SUSCEPTIBILITY OF THE WATER BUFFALO *(Bubalus bubalis)* TO ENZOOTIC BOVINE LEUKOSIS VIRUS[1]

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Fluidos de culturas celulares infectadas com o vírus da leucose enzoótica bovina (VLB) e sangue total heparinizado obtido de uma vaca virologicamente e sorologicamente positiva ao VLB, foram inoculados intra-abdominalmente em 14 búfalos (*Bubalus bubalis*). Depois de oito meses de observação, quatro búfalos inoculados com sangue possuiam anticorpos específicos para a glicoproteína maior (gp55) do VLB, os quais foram detectados através da prova de imunodifusão para o VLB.

Culturas de leucócitos isolados do sangue periférico dos búfalos sorologicamente positivos, continham partículas virais de Tipo C, confirmando-se assim, a susceptibilidade experimental de *Bubalus bubalis* ao VLB. Por outro lado, um levantamento para anticorpos realizado no soro de 234 búfalos, não forneceu evidência da infecção natural com o VLB. Estes resultados indicam que *Bubalus bubalis* é susceptível à infecção experimental com o VLB, mas o VLB não parece circular naturalmente nesta espécie.

TERMOS DE INDEXAÇÃO: Leucose enzoótica bovina, vírus, *Bubalus bubalis*, búfalos.

ABSTRACT.- Tissue culture fluids containing enzootic bovine leukosis virus (BLV) and heparinized whole blood obtained from a virologically and serologically BLV-positive cow, were inoculated intra-abdominally into 14 water buffaloes (*Bubalus bubalis*). After eight months of observation, four buffaloes inoculated with blood had specific antibodies against the major glycoprotein (gp55) of BLV, as detected by the immunodiffusion test.

Cultures of leukocytes isolated from the peripheral blood of the serologically positive buffaloes contained Type-C viral particles, confirming the experimental susceptibility of *Bubalus bubalis* to BLV. On the other hand, a survey for antibodies performed on the sera of 234 buffaloes did not provide evidence of natural infection with BLV. These results indicate that *Bubalus bubalis* is susceptible to experimental infection with BLV, but that BLV does not seem to cycle naturally in this species.

INDEX TERMS: Enzootic bovine leukosis, virus, *Bubalus bubalis*, buffaloes.

INTRODUCTION

Enzootic bovine leukosis is a neoplasia of the lymphoid tissue of cattle caused by a Type-C virus (Miller et al. 1969), classified as an RNA tumor virus and currently known as bovine leukosis virus (BLV). The bovine seems to be the only natural host of BLV, although sheep (Wittmann & Urbanek 1969) and goats (Hoss & Olson 1974) can be experimentally infected. Outbreaks of leukosis of unknown etiology have been described in sheep (Boyt et al. 1976) and in water buffaloes (Singh et al. 1979). There is, at this time however, no serological or virological evidence to show that the water buffalo, *Bubalus bubalis*, is susceptible to natural or experimental infection with BLV. We present herein, the results of experiments performed to determine whether the water buffalo can be experimentally infected with BLV and the results of a preliminary sero-epidemiological study to determine if BLV naturally occurs in this species.

MATERIALS AND METHODS

Enzootic bovine leukosis antigen. Standard antigen for use in the immunodiffusion test, containing both p24 (Miller & Olson 1972) and gp55 (Onuma et al. 1975), was prepared from fluids of a fetal lamb kidney (FLK) cell line, persistently infected with BLV (Van Der Maaten et al. 1974), as previously described (Romero & Rowe 1981).

Enzootic bovine leukosis antiserum. Positive reference serum was obtained from a cow with terminal lymphosarcoma. This serum gave two lines of identity with a serum from a sheep that had been experimentally infected with BLV (Onuma et al. 1975), when reacted against the standard antigen.

Immunodiffusion test. Specific antibodies to BLV antigens (Miller & Van Der Maaten 1977) were detected in a double immunodiffusion test using 0.7% agar. Test conditions have been previously described (Romero & Rowe 1981).

Short-term leukocyte cultures. Thirty ml of blood were

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collected by jugular vein puncture directly into sterile tubes containing heparin (200U), penicillin (20,000U), streptomycin (20,000μg) and 0.2ml of phytohemagglutinin M, kept in an upright position and transported to the laboratory for processing. Six hours later, the plasmas and buffy coats were transferred to another tube and incubated overnight at 37°C. The following morning, the plasmas containing the leukocytes and some erythrocytes were centrifuged at 1,200rpm for 12 minutes at 4°C. Plasmas were harvested and the cell pellets subjected to hypotonic shock in 15ml of sterile distilled water for 20 seconds to lyse the erythrocytes. Then, 15ml of 2X phosphate buffered saline and 3ml of BLV antibody-free calf serum were added to restore tonicity and maintain cell viability, respectively. The cell suspensions were centrifuged as above, the supernatants discarded and the leukocytes resuspended in tissue culture medium RPMI 1640 containing 10% autologous plasma, 20% fetal calf serum, 200U of penicillin, 200μg of streptomycin, 25U of mycostatin and 0.01 ml of phytohemagglutinin M per ml of medium. Leukocytes were counted and cell suspensions adjusted to contain $1x10^6$ cells/ml. Ten ml of each suspension were grown in glass milk dilution bottles for 24 hours at 37°C before being processed for ultrathin sectioning.

Electron microscopy. Leukocyte cultures were fixed for 20 minutes by adding an equal volume of 6% glutaraldehyde, centrifuged at 2,000rpm for 10 minutes, fixed in 1% osmium tetraoxide for 45 minutes, stained with 0.5% uranyl acetate, dehydrated in ethanol and embedded in polylite (Coiro & Brunner 1972). Sections were cut, stained with 0.5% uranyl acetate and lead citrate (Reynolds 1963) and examined with a Phillips 301 electron microscope at 80kV.

Experimental infection. Twenty-one 16 month old water buffaloes, free of antibodies to BLV were randomly allotted to three treatment groups of seven buffaloes each. Buffaloes in one group were inoculated intra-abdominally with 100ml of tissue culture fluids, harvested from the FLK-BLV infected cell line and filtered through a Seitz No.4664 filter pad to remove all cells. A second group of seven buffaloes was inoculated with whole heparinized fresh blood in quantities that varied from 50 to 80ml. The blood donor was an adult cow with persistent lymphocytosis and persistent antibodies to gp55, whose peripheral blood leukocytes produced large numbers of Type-C viral particles after stimulation with phytohemagglutinin M. The seven remaining buffaloes were left uninoculated. In order to verify the infectivity of the inocula, three antibody-free eight month old calves were inoculated intra-abdominally with 100ml of the tissue culture fluids and three others with 50 ml of the whole blood. An additional three antibody negative calves of the same age were left as uninoculated controls. All buffaloes and calves were bled before inoculation and thereafter at monthly intervals for a period of eight months. Serum obtained was tested in the double immunodiffusion test. The development of specific antibodies was considered as evidence of infection with BLV. Eight months after the experimental inoculation, short-term leukocyte cultures were prepared from three serologically positive buffaloes and examined by electron microscopy for the presence of Type-C viral particles.

Natural infection. Blood was obtained from water buffaloes of the Brazilian States of Amapá, Pará and Rio de Janeiro. The serum was tested for the presence of specific antibodies to BLV antigens in the immunodiffusion test.

RESULTS

Reference antiserum produced lines of precipitation against p24 and gp55 contained in the reference antigen. Sera from experimentally infected calves and buffaloes produced only one line of precipitation that corresponded to gp55, when reacted against the reference antigen (Figure 1).

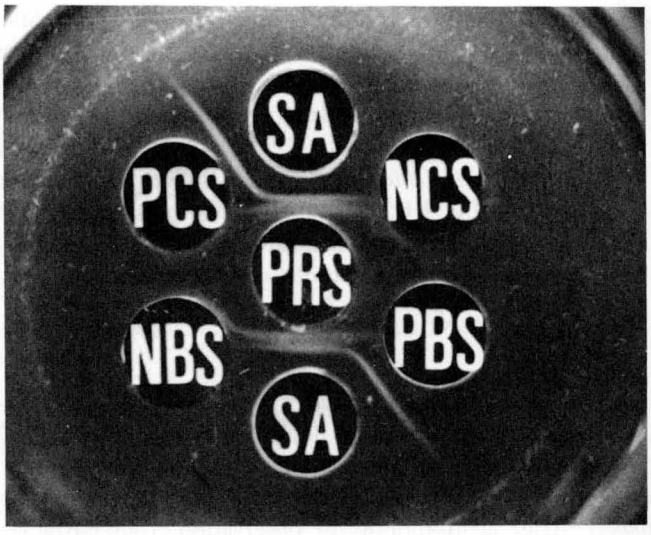

Fig. 1. *Immunodiffusion test for antibodies to BLV antigens. PRS-Positive reference serum; SA-Standard antigen; PCS-Positive calf serum; PBS-Positive buffalo serum; NCS-Negative calf serum; NBS-Negative buffalo serum.*

The experimental inoculation of calves with either filtered tissue culture fluids or heparinized whole blood resulted in the development of specific antibodies by three and one months post-inoculation, respectively, thus confirming the infectivity of both inocula. Attempts to infect seven buffaloes with tissue culture fluids were not successful. On the other hand, four of six buffaloes inoculated with whole blood were infected with BLV at the end of the eight month observation period (Table 1).

Electron microscopic examination of ultrathin sections of all three leukocyte cultures revealed the presence of small numbers of Type-C viral particles within intracytoplasmic vacuoles and in the cell debris surrounding the leukocytes (Figure 2). Budding particles were not observed.

All 234 buffalo serum samples collected as part of the sero-epidemiological survey, tested in the immunodiffusion test, were negative for specific antibodies to BLV antigens (Table 2).

Table 1. *Development of precipitating antibodies to gp55 of BLV in* Bubalus bubalis *and* Bos sp. *experimentally inoculated with infectious BLV*

Genus	Inoculum	Antibodies to BLV in the serum							
		0[a]	1	2	3	4	5	7	8
Bubalus	FLK fluids	0/7[b]	0/7	0/7	0/7	0/7	0/7	0/7	0/7
Bos	"	0/3	0/3	0/3	2/3	2/3	2/3	2/3	2/3
Bubalus	BLV-blood	0/7	0/7	0/7	1/7	1/6	1/6	2/6	4/6
Bos	"	0/3	1/3	1/3	2/3	2/3	2/2	2/2	2/2
Bubalus	None	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7
Bos	"	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

(a) Months after experimental inoculation.

(b) Number with precipitating antibodies/number tested.

Table 2. *Lack of serological evidence of natural infection with BLV in* Bubalus bubalis

State	Age in months				Total
	13–18	19–30	31–48	49+	
Amapá	0/9[a]	0/79	0/36	0/43	0/167
Pará	–	0/14	0/16	0/16	0/46
Rio de Janeiro	0/21	–	–	–	0/21

(a) Number with precipitating antibodies/number tested.

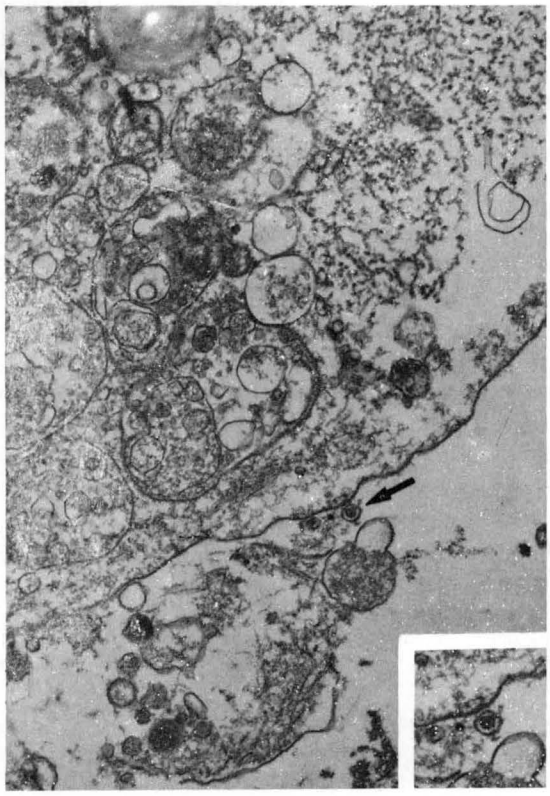

Fig. 2. *Electron micrograph of a thin section of a leukocyte culture from a BLV-serologically positive buffalo showing a Type-C viral particle. 31000x. The inset shows the same viral particle at higher magnification. 52000x.*

DISCUSSION

The results of our study have shown that both the filtered tissue culture fluids from the FLK-BLV cell line and the whole heparinized blood from a cow with serum antibodies to gp55, contained infectious BLV, as demonstrated by the seroconversion of experimentally inoculated calves. Buffaloes inoculated with the tissue culture fluids did not become infected with BLV during the same observation period. It is not known why the infectious fluids failed to establish BLV infection in any of the seven buffaloes inoculated. Similar inocula used by Van Der Maaten and Miller (1975) proved to be infectious for ewes and lambs. In our experiments we inoculated buffaloes, both older and considerably larger in size than any previously described susceptible host. There is also the possibility that the Seitz filter pad may have retained part of the infectivity of the FLK-BLV fluids. We speculate that a combination of these factors may have contributed to the lack of serological response in these buffaloes. On the other hand, the antibody response demonstrated in two of the three calves inoculated with identical material indicates a lower threshold of susceptibility of the young *Bos* calf. Four of the six surviving buffaloes inoculated with BLV-infected blood were reactive by the end of the experimental period. The success of the whole blood inoculum in the transmission of BLV infection could be attributed to the presence of viable BLV-infected leukocytes, that may have gone on to actively synthesize infectious virus, thereby improving the conditions for establishing a permanent infection in these buffaloes. Further evidence of the establishment of the infection in serologically positive buffaloes was obtained when Type-C viral particles were visualized with the aid of the electron microscope. The number of particles observed was relatively small, a finding that may have been associated with the fact that the infection was in an early stage of development.

Our limited serological survey to detect antibodies to BLV in the sera of 234 buffaloes from three regions of Brazil showed that natural infection with BLV in buffaloes is either rare or does not occur. Singh et al. (1979) examined approximately 51,000 buffalo carcasses and suspected lymphosarcoma in 1,277 cases, on the basis of gross lesions. Histopathological examination confirmed the lymphosarcoma diagnosis in 170 out of 283 cases processed. Since no serological testing was performed on any of these buffaloes, it is impossible to establish the role that BLV may have played in the occurrence of the lymphosarcomas. As there has been recent interest in buffalo meat as a new source of protein, it would be interesting to undertake similar studies here in Brazil, performing the serological tests necessary to determine whether there is a link between the lymphosarcoma and the

causative agent of enzootic bovine leukosis in buffaloes.

Our studies indicate that although the water buffalo (*Bubalus bubalis*) is susceptible to experimental infection, BLV does not seem to cycle naturally in this species.

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REFERENCES

Boyt W.P., MacKenzie P.K.I & Emslie V.W. 1976. Enzootic leucosis in a flock of sheep in Rhodesia. Vet. Rec. 98:112-115.

Coiro J. & Brunner Jr A. 1972. Polylite 8001: A new embedding medium for electron microscopy. Revta Microscopia Eletrônica 1:12.

Hoss H.E. & Olson C. 1974. Infectivity of bovine C-type (leukemia) virus for sheep and goats. Am. J. Vet. Res. 35:633-637.

Miller J.M., Miller L.D., Olson C. & Gillette K.G. 1969. Virus-like particles in phytohemagglutinin-stimulated lymphocyte cultures with reference to bovine lymphosarcoma. J. Natl Cancer Inst. 43:1297-1305.

Miller J.M. & Olson C. 1972. Precipitating antibody to an internal antigen of the C-type virus associated with bovine lymphosarcoma. J. Natl Cancer Inst. 49:1459-1462.

Miller J.M. & Van Der Maaten M.J. 1977. Use of glycoprotein antigen in the immunodiffusion test for bovine leukemia virus antibodies. Europ. J. Cancer 13:1369-1375.

Onuma M., Olson C., Baumgartener L.E. & Pearson L.D. 1975. An ether-sensitive antigen associated with bovine leukemia virus infection. J. Natl Cancer Inst. 55:1155-1158.

Reynolds E.S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. 17:208.

Romero C.H. & Rowe C.A. 1981. Enzootic bovine leukosis virus in Brazil. Trop. Anim. Hlth Prod. 13:107-111.

Singh B., Singh K.P., Parihar N.S., Bansal M.P. & Singh C.M. 1979. Clinicopathological studies on lymphosarcoma in Indian buffaloes (*Bubalus bubalis*). Zbl. VetMed. 26A:468-481.

Van Der Maaten M.J., Miller J.M. & Boothe A.D. 1974. Replicating Type-C virus particles in monolayer cell cultures of tissues from cattle with lymphosarcoma. J. Natl Cancer Inst. 52:491-497.

Van Der Maaten M.J. & Miller J.M. 1975. Induction of lymphoid tumors in sheep with cell-free preparations of bovine leukemia virus. Bibl. Haemat. 43:377-379.

Wittmann W. & Urbanek D. 1969. Untersuchungen zur Aetiologie der Rinderleukose. 8. Uebertragungsversuche mit Blutleukose-kranker Rinder auf Schaflaemmer (Kurzmitteilung). Arch. Exp. VetMed., B, 23:709-713.