EXPERIMENTAL TRANSMISSION OF ENZOOTIC BOVINE LEUCOSIS VIRUS WITH BLOOD AND MILK IN THE TROPICS[1]

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Foi testada a infectividade de sangue total, plasma e leucócitos periféricos de uma vaca adulta com anticorpos para gp55 do vírus da leucose bovina (VLB), cujos leucócitos em cultura produziram numerosas partículas virais de tipo-C, e de misturas de leite de vacas livres e infectadas com o VLB, utilizando-se um bioensaio em bezerros. Durante um período de observação de 6 meses, o desenvolvimento de anticorpos para gp55 nos bezerros inoculados foi considerado como evidência de infecção adquirida com o VLB.

Sangue total inoculado subcutaneamente, em quantidades variando entre 0,5ml e 8,0ml, transmitiu o VLB a quase 50% dos bezerros. Leucócitos do sangue periférico inoculados em quantidades de 10^3, 10^4 e 10^5 por bezerro não transmitiram o VLB, porém, 1 de cada 2 bezerros inoculados com 10^6 e 10^7 leucócitos adquiriram a infecção. Os bezerros inoculados com plasma assim como os bezerros testemunhas não inoculados não adquiriram a infecção.

Bezerros nascidos de vacas livres do VLB permaneceram sorologicamente negativos após a inoculação intra-abdominal de mistura de leite de vacas isentas do VLB e não foram observadas partículas virais de tipo-C em culturas dos seus leucócitos. Bezerros similares inoculados com uma mistura de leite de vacas infectadas com o VLB estavam todos infectados 3 meses mais tarde e seus leucócitos em cultura continham um pequeno número de partículas virais de tipo-C. Quatro de 5 bezerros nascidos de vacas infectadas com o VLB permaneceram sorologicamente negativos após a inoculação de mistura de leite de vacas livres do VLB. Por outro lado, 5 de 7 bezerros também nascidos de vacas infectadas com o VLB desenvolveram anticorpos para gp55 após a inoculação de mistura de leite de vacas infectadas com o VLB.

Conclue-se que o VLB pode ser transmitido iatrogenicamente através da inoculação de sangue fresco, que os linfócitos de animais infectados produzem partículas virais de tipo-C e são provavelmente as células responsáveis pela infectividade, que não existe uma viremia verdadeira como foi determinado pela falta de infectividade do plasma e que o leite de vacas infectadas contém o VLB em estado infeccioso.

TERMOS DE INDEXAÇÃO: Leucose enzoótica bovina, vírus, transmissão, sangue, leite.

ABSTRACT.- Whole blood, plasma and peripheral blood leukocytes from an adult cow with antibody to gp55 of bovine leukosis virus (BLV), whose cultured leukocytes produced numerous type-C viral particles, and pools of milk from BLV-free and BLV-infected cows were assayed for infectivity in experimental transmission studies utilizing a calf bioassay. During an observation period of 6 months, the development of antibody to gp55 in the inoculated calves was considered as evidence of acquired infection with BLV.

Whole blood inoculated subcutaneously in quantities that varied from 0.5ml to 8.0ml transmitted BLV to almost 50% of the calves. Peripheral blood leukocytes inoculated in quantities of 10^3, 10^4 and 10^5 per calf did not transmit BLV. However, 1 of every 2 calves inoculated with 10^6 and 10^7 leukocytes acquired the infection. Calves inoculated with plasma as well as uninoculated control calves did not become infected.

Calves born to BLV-free dams remained serologically negative after being inoculated intra-abdominally with a pool of milk from BLV-free cows and type-C viral particles were not observed in cultures of their leukocytes. Similar calves inoculated with a pool of milk from BLV-infected cows were all infected 3 months later and their cultured leukocytes contained small numbers of type-C viral particles. Four of 5 calves born to BLV-infected dams remained serologically negative after the inoculation of a pool of milk from BLV-free

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cows. Conversely, 5 of 7 calves also born to BLV-infected dams developed antibody to gp55 after being inoculated with a pool of milk from BLV-infected cows.

It is concluded that BLV can be transmitted iatrogenically through the inoculation of fresh blood, that lymphocytes from infected animals produce type-C viral particles and are probably the cells responsible for infectivity, that there is no true viremia as shown by the lack of infectivity of plasma and that the milk from infected cows contains infectious BLV.

INDEX TERMS: Enzootic bovine leukosis, virus, transmission, blood, milk.

INTRODUCTION

The presence of enzootic bovine leukosis (EBL) in Brazil in either the tumoral form (Dacorso Filho et al. 1966, Freire & Freitas 1966) or as a subclinical infection (Alencar Filho et al. 1979, Romero & Rowe 1981) is well documented. There is substantial evidence to indicate that EBL is infectious in nature and is caused by a type-C oncovirus (Miller et al. 1969), classified as a retrovirus, commonly known as bovine leukosis virus (BLV).

Although approximately 18% of infected cows vertically infect their developing fetus *in utero,* infection of susceptible cattle mainly occurs horizontally by contact with infected animals (Piper et al. 1979). The mechanisms involved in horizontal transmission, however, are ill-defined. The only known target cell for BLV infection is the lymphocyte (Baliga & Ferrer 1977). In infected cattle, BLV probably exists in these lymphocytes as non-infectious proviral DNA (Kettmann et al. 1978), since infectivity cannot be demonstrated until after they are grown in culture for a few hours (Stock & Ferrer 1972). This phenomenon makes the explanation of horizontal transmission rather difficult.

In the tropics, the epidemiology of EBL may be modified due to the abundance of potential mechanical and biological vectors such as ticks, biting flies, mosquitoes and even vampire bats. Premunition of young calves with fresh blood obtained from adult cattle to induce immunity against Anaplasma and Babesia organisms, is also common practice. Since antibodies to the major glycoprotein of BLV are widespread in dairy herds in Brazil (Romero & Rowe, in preparation), work has been started to determine which factors are facilitating the dissemination of this infection, in order to clarify the epidemiology of EBL and its causal agent, BLV.

In this report we present evidence on the infectivity of blood and milk obtained from BLV-infected cattle and associate these results to common animal husbandry practices.

MATERIALS AND METHODS

Detection of BLV antibodies. Antibodies to BLV in the serum and milk were assayed for in the agar gel precipitin (AGP) test (Miller & Van Der Maaten 1977). Milk casein was precipitated with 1N HCl. After centrifugation, the clear portion was removed, the pH adjusted to 7.2-7.4 with 1N NaOH and tested for antibodies. Reference sera, reference antigen and test conditions have been previously described (Romero & Rowe 1981, Romero et al. 1981).

BLV-infected blood donor. Blood for the transmission studies was obtained from an adult cow (Holstein x Brazilian Gir) with persistent lymphocytosis, antibodies to gp55, and whose peripheral blood leukocytes produced large numbers of type-C viral particles after a few hours in culture (Fig. 1).

BLV transmission with blood, plasma and leukocytes. This experiment was carried out on an island where a small number of cattle, random crosses of Holstein, Nelore and Brazilian Gir, was being raised extensively on large hilly pastures. A preliminary survey for BLV antibodies in the adult animals had indicated an infection rate of less than 5%. During the experimental period there was no direct contact between the calves used and any of the adult cattle on the island. Thirty-seven BLV antibody negative calves, between 1 and 11 months of age were randomly allotted to three experimental groups of 10 calves each, 7 calves being kept as uninoculated controls to monitor horizontal transmission of BLV. Two hundred and fifty ml of blood were collected from the donor by needle puncture of the jugular vein, directly into a flask containing 5000U of heparin. A portion of this whole blood was utilized after storage during 18 hours at 4°C. A second portion was centrifuged at 4°C, at 2500rpm for 20 minutes to sediment all cells. The clear plasma was used as inoculum after storage at 4°C for 18 hours. The remaining blood was processed to prepare short-term leukocyte cultures. Leukocytes were grown for 18 hours at 37°C. Viable cell counts were made using trypan blue and cell suspensions containing 10^3, 10^4, 10^5, 10^6 and 10^7 viable leukocytes were prepared in tissue culture medium RPMI 1640 containing 20% calf serum free of antibodies to BLV. Ten calves were injected subcutaneously with the heparinized whole blood; quantities of 0.5ml, 1.0ml, 2.0ml, 4.0ml or 8.0ml were inoculated into each of 2 calves. A second group of 10 calves were similarly inoculated with 0.5ml, 1.0ml, 2.0ml, 4.0ml or 8.0ml of plasma. The last 10 calves received 10^3, 10^4, 10^5, 10^6 or 10^7 viable leukocytes in the same manner. All calves were bled prior to inoculation and every month during 6 consecutive months. The sera were tested in the AGP test for the presence of antibodies to BLV as evidence of acquired infection.

Milk donors. Six cows in their 5th to 7th month of lactation served as negative milk donors. They had been shown to be free of antibodies to BLV in both the serum and milk as determined by the AGP test. Another 6 cows, also in their 5th to 7th month of lactation, that possessed antibodies to gp55 of BLV in their serum but not in their milk, were used as BLV-infected milk donors. All cows were a cross of Holstein and Brazilian Gir.

Transmission with milk. Calves were allowed to suckle colostrum from their dams during the first 5 days of life, after which both calves and dams were bled for serum. Serum antibodies to BLV were assayed for in the AGP test. Calves were identified as either antibody negative calves born to antibody negative dams or antibody positive calves born to antibody positive dams. All calves were given daily, for a period of 2 months, 2 liters of milk obtained from cows that were considered to be BLV-free, on the basis of 2 consecutive AGP tests 2 months apart. Calves were confined to individual

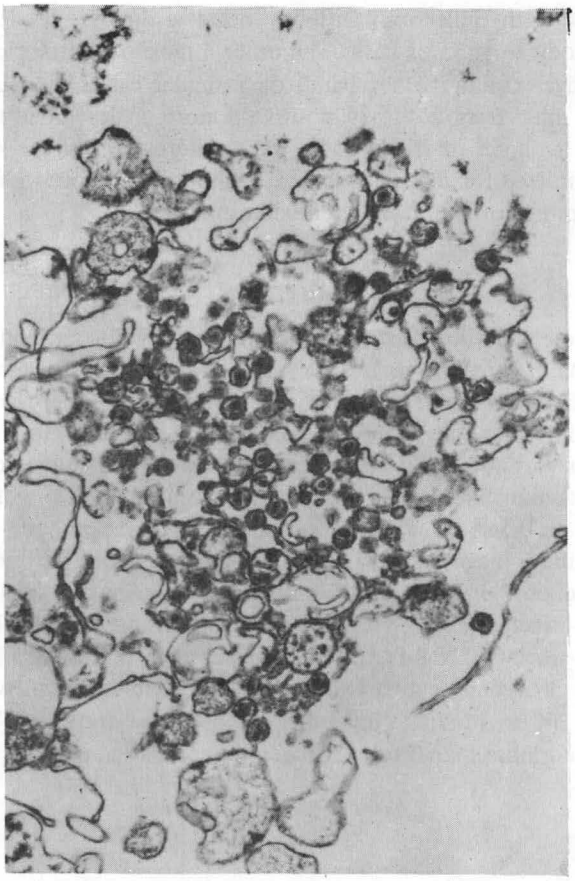

Fig. 1. *Electron micrograph of a leukocyte culture from a cow utilized in the blood-transmission experiments showing numerous extracellular type-C viral particles in the cell debris. 45,000x.*

Table 1. *Transmission of BLV to calves[(a)] inoculated subcutaneously with whole blood, plasma or leukocytes obtained from a BLV-infected cow*

Inoculum		Months after inoculation						
Type	Quantity	0	1	2	3	4	5	6
Whole blood	0.5ml	0/2[(b)]	1/2	1/2	1/2	1/2	1/2	1/2
	1.0	0/2	0/2	1/2	1/2	1/2	1/2	1/2
	2.0	0/2	0/1	1/2	1/2	ND[(c)]	ND	ND
	4.0	0/2	0/2	0/2	0/2	0/2	0/2	0/2
	8.0	0/2	0/2	1/1	1/1	1/1	1/1	1/1
Plasma	0.5ml	0/2	0/2	0/2	0/2	0/2	0/2	0/2
	1.0	0/2	0/2	0/2	0/2	0/2	0/2	0/2
	2.0	0/2	0/2	0/2	0/2	0/2	0/2	0/2
	4.0	0/2	0/2	0/2	0/2	0/2	0/2	0/2
	8.0	0/2	0/2	0/2	0/2	0/2	0/2	0/2
Leukocytes[(d)]	10^3	0/2	0/2	0/2	0/2	0/2	0/2	0/2
	10^4	0/2	0/2	0/2	0/2	0/2	0/2	0/2
	10^5	0/2	0/2	0/2	0/2	0/2	0/2	0/2
	10^6	0/2	1/2	1/2	1/2	1/2	1/2	1/2
	10^7	0/2	0/2	1/2	1/2	1/2	1/2	1/2
None	– –	0/7	0/7	0/7	0/6	0/5	0/5	0/5

(a) One to eleven month old calves negative for BLV antibodies.

(b) Number of calves with antibody to gp55 of BLV/Number of calves tested.

(c) Not done.

(d) Viable leukocytes inoculated after 18 hours in culture.

boxes during the first 2 months of life, weaned and then reared on pasture in an open field as is common in the tropics. Seven antibody negative calves between 1 and 5 months of age, born to antibody negative dams, were inoculated intra-abdominally with 100ml of milk from a pool obtained from either negative or positive donors. Total and differential leukocyte counts were performed every month on the blood of these calves and the absolute number of lymphocytes was calculated. In addition, short-term leukocyte cultures were grown from these calves at the end of the experimental period and processed for electron microscopy as previously described (Romero et al. 1981). Twelve calves born to antibody positive dams were assayed periodically until no maternal antibody was found in their serum. When these calves were between 3 and 5 months of age they were inoculated intra-abdominally with 100ml of milk from pools obtained from the same donor cows. All calves were bled monthly during 6 consecutive months and their serum tested for the presence of AGP antibodies to detect an acquired infection.

Short-term leukocyte cultures. Forty ml of blood were obtained from each calf by jugular vein puncture, collected directly into 50ml tubes containing 600U of heparin and centrifuged at 1200rpm for 12 minutes. The plasma, together with the buffy coat containing some erythrocytes, was transferred to another 50ml tube and centrifuged as before. After the plasma was discarded, the erythrocytes were lysed by hypotonic shock for 30 seconds using 8ml of distilled water. Isotonicity was restored by the addition of 8ml of 2X phosphate buffered saline. This preparation was centrifuged at 4°C, at 1500rpm for 12 minutes, the supernatant discarded and the leukocyte pellet resuspended in 5ml of growth medium RPMI 1640 containing 10% calf serum negative for antibodies to BLV, penicillin 200U/ml, streptomycin 200μg/ml and mycostatin 50U/ml. Leukocytes were counted and the concentration adjusted to 1.5 x 10^6 leukocytes per ml. Ten ml of the leukocyte suspension were cultured in tubes at 37°C for 48 to 72 hours.

RESULTS

BLV transmission with blood. Calves inoculated subcutaneously with heparinized whole blood obtained from a BLV-infected cow, developed antibodies to gp55 of BLV as early as 1 month after inoculation. Although BLV infection was established with as little as 0.5ml, not all inoculated calves were infected at the end of the 6 month experimental period (Table 1).

BLV transmission with plasma. All 10 calves inoculated with varying amounts of plasma remained serologically negative during the experimental period (Table 1).

BLV transmission with leukocytes. One of each of the 2

calves inoculated with 10^6 or 10^7 viable leukocytes developed antibodies to gp55 of BLV. The 6 calves inoculated with 10^3 to 10^5 leukocytes remained serologically negative (Table 1).

Monitoring of horizontal transmission. Two calves of the control group that had remained negative for BLV antibodies died of clostridial infections during the 3rd and 4th months of the experiment. The 5 remaining calves were serologically negative to BLV at the end of 6 months (Table 1).

BLV transmission with milk. Four calves born to antibody negative cows inoculated with milk from antibody negative donors did not develop antibodies to BLV. However, 3 calves also born to antibody negative dams developed antibody to gp55 of BLV after being inoculated with milk from antibody positive donors (Table 2). The distribution of the absolute lymphocyte counts of these 7 calves according to Bendixen's hematological key during 6 consecutive months is presented in Figure 2. Type-C viral particles were observed in ultrathin sections of leukocytes obtained from the 3 calves that had developed antibody specific for gp55 of BLV. These particles were usually observed in the cell debris around intact leukocytes and inside cytoplasmic vacuoles (Fig. 3 and 4). Budding of viral particles was not observed. Type-C viral particles were not observed in leukocytes from serologically negative calves. One of 5 calves born to antibody positive cows, inoculated with milk from antibody negative donors, developed antibody to gp55 of BLV. The other 4 remained serologically negative. On the other hand, of 7 similar calves inoculated with milk from antibody positive donors, 5 developed antibody to gp55 of BLV (Table 2). A photograph of the AGP test utilized for the detection of antibody to BLV as evidence of acquired infection is presented in Figure 5.

DISCUSSION

In these experiments, the development of specific antibody to the major glycoprotein of BLV, gp55, was taken as evidence of acquired infection in calves inoculated with either blood, plasma, leukocytes or milk. The infectivity of whole blood and leukocytes obtained from an antibody and virus positive donor was demonstrated. The success in transmitting BLV with as little as 0.5ml of whole blood confirms the hypothesis that premunition against Anaplasma and Babesia organisms by the inoculation of fresh blood obtained from clinically healthy, BLV-infected cattle, is one factor responsible for the high incidence of BLV infection in Brazilian dairy herds. In a field situation, young calves are usually inoculated subcutaneously with 2ml to stimulate immunity against these organisms. This amount more than insures iatrogenic transmission of BLV. Our

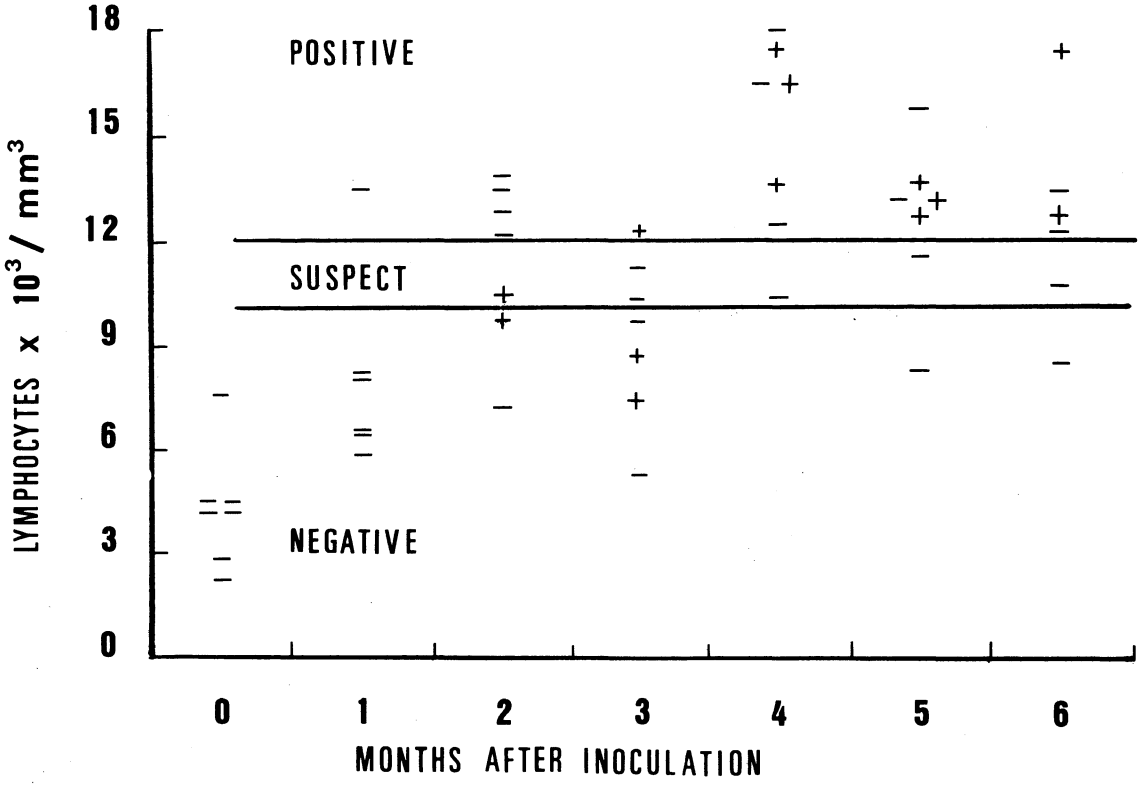

Fig. 2. *Distribution of the absolute lymphocyte counts according to Bendixen's hematological key, of calves serologically negative (–) or positive (+) after the experimental inoculation of milk from cows free of or infected with BLV.*

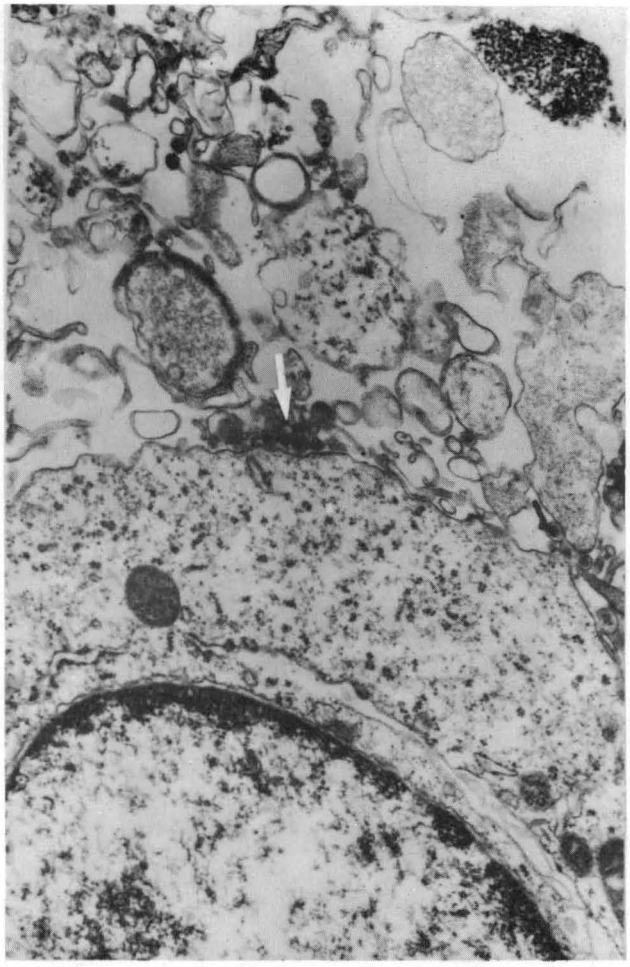

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Fig. 3. *Electron micrograph of a leukocyte culture from a calf that developed antibody to gp55 after the intra-abdominal inoculation of milk from BLV-infected cows. The arrow shows characteristic type-C viral particles along the cell membrane of a lymphocyte. 20,000x.*

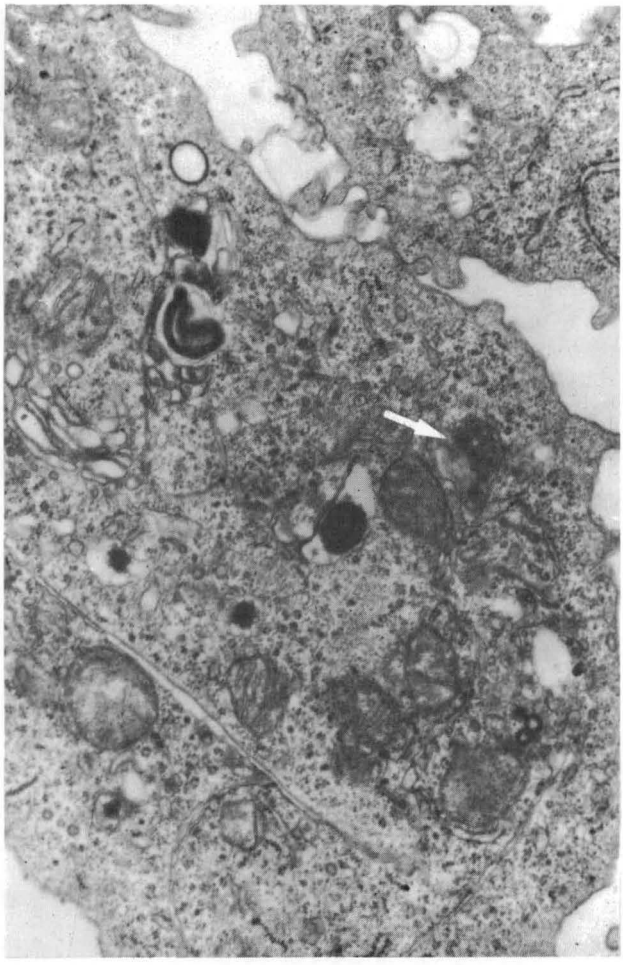

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Fig. 4. *Electron micrograph of a leukocyte culture from a calf that developed antibody to gp55 after the intra-abdominal inoculation of milk from BLV-infected cows. The arrow shows type-C viral particles inside a cytoplasmic vacuole. 21,000x.*

results did not demonstrate transmission of BLV when plasma was used as inoculum. It has been previously shown that there is no true viremia in BLV infection and that BLV is strictly lymphocyte-associated (Baliga & Ferrer 1977). Our results lend support to those findings. Transmission of BLV was successful with 10^6 and 10^7 viable leukocytes. Smaller numbers of leukocytes failed to transmit the BLV infection during the 6 month period. Van Der Maaten and Miller (1977) reported that as few as 2.5 x 10^3 leukocytes were required to transmit BLV infection to susceptible calves of European origin. The higher number of leukocytes required for transmission in our experiments could have been due to the utilization of genetically different calves, random crosses of Nelore, Gir and Holstein that may be more resistant to the infection. The leukocytes we used were cultured for 18 hours prior to being inoculated, which could have affected their infectivity. Success in the transmission with blood raises the issue of whether blood sucking arthropods and insects, so abundant in the tropics, could also be contributing towards the dissemination of BLV in our herds. Bech-Nielsen et al. (1978) demonstrated the presence of infectious BLV in horseflies (*Tabanus nigrovittatus* Macquart) that had been allowed to feed on a BLV-infected cow and observed a higher frequency of contact infection during the summer months when insects in general are more active. However, direct evidence of BLV transmission by tabanids, mosquitos, ticks and possibly hematophagous bats is lacking. No evidence of horizontal transmission was obtained from the uninoculated control calves.

Milk obtained from cows with serum antibody to gp55 contained infectious BLV as shown by the development of specific antibody in experimentally inoculated calves. All calves born to antibody negative cows were infected with BLV 3 months after the inoculation of this milk and short-term leukocyte cultures prepared from these animals at the end of 6 months contained characteristic type-C viral particles. Similar calves inoculated with milk from antibody negative cows did not become infected during the same 6 months and their leukocytes did not have viral particles. When the absolute lymphocyte counts of all these calves, recorded monthly for 6 months, were superimposed on a chart representative of Bendixen's hematological key corrected for age (Bendixen 1960), it was noted that of the 34 AGP antibody negative

Table 2. *Transmission of BLV to calves[(a)] inoculated intra-abdominally with milk from non-infected and BLV-infected cows*

BLV antibody		Months after inoculation of milk							% infected
Dams	Milk donors	0	1	2	3	4	5	6	
–	–	0/4[(b)]	0/4	0/4	0/4	0/4	0/4	0/4	0.0
–	+[(c)]	0/3	0/3	2/3	3/3	3/3	3/3	3/3	100.0
+[(d)]	–	0/5	0/5	0/5	1/5	1/5	1/5	1/5	20.0
+	+	0/7	0/7	1/7	3/7	4/7	4/7	5/7	71.4

(a) One to five month old calves injected with 100ml of milk.

(b) Number of calves with antibody to gp55 of BLV/Number of calves tested.

(c) Antibody to gp55 of BLV in the serum, but not in the milk.

(d) Antibody to gp55 of BLV in the serum one month after calving.

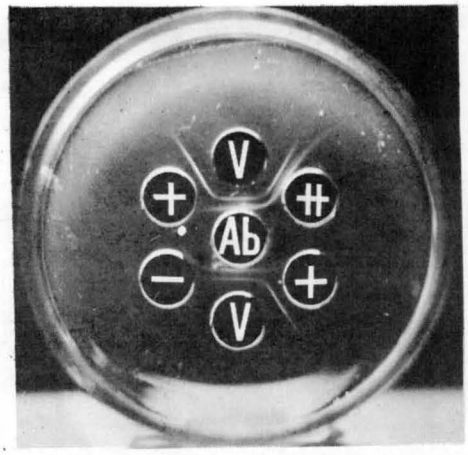

Fig. 5. *The agar gel precipitin test to assay for antibody to BLV. The positive reference serum in the central well (Ab) reacts with the dual antigen (V) giving 2 lines of precipitation. Test sera are located in the lateral wells. The wells marked + contain serum with antibody to gp55. The well marked ++ contains a serum with antibody to both gp55 and p24. The well marked – contains a serum without antibody.*

serum samples, 12 (35.3%) would have been considered positive and 5 (14.7%) suspect for BLV infection on the basis of hematology alone. Conversely, of 13 serologically positive sera, 3 were identified as negative and 1 as suspect, as judged by absolute lymphocyte counts. These results demonstrate the low sensitivity of hematological keys in the proper identification of cattle free of or infected with BLV.

Less than 50% of calves born to antibody positive cows, that had been inoculated with milk from infected donors, developed antibody to gp55 by 3 months and only 71.4% were positive by the 6th month. These results indicate that the progeny of antibody positive cows are more resistant to BLV infection through milk than the progeny of antibody negative cows. A possible explanation for this finding could be the persistence of passively acquired neutralizing antibody in the absence of detectable precipitating antibody and subsequent neutralization of part or all of the infectivity of the milk inoculated. Previous experiments with BLV-infected milk using a sheep bioassay (Miller & Van Der Maaten 1979) showed that only 36.4% of the inoculated sheep acquired the infection 6 months after inoculation. We suspect that sheep may be less susceptible than calves to experimental BLV infection. One of 5 calves born to antibody positive cows developed antibody to gp55 3 months after the inoculation of milk obtained from negative donors. This was very likely due to either intrauterine or perinatal infection with BLV, an explanation supported by the knowledge that approximately 18% of infected cows infect their progeny *in utero* (Piper et al. 1979).

Although the importance of milk in the natural transmission of BLV was not evaluated in the present work, the demonstration of BLV infectivity in fresh milk points to the transmission through ingestion of BLV-contaminated milk. Studies done by Ferrer & Piper (1978) showed that 17% of calves nursed on milk from their infected dams acquired BLV infection over an observation period of 18 months. Our preliminary results from experiments in progress suggest that feeding calves from a pool of milk from infected cows may play a more important role in the transmission of BLV than previously suspected. When the infection is endemic, as seems to be the case in our dairy herds, BLV transmission through milk should not be underestimated. This is especially true of dairy herds where young calves are fed from pools of freshly drawn, unheated milk obtained from BLV-infected, clinically healthy cows in an advanced stage of lactation. It has been shown that BLV infectivity of milk-virus mixtures is destroyed after a short-time pasteurization procedure (Baumgartener et al. 1976). However, it is not known whether infectivity of BLV-infected leukocytes in milk is similarly destroyed. This raises questions related to human health when raw milk is consumed, although limited serological surveys indicate the absence of antibody in persons under "high risk" (Olson & Driscoll 1978).

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