-h-stimulated PBMC from three BLV-infected rabbits; lane C, RNA isolated from 96-h-stimulated BLV-infected rabbit PBMC; lane D, RNA isolated from 48-h-stimulated uninfected rabbit PBMC. px, Region containing the *trans*-activating gene.

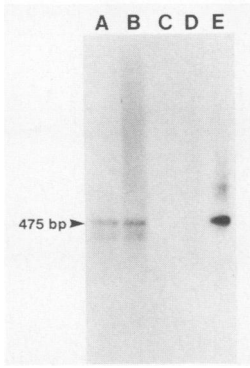

FIG. 2. Amplification analysis of PBMC DNA from BLV-infected rabbits. PCR was performed on DNA isolated from PBMC from BLV-infected and control rabbits. The autoradiogram yielded the diagnostic 475-base-pair (bp) BLV-specific fragment. Lanes A and B, PCR-amplified DNA from a BLV-infected rabbit; lane C, PCR-amplified DNA from a different BLV-infected rabbit; lane D, PCR-amplified DNA from an uninfected rabbit; lane E, PCR-amplified DNA from a pBR322 plasmid containing the full-length BLV genome.

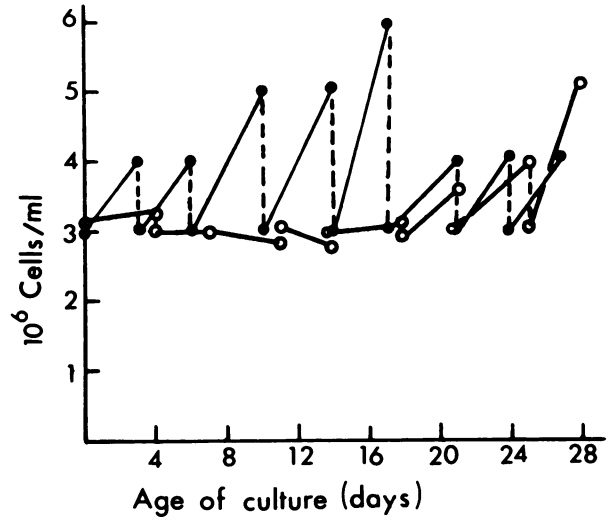

FIG. 4. Typical growth in continuous culture of PBMC from BLV-infected and control rabbits. The data represent cell growth from one control (solid circles) and one infected (open circles) rabbit. Similar growth patterns were obtained for four additional control and seven additional infected rabbits. The vertical dashed lines indicate times and cell densities at which cultures were either divided or concentrated.

infected rabbits had altered growth characteristics in long-term culture, PBMC from BLV-infected and control rabbits were stimulated with phytolectin and maintained in culture with human recombinant IL-2. During the first 20 to 30 days of culture, major differences were observed in the growth of infected-rabbit PBMC compared with PBMC from normal animals. Specifically, beginning at 4 to 5 days of culture, giant multinucleated cells formed in cultures initiated from BLV-infected rabbits (Fig. 3). In some of the giant cells, as many as 25 to 30 nuclei were observed. Figure 4 shows growth patterns during the first 28 days in culture of PBMC from a normal rabbit and PBMC from a BLV-infected rabbit. During that period, while giant cells formed in the infected-PBMC cultures, total cell numbers did not increase. IL-2-dependent cell proliferation could be detected in BLV-infected cultures only after giant-cell formation ceased (by 30 days).

BLV production in cultures of long-term cells. The use of PBMC to show that BLV-inoculated rabbits were infected with the virus indicated that virus could be demonstrated in circulating leukocytes. However, PBMC contain monocytes as well as lymphocytes. Therefore, to determine whether

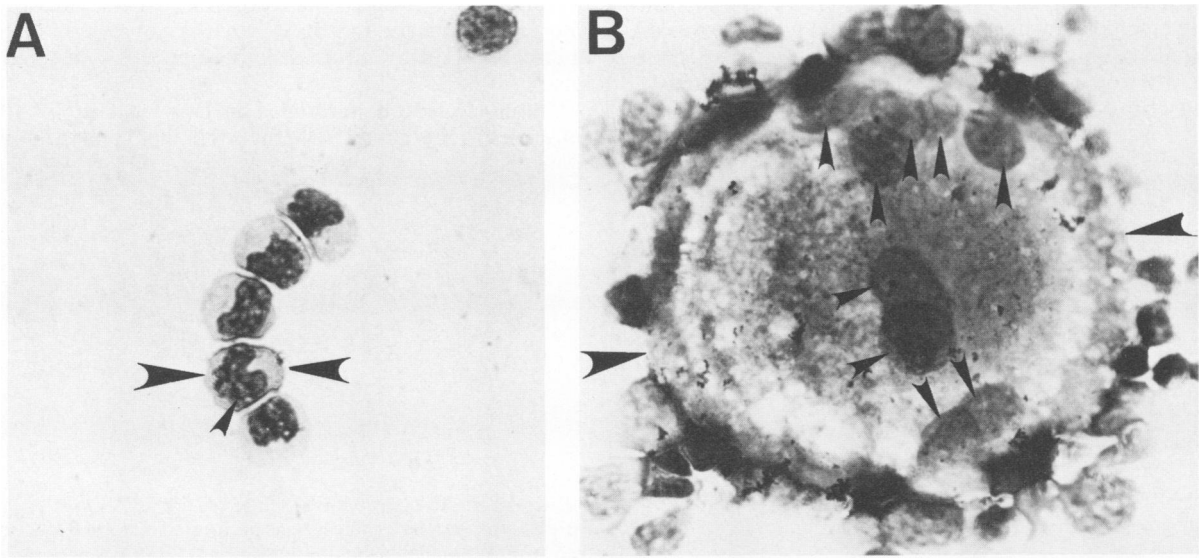

FIG. 3. Multinucleated giant-cell formation in BLV-infected rabbit PBMC cultured with recombinant IL-2. BLV-infected and control rabbit PBMC were stimulated with ConA and cultured with human recombinant IL-2. To evaluate cell morphology, 1×10^5 to 2×10^5 cells were centrifuged onto glass slides (Cytospin, Shandon Southern Instruments, Sewickley, Pa.) and stained with Wright-Giemsa stain. (A) Cells obtained from a 3-week-old culture of control rabbit PBMC. (B) Cells obtained from a 3-week-old culture of BLV-infected rabbit PBMC. The large arrowheads point to the cytoplasmic margin of a single cell. Each small arrowhead points to a nucleus within that cell. Magnification, ×200.

TABLE 5. Induction by cytokine-containing medium of BLV expression in infected lymphocytes in long-term culture

Expt no.	BLV inoculated[b]	No. of syncytia/cm^2 [a]		
		CM	Non-CM	CM + anti-BLV serum
1	Y	24	2	0
2	Y	24	0	NT[c]
	Y	25	2	NT
3	Y	25	8	NT
	N	0	2	NT

[a] CM, Cytokine-containing medium.
[b] Y and N, BLV-infected (Y) and control uninfected (N) lymphocytes.
[c] NT, Not tested.

BLV could be produced by lymphocytes from infected rabbits, IL-2-dependent lymphocyte cultures were derived from PBMC of infected rabbits. BLV expression was induced from lymphocytes maintained in long-term culture for 3 months or longer by adding cytokine-containing supernatants from phytolectin-stimulated PBMC from uninfected rabbits to the long-term cultures of IL-2-dependent lymphocytes derived from BLV-infected rabbits. Virus expression was monitored by induction of syncytium formation on susceptible fibroblasts (Tb1Lu) (Table 5). In three separate experiments, lymphocytes from three different BLV-infected rabbits induced increased numbers of syncytia in the presence of supernatant compared with the numbers seen either in the absence of supernatant, in the presence of anti-BLV antiserum, or in comparison with cells from an uninfected rabbit.

Response of peripheral blood leukocytes to phytolectin stimulation. To assess whether BLV infection was affecting rabbit T-lymphocyte function, a method which measures general responsiveness of T lymphocytes was used. Circulating leukocytes from the first 11 rabbits to receive BLV-infected bovine lymphocytes and the first two rabbits to receive BLV-FLK cells were evaluated at regular intervals for response to stimulation by the T-lymphocyte-stimulatory phytolectins PHA and ConA. The stimulation obtained with BLV-inoculated rabbit leukocytes was compared with the stimulation obtained with age-matched control rabbit leukocytes run at the same time. The BLV-inoculated rabbits were evaluated for 18 to 24 months or until they died. The data (Fig. 5) revealed that leukocyte responses to phytolectin stimulation fluctuated in an abnormal and apparently random manner which was different for each BLV-infected rabbit.

Specifically, analysis of the data after 6 months showed that responses of five animals had become abnormally low, five had remained normal, and three had become elevated. Figure 5 shows the responses to PHA of the five animals which had abnormally low responses (panel A) and those with responses within the normal range (panel B) in the first 6 months postinoculation. The shaded areas show the range of responses of age-matched control animals. Of the five rabbits with abnormally low responses by 6 months postinoculation (panel A), one animal was killed at 12 months postinoculation (see Pathology, below). The responses of the other four returned to normal and have fluctuated between normal and abnormally low. Three had abnormally low responses by 20 months (1, 8, and 44% of normal), while the fourth animal appeared normal. Of the five animals with PHA responsiveness within the normal range for the first 6 months postinoculation (panel B), two developed abnormally high responses over the next 6 to 8 months, and a third

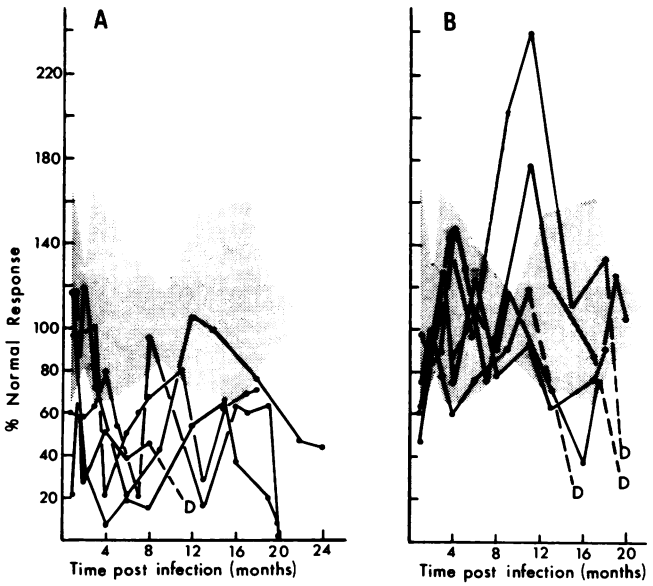

FIG. 5. Response to phytolectin stimulation of peripheral blood leukocytes from BLV-infected rabbits. Heparinized blood was diluted and cultured in the presence of PHA-P for 3 days. Cell proliferation was determined by uptake of [*methyl*-^{3}H]thymidine. Panels A and B show the phytolectin responsiveness of BLV-infected rabbits over 18 to 24 months postinoculation. The shaded areas represent the range of responses of leukocytes from age-matched normal rabbits run at the same time as the BLV-infected rabbits. (A) Responsiveness of five infected rabbits which had abnormally low responses by 6 months postinoculation. (B) Responsiveness of five infected rabbits which had normal responses by 6 months postinoculation. A dashed line followed by a D indicates the death of the rabbit being evaluated. Results are expressed as a percentage of the responsiveness of leukocytes from an age-matched control rabbit run side by side with those from the infected animal. Uptake of [*methyl*-^{3}H]thymidine into PHA-stimulated control rabbit leukocytes ranged between 20,000 and 45,000 cpm for control rabbits less than 18 months old and between 6,000 and 15,000 cpm for control rabbits over 18 months old. Unstimulated leukocytes incorporated between 100 and 700 cpm regardless of the age of the animal.

animal was killed (see Pathology). At 20 months postinoculation, the two rabbits which had developed abnormally high responses within 6 to 14 months postinoculation were found dead (see Pathology). The remaining two rabbits have responses within the normal range at this time. The three animals with responses above normal within the first 6 months still have abnormally high responses after 19 to 20 months (148, 225, and 286% of normal). Together, these data show that most BLV-infected rabbits develop unique and abnormal patterns of T-lymphocyte responsiveness.

Pathology. Within 12 to 18 months of inoculation, three rabbits displayed marked weight loss (one of these rabbits, no. 5, was euthanized). In six additional rabbits, there were clinical indications of upper respiratory disease. The clinical indications of upper respiratory disease most often found were conjunctival and nasal exudates. The extent of weight loss in one of the BLV-infected rabbits is shown in Fig. 6. The additional two rabbits with marked weight loss had similar appearance. Currently, of the nine infected rabbits exhibiting either weight loss or clinical disease, five have died (two rabbits, nos. 2 and 5, were killed due to decline in their clinical condition, and three were found dead). This represents a mortality rate of 28% (5 of 18) for rabbits

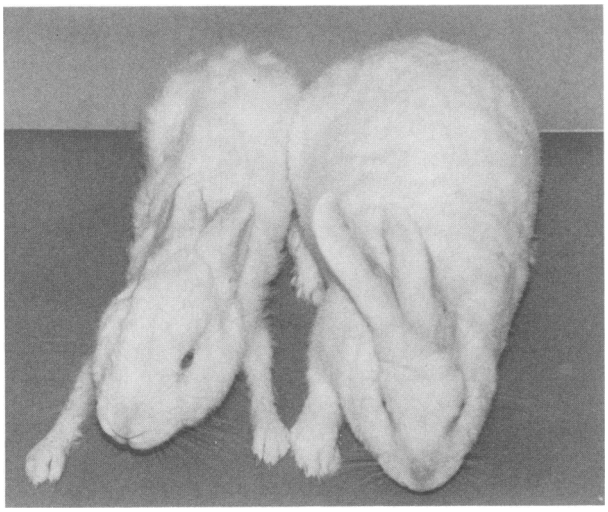

FIG. 6. Severe weight loss in a BLV-infected rabbit. Appearance of a BLV-infected rabbit (left) compared with that of an age-matched control rabbit (right). The BLV-infected rabbit had received BLV-infected cells 23 months earlier. Both animals were maintained under the same environmental conditions.

inoculated with BLV. During the same time period, no illnesses or deaths were observed in our colony of 25 uninfected rabbits held under the same environmental conditions.

Gross and histological examination of these five rabbits revealed inflammatory processes of the colon in two rabbits (nos. 1 and 2). Rabbit 1 exhibited a moderate plasmacytic and lymphocytic colitis, and rabbit 2 had a focal chronic transmural colitis. The colonic lesion of rabbit 2 was associated with a focal perforation. Although we hypothesized that a foreign body might have caused the perforation, a foreign body was not found. Inflammatory changes of the epicardium, pulmonary pleura, kidney, and liver were found in rabbits 3 and 4. Rabbit 3 exhibited a fibrinous and suppurative pleuritis and pericarditis, and rabbit 4 had moderate interstitial lymphocytic nephritis and periportal lymphocytic hepatitis. *Pasteurella multocida* was isolated from the pericardial and pleural lesions; however, the cause of the hepatic and renal inflammation was not determined. Rabbit 5, the last one necropsied, had only a moderate increased cellularity of peribronchial lymphoid tissue.

The gross and histological examinations of rabbits 2 and 3 revealed processes of adequate severity to explain the clinical course in these BLV-infected rabbits. Furthermore, the pleural and pericardial lesions caused by *P. multocida* in rabbit 4 certainly contributed to the death of this rabbit; however, the reason for the proliferation of this common rabbit pathogen in this rabbit was not determined. Although the gross and histological examinations of the five rabbits necropsied did not reveal neoplastic, degenerative, or inflammatory processes that could be directly attributable to BLV infection, an indirect contribution of BLV to the clinical deterioration and death seen in these rabbits cannot be ruled out.

DISCUSSION

In this study, we inoculated homogeneous preparations of the type C lymphotropic retrovirus BLV or circulating lymphocytes from a cow infected with BLV into 18 young adult rabbits to determine whether rabbits could become persistently infected with the virus. We found that all rabbits receiving BLV preparations produced persistent antibody to BLV and had PBMC which induced syncytia in BLV-susceptible Tb1Lu fibroblasts when tested as long as 16 months after inoculation. Additional tests on PBMC from randomly selected rabbits revealed the presence of RT activity, BLV mRNA, and BLV proviral DNA. Long-term IL-2-dependent lymphocyte cultures initiated from BLV-infected rabbits developed multinucleated giant cells within the first 30 days of culture, during which time no increase in cell numbers was observed. After IL-2-dependent cell proliferation commenced, lymphocyte cultures were shown to contain cells which could induce syncytia in Tb1Lu fibroblasts upon stimulation with a cytokine-containing supernatant from PBMC of control rabbits. Abnormally low responses to phytolectin stimulation of circulating leukocytes developed within 6 months in 38% of the rabbits tested and subsequently fluctuated between normal and abnormally low. Forty-four percent of BLV-infected rabbits suffered recurrent conjunctivitis and rhinitis, and 28% of the animals were found dead or were killed due to poor clinical condition. None of the control rabbits has had clinical signs, and none have died.

The major conclusion to be drawn from these cumulative observations is that all rabbits inoculated with BLV preparations become persistently infected with the virus. The persistent presence of anti-BLV antibody and titers of anti-BLV antibody which rise and fall in the absence of administration of additional BLV antigen in and of themselves suggest that the virus which initiates antibody production is being made in the inoculated rabbits. In addition, the capacity of PBMC to induce syncytia in Tb1Lu monolayers when tested up to 1 year after inoculation with BLV indicates that BLV-infected cells are present among the circulating PBMC. The finding of persistent BLV infection in rabbits receiving BLV-infected cells is consistent with several studies which have demonstrated the presence of infectious BLV in lymphocytes from infected cattle and in infected cell lines (1, 9, 35, 38).

There are two important findings in these studies. First, two-thirds of the BLV-infected rabbits showed one or more of the following changes: low response to phytolectin stimulation by T-cell mitogens, clinical indications of respiratory disease, severe weight loss, or death. None of these changes has been seen among the control rabbits, strongly suggesting that BLV infection is involved in the development of these abnormalities. The decreased in vitro T-cell responses to phytolectin stimulation that we saw are similar to reports for other *trans*-acting lymphotropic retrovirus systems, such as HTLV-I-induced chronic T-cell lymphocytosis (26) and HIV-1-induced acquired immunodeficiency syndrome (10). Furthermore, our observations of recurrent clinical indications of respiratory disease are suggestive of an increased incidence of secondary infections in BLV-infected rabbits. Secondary infections have been found both in patients with HIV-1-induced acquired immunodeficiency syndrome and those with HTLV-I-induced leukemia (reviewed in references 17 and 41).

Second, long-term IL-2-dependent lymphocyte cultures initiated from BLV-infected rabbits initially showed abnormal growth characteristics consistent with the presence of a *trans*-acting retrovirus. Multinucleated giant cells similar to those seen in our rabbit PBMC cultures have been observed in HTLV-I-, HTLV-II-, and HIV-1-infected human cell cultures (30, 37, 41, 42). The presence of such cells suggests

that BLV expression induced by stimulating infected cells resulted in their subsequent fusion. Preliminary results indicate the presence of intensely stained nonspecific esterase-positive cells in the fusions, suggesting that some monocytes might be infected with the virus. Studies are in progress to evaluate BLV expression in these fused cells.

Furthermore, once fusion has ceased and IL-2-dependent growth has become established, a portion of the remaining lymphocytes must carry the virus. Our data show that BLV expression can be induced from cultured long-term IL-2-dependent lymphocytes when those lymphocytes are incubated with a cytokine-containing medium derived from PBMC of control rabbits. IL-2 does not appear to be sufficient to induce virus expression, because cultures containing IL-2 without cytokine-containing medium did not induce syncytium formation. Our results are similar to those reported for induction of HIV-1 from a monocyte cell line by cytokine-containing medium (13) in that long-term-cultured, infected cells require cytokine stimulation in order to express virus. These findings are important because they indicate that both monocytes and lymphocytes can be reservoirs of BLV in infected rabbits.

While all BLV-inoculated rabbits showed persistent BLV infection, the confirming tests for BLV (RT assay, Northern blot analysis, and PCR) done on PBMC did not show the presence of virus in all animals tested. It is interesting that RT activity was detected in cells from animals that had been infected for longer than 1 year and were showing signs of disease. Two animals which were RT negative at 6 months postinoculation were RT positive over 1 year later, suggesting that too few infected cells were present in those rabbits early in the infection to produce enough virus to detect in this assay. Failure to consistently detect BLV message by Northern blot analysis and BLV proviral sequences by PCR could also be due to the presence of very few infected cells in the preparations from which the nucleic acids were extracted. The presence of very few circulating infected cells has been a typical observation by other investigators studying lymphotropic retroviruses. For example, although sheep are very sensitive to infection with BLV (22), BLV-infected sheep have been estimated to have between 1 in 2×10^3 and 1 in 5×10^5 infected circulating cells early in infection (25). Similarly, HIV-1-infected people have fewer than 1 in 10^4 infected circulating lymphocytes (20, 34). These findings are consistent with our observations.

Recently, attention has been given to the presence of an additional bovine retrovirus, which has been classified as a lentivirus. Tentatively named bovine immunodeficiencylike virus, or BIV (18), it was isolated from some cattle with persistent lymphocytosis, wasting, and central nervous system signs (39). While BIV infection does not appear to be widespread among cattle in the United States (M. A. Gonda, 10th Annu. W. Food Anim. Dis. Res. Conf., 1989, abstr.), it is of concern that some of our rabbits, notably those that received lymphocytes from a BLV-seropositive, persistently lymphocytotic cow, could have received a mixed BLV-BIV infection. While we know that all of our rabbits became infected with BLV, we do not believe that the changes observed in our rabbits were the result of a superimposed BIV lentivirus infection. BLV-infected rabbits developed clinical signs of disease whether the animals were given homogeneous BLV preparations (BLV-FLK cells or purified BLV) or bovine lymphocytes. In fact, homogeneous BLV preparations appeared to be more effective in inducing disease signs in rabbits (six of six rabbits with clinical signs) than were bovine lymphocyte suspensions (6 of 12 rabbits with clinical signs) (Table 1). To date, we have no evidence to support infection by BIV in rabbits. Furthermore, no direct relationship between BIV infection and disease in cattle has been demonstrated (M. C. Thurmond, J. Stott, G. Theilen, and J. P. Picanso, 10th Annu. W. Food Anim. Dis. Res. Conf., abstr.; M. J. van der Maaten, C. A. Whetstone, J. A. Roth, and J. M. Miller, 10th Annu. W. Food Anim. Dis. Res. Conf., abstr.). Based on the abundance of evidence that BLV causes disease in cattle, we suggest that the changes in parameters associated with immunity that occurred in our rabbits are more consistent with infection by BLV.

A possible alternative explanation for the depression in immune function seen in our BLV-infected rabbits might be that infection with BLV activated a latent, endogenous rabbit retrovirus, and the changes seen in the rabbits were due to the activated rabbit virus. Such an explanation cannot be unequivocally ruled out because of the prevalence of endogenous retroviruses in a large number of other animal species (Infectious Diseases of Rodents, short course, Massachusetts Institute of Technology, May 1986). However, we believe that endogenous retrovirus activation does not explain our findings because endogenous retroviruses have not yet been confirmed in rabbits. In fact, rabbits have been recommended as experimental hosts to study murine retroviruses (MIT short course, 1986), suggesting that activation of endogenous retroviruses in rabbits is an unlikely event.

We have demonstrated that BLV infects rabbits, and we suggest that this viral infection can result in immune dysfunction which may predispose infected animals to secondary infections and death. Because BLV is one of a unique group of *trans*-acting lymphotropic retroviruses, the use of BLV infection in rabbits provides a safe and relatively inexpensive in vivo system for studies of expression of viruses which contain *trans*-acting genes.

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