Biochemical and Epidemiological Studies on Bovine Leukemia Virus (BLV)

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Abbreviations Used

ID gp 60:	Immunodiffusion test based on BLV glycoprotein (MW = 60000) as antigen
RIA gp 60:	Radioimmunoassay test based on BLV glycoprotein (MW = 60000) as antigen
RIA p 24:	Radioimmunoassay test based on BLV internal protein p 24 (MW = 24000) as antigen
BLV:	Bovine Leukemia Virus
PL:	Persistent Lymphocytosis
EBL:	Enzootic Bovine Leucosis
SBL:	Sporadic Bovine Leucosis

1. Enzootic Bovine Leucosis: The Disease

One generally distinguishes two types of bovine leucoses: an enzootic type and a sporadic type [3].

Here, we will be solely dealing with the enzootic type, the so-called Enzootic Bovine Leucosis (EBL) The basic features of this lymphoproliferative disease are:

- it is contagious; it spreads within a herd through contacts, saliva, milk, ... and from herd to herd mainly through commercial exchanges;
- it is induced by Bovine Leukemia Virus (BLV) a retrovirus *exogenous* to the bovine species [10];
- it involves the B lymphocytes [15, 17]
- it can be easily transmitted by the virus to cattle or sheep. Experimental BLV infection (but no clinical disease, sofar) has been obtained in goats and chimpanzees. No natural transmission of BLV to man seems to occur [3, 6];
- BLV infected animals develop a humoral response directed against the viral antigens.

Enzootic bovine leucosis is a chronic disease that develops over a long period of time (several years generally). In natural conditions, very few cattle less than 2 years of age harbor antibodies to BLV antigens [11]. The same is true where BLV is searched for by its biological property of inducing syncytia [7] or early polykaryocytosis. If, however, a search is made *among the off-*

spring of BLV infected parents, it appears that as much as *14% of calves are infected at birth.* As discussed in [3], this situation reflects congenital infection by BLV and not true vertical transmission. BLV infection always induces a humoral antibody response and sometimes induces an hematological disorder called "Persistent Lymphocytosis" (PL). In such cases, the lymphocyte population is made of normal cells and a variable percentage of tumor cells as proven by molecular hybridization studies [3, 10]. PL has a genetic background [1] being much more frequent in some families within a breed than in other families of the same breed. With time, tumor development may occur, a phenomenon very poorly understood at the present time. Tumors may appear practically everywhere, in the digestive tract, the respiratory tract, muscles, ... but they are always lymphoïd. Most lymph nodes are enlarged, sometimes some of them only [2, 14, 19].

2. BLV: The Causative Agent of EBL

BLV is a retrovirus [3] produced in large quantities by essentially two cell culture systems, the Fetal Lamb Kidney cell-line [18] and the Tb_1Lu, a bat cell-line [13]. Morphologically the virus can be considered as a C-type although it displays some unusual peculiarities [3].

2.1. BLV Genome

It is a 60–70 S RNA molecule associated with reverse transcriptase. The number of genes and their order along the RNA molecule are not precisely known so far. DNA complementary to the RNA genome and representative of it, has been synthesized and extensively used in molecular hybridization experiments. The results of these studies are illustrated in Fig. 1 A and 1 B and call for the following comments:

If we take salmon sperm DNA as a control (histogram 11) it appears that normal bovine DNA hybridizes some 4% better than the control. We now know that this is due to contamination of 70S RNA used as template by 28S ribosomal RNA. DNAs from FLK-BLV producing cells (histogram 2), from buffy coat cells of an animal in persistent lymphocytosis with tumor (histogram 4) and from bovine enzootic tumor (histogram 5) hybridize with a maximum of 45% of the probe at a Cot value of 30000. This result is compatible with one proviral DNA copy per haploïd genome, if every cell contains the viral information. DNAs from tissues infiltrated with tumorous lymphocytes, hybridize to BLV c DNA to an extent that is roughly proportional to the degree of infiltration (histograms 3, 6 and 7). Sheep infected by BLV (histograms 9 and 10) show the same pattern of hybridization as cattle do. DNAs from human leukemic cells do not anneal to BLV c DNA.

That BLV is exogenous to the bovine genus was definitely established by recycling the ^3H BLV c DNA probe on normal bovine DNA. Results are illustrated in Fig. 1 B. They prove 1°) that BLV is largely if not totally exogenous to the bovine genus, 2°) that EBL is an infections disease, thus amenable to eradication.

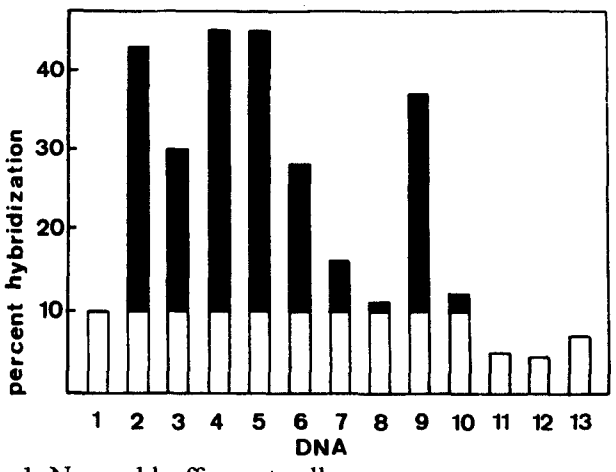

Fig. 1A. Hybridization of BLV ^3H cDNA to various bovine, ovine and human cellular DNAs. Hybridizations between 2400 cpm of ^3H cDNA (specific activity: $1{,}8 \times 10^7$ cpm/μg) and 250 μg of cellular DNA were performed in 0,4 M phosphate buffer (pH = 6,8) and 0,05% SDS in a final volume of 85 μl at 68°C. At a Cot value of 30000. samples were assayed for S_1 resistance. Source of DNA:

1. Normal buffy coat cells.
2. FLK cell line.
3. Buffy coat cells from a cow in persistent lymphocytosis without tumors.
4. Buffy coat cells from a cow in persistent lymphocytosis with tumors.
5. EBL tumor.
6. Liver moderately infiltrated with lymphocytes (EBL).
7. Kidney slightly infiltrated with lymphocytes (EBL).
8. Tumorous lymphnode from an SBL case.
9. Cutaneous tumor from a sheep infected with BLV.
10. Liver from the same leukemic sheep.
11. Salmon sperm.
12. Human chronic lymphatic leukemia.
13. Human chronic lymphatic leukemia.

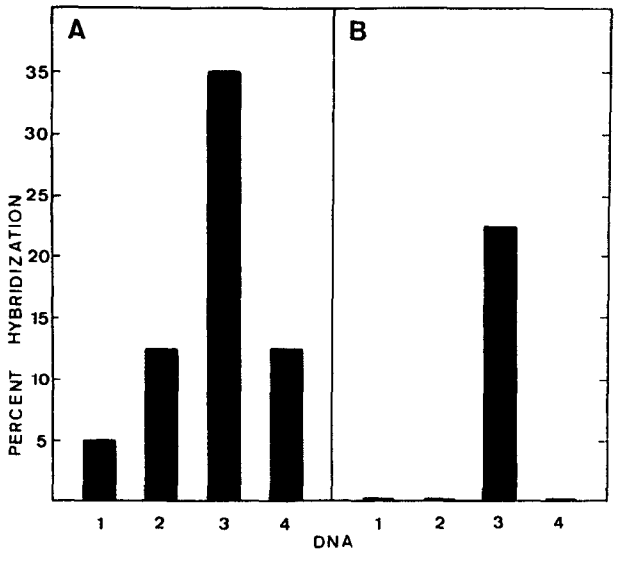

Fig. 1B. Hybridization of BLV ^3H cDNA (panel A) and recycled BLV ^3H cDNA (panel B) to the following cellular DNAs: 1. Salmon sperm; 2. normal bovine buffy coat cells; 3. EBL tumor; 4. SL tumorous lymph node. 2400 cpm of BLV ^3H cDNA (or recycled cDNA) and 250 μg of cellular DNA were hybridized in 0,4 M phosphate buffer (pH = 6,8) and 0,05% SDS. At a cellular Cot value of 30000 samples were assayed for S_1 resistance

2.2. BLV Proteins

BLV virions, at least, contain:
2 glycoproteins: gp60 and gp30 linked together within the virus envelope
4 non-glycosylated polypeptides p24, p15, p12 and p10
one reverse transcriptase, MW = 58000–70000 ([4] and Drescher et al., in preparation)

That the above mentioned proteins are indeed viral antigens rests upon two lines of evidence:

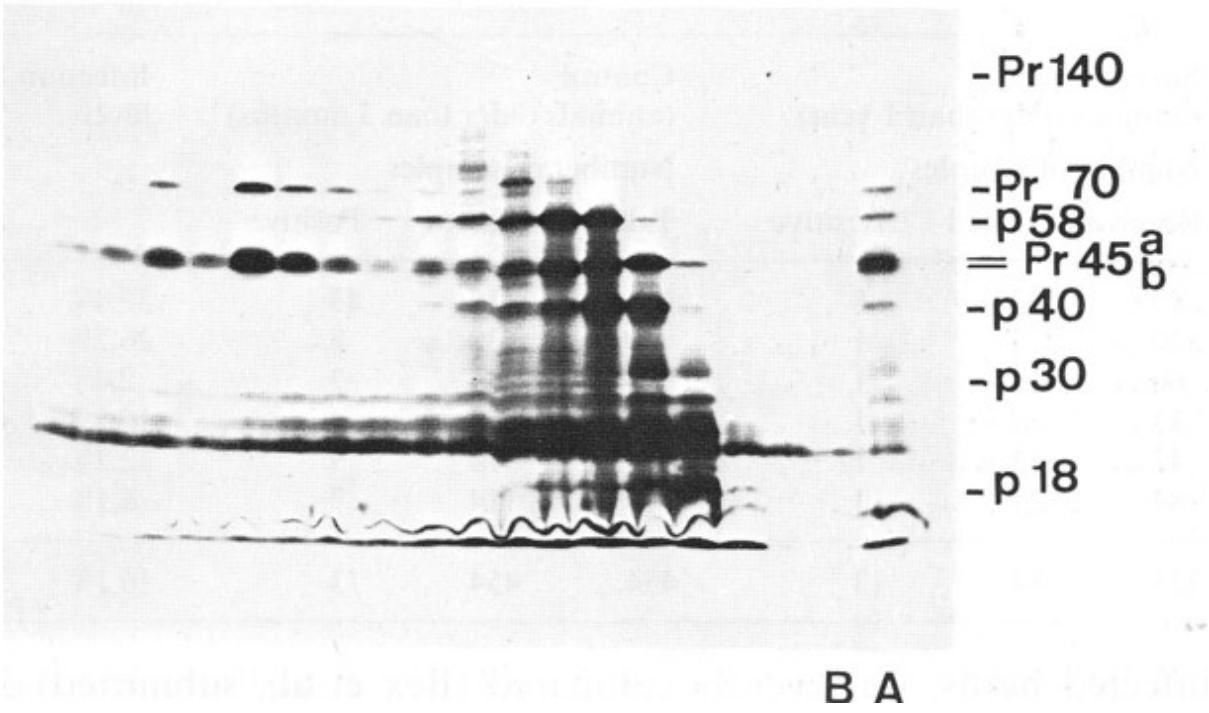

Fig. 2. Fluorograph of SDS-polyacrylamide gel of immune precipitates. Oocytes microinjected with a 1 mg/ml solution of 30–40S BLV RNA were labeled for 20 hours in Barth medium with 2 mCi/ml of ^{3}H leucine, lysed immediately (D) or chased in culture medium containing excess unlabeled leucine for 100 hours (E) and 300 hours (F) and then lysed. Non injected control oocytes were incubated in parallel (A–C) and indirect immune precipitation carried out on the same amount of homogenate with 4 µl of polyvalent anti-BLV serum. After 2 hours at 37°C and overnight incubation at 4°C, 100 µl of a 10% W/V Staphylococcus aureus suspension was added and incubation continued for another 4 hours at 4°C. Bacterial suspension was collected by centrifugation and washed. Immune complexes were separated by boiling 2 minutes in SDS containing buffer and analyzed by electrophoresis on a 15% polyacrylamide slab gel in the presence of SDS. G: ^{3}H amino acids labeled BLV marker

1. BLV infected animals synthesize antibodies directed against at least 4 of them (gp60, gp30, p24, p15).
2. BLV infected cells synthesize protein precursors to the gag group (p24, p15), a presumed gag-pol precursor and a precursor to BLV glycoproteins. In vitro protein synthesizing systems programmed with BLV 30–40S viral RNA synthesize gag precursors and the putative gag-pol precursor (Fig. 2). In adequate systems, these precursors mature into viral structural antigens [8]. Subgenomic fractions of BLV 35S RNA code for a number of polypeptides with molecular weights as 58000, 45000, 40000, 35000, 18000, ... (Fig. 3).

The 58000, 45000 and 40000 Molecular weight polypeptides are coded by m RNAs sedimenting in sucrose gradients in the 16S to 18S region and do not seem to be related one to the other by fingerprint analysis. Apparently, they are not of viral origin as preannealing of RNAs with BLV 35S-c DNA does not block their synthesis in reticulocyte cell-free systems.

On the other hand, polypeptide 18000 is undoubtedly of viral origin. Its biosynthesis in reticulocyte lysates is blocked if 16S to 18S m RNA is preannealed with BLV 35S-c DNA. Our present efforts are attempting to identify the region of the BLV genome coding for polypeptide 18000.

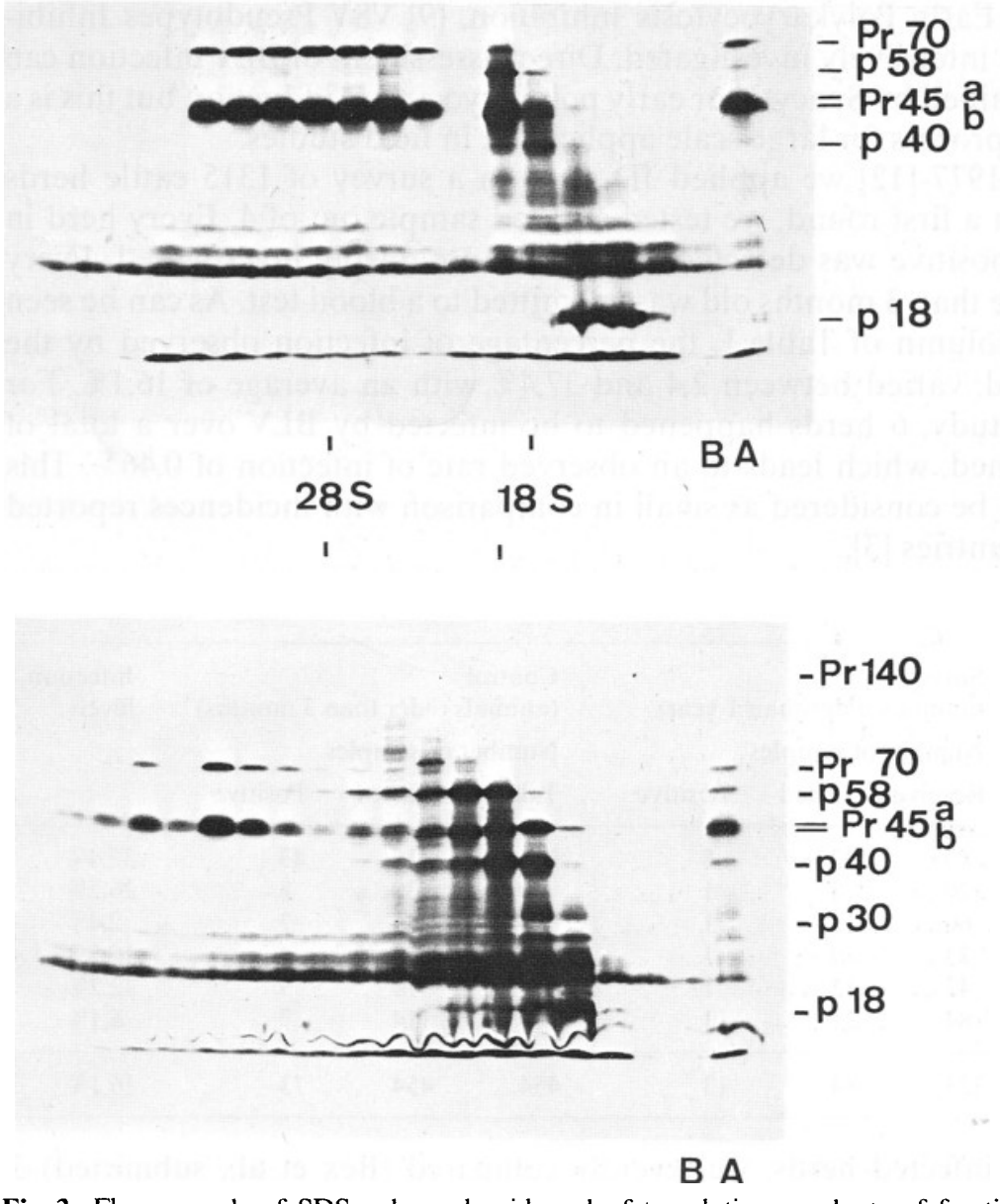

Fig. 3. Fluorograph of SDS-polyacrylamide gel of translation products of fractionated BLV virion RNA. Heat denaturated (95°C for 1 minute in Tris-HCl, 10^{-2} M, pH = 7,4, EDTA 10^{-3} M) BLV 60–70S RNA (80 μg) was fractionated by oligo dT cellulose chromatography and the poly-A-containing fraction (lower panel) or poly-A-deficient fraction (upper panel) were sedimented in a linear 15–30% glycerol gradient in Tris-HCl 10^{-2} M, pH = 7,4, NaCl 0,1 M, EDTA 0,01 M in a SW 41 rotor at 40000 rpm for 4 hours at 20°C. The RNA of each fraction was precipitated twice with ethanol, calf liver t-RNA being added as a carrier. One fourth of the RNA of each fraction was used to program protein synthesis in a messenger-dependent reticulocyte cell-free lysate. Analysis of translation products is made on a 15% SDS-polyacrylamide slab gel.
Track A: complete translation product of poly A-containing (lower panel) or poly A-deficient (upper panel) BLV RNA.
Track B: control, no RNA added

3. Epidemiology of BLV

Search for antibodies to BLV structural antigens is the present basis of all epidemiological investigations and eradication campaigns [3]. The presently most popular serological method is agar gel immunodiffusion based on BLV gp60, but other techniques such as radioimmunoassays ([5] and Bex et al.,

submitted), Early Polykaryocytosis Inhibition, [9] VSV Pseudotypes Inhibition [20] are intensively investigated. Direct assessment of BLV infection can even be obtained by Syncytia or early polykaryocytosis induction but this is a too tedious process for large scale application in field studies.

In 1976–1977 [12] we applied JD gp60 in a survey of 1315 cattle herds (Table 1). In a first round, we tested 1 blood sample out of 4. Every herd in which one positive was detected was then more deeply investigated. Every animal more than 3 months old was submitted to a blood test. As can be seen in the last column of Table 1, the percentage of infection observed by the method used, varied between 2,4 and 37,4% with an average of 16,1%. For the whole study, 6 herds happened to be infected by BLV over a total of 1315 examined, which leads to an observed rate of infection of 0,46%. This number can be considered as small in comparison with incidences reported for other countries [3].

Table 1. Survey of 1315 herds by ID gp60 and Control of this survey

Herd n°	Survey (animals older than 1 year) Number of samples			Control (animals older than 3 months) Number of samples			Infection levels
	Received	Tested	Positive	Taken	Tested	Positive	
1	80	22	8	115	115	43	37,4%
2	20	5	1	30	30	8	26,7%
3	60	15	1	83	83	2	2,4%
4	33	9	1	38	38	4	10,5%
5	42	12	1	74	74	9	12,2%
6	84	21	1	114	114	7	6,1%
Total	323	84	13	454	454	73	16,1%

Within infected herds, we recently compared (Bex et al., submitted) 3 serological tests, namely ID gp60, RIA gp60 and RIA p24. Over 345 animals tested, 104 were positive in RIA gp60, 101 in ID gp60 and 99 in RIA p24. The investigated herds were most probably foci of old BLV infections in which most "susceptible" animals had reached such antibody levels that ID gp60 was almost as sensitive as RIA gp60 and, indeed, more sensitive than RIA p24.

It should perhaps be mentioned here that the Commission of European Communities recently recommended to eradicate Enzootic Bovine Leucosis. The strict exogenous character of BLV and its apparent low progression power make the European recommendation quite feasible.

4. Host-Virus Relationship

The incoming of BLV into a recipient immediately elicits an antibody response to BLV structural antigens. The intensity of this response probably depends on age of the host and its genetic make-up, virus dose, health status, environ-

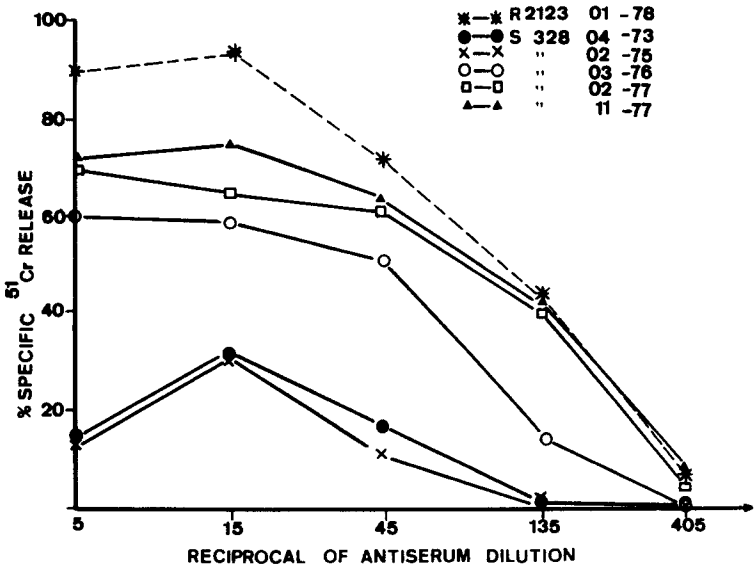

Fig. 4. Evolution of cytotoxic activity of a sheep serum (animal n° 328) during the last two years of the animal's life. Target cells were Fetal Lamb Kidney cells infected by and producing BLV (These cells are most probably not transformed by BLV). One of our best cytotoxic bovine sera (R2123) was used here as a reference

ment, ... In a recent study (Bex et al., submitted) we followed six sheep inoculated at birth, by the oral route, by whole leukemic bovine blood. As a rule, antibody levels to BLV gp60 and p24 rised steadily until the animal's death. In parallel we followed the complement dependent cytotoxicity of these sheep sera toward a BLV-producer cell line Fetal Lamb Kidney cells [16]. Results examplified in Fig. 4 clearly show that serum cytotoxicity increased with time, reaching a maximum level in the tumor phase of the disease at the animal's death. Immunoglobulins active in the cytotoxic reaction belong to the Ig G_1 sub-class.

5. Conclusions

The lymphoproliferative disease, Enzootic Bovine Leucosis, is an infectious disease caused by a retrovirus called BLV (Bovine Leukemia Virus).

The nature and mode of action of virus gene products and the mechanisms involved in the host-virus interplay are presently under intense investigation.

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References

1. Abt, D.A., Marshak, R.R., Ferrer, J.F., Piper, C.E., Bhatt, D.M.: Studies on the development of persistent lymphocytosis and infection with the bovine C-type leukemia virus (BLV) in cattle. Vet. Microbiol. **1**, 287–300 (1976)
2. Anonymous: Criteria for the determination of the normal and leukotic state in cattle. Prepared by an international committee on bovine leukosis. J. Natl. Cancer Inst. **41**, 243–263 (1968)
3. Burny, A., Bex, F., Chantrenne, H., Cleuter, Y., Dekegel, D., Ghysdael, J., Kettmann, R., Leclercq, M., Leunen, J., Mammerickx, M., Portetelle, D.: Bovine leukemia virus involvement in enzootic bovine leucosis. Adv. Cancer Res. **28**, 251–311 (1978)
4. Burny, A., Bex, F., Bruck, C., Cleuter, Y., Dekegel, D., Ghysdael, J., Kettmann, R., Leclercq, M., Mammerickx, M., Portetelle, D.: Biochemical studies on enzootic and sporadic types of bovine leucosis. In: Anti-viral mechanisms in the control of neoplasia. Chandra, P. (ed.). New York: Plenum Press 83–99 (1979)
5. Devare, S.G., Stephenson, J.R.: Biochemical and immunological characterization of the major envelope glycoprotein of bovine leukemia virus. J. Virol. **23**, 443–447 (1977)
6. Donham, K.J., van der Maaten, M.J., Miller, J.M., Kruse, B.C., Rubino, M.J.: Seroepidemiologic studies on the possible relationships of human and bovine leukemia. J. Natl. Cancer Inst. **59**, 851–854 (1977)
7. Ferrer, J.F., Piper, C.E., Baliga, V.: Diagnosis of BLV infection in cattle of various ages. In: Bovine leucosis: Various methods of molecular virology. Burny, A. (ed.). pp. 323–336. Luxembourg: Commission of the European Communities 1977
8. Ghysdael, J., Hubert, E., Cleuter, Y.: Biosynthesis of bovine leukemia virus major internal protein (p24) in injected cells and xenopus laevis oocytes microinjected with BLV 60–70S RNA. Arch. internat. Physiol. Biochim. **85**, 978–979 (1977)
9. Guillemain, B., Mamoun, R., Levy, D., Astier, T., Parodi, A.L.: Serological diagnosis of bovine leukemia virus infection by early polykaryocytosis inhibition (EPI). Vet. Microbiol. 1978 (in press)
10. Kettmann, R., Burny, A., Cleuter, Y., Ghysdael, J., Mammerickx, M.: Distribution of bovine leukemia virus proviral DNA sequences in tissues of animals with enzootic bovine leucosis. Leukemia Research 2/23–32 (1978)
11. Mammerickx, M., Burny, A., Dekegel, D., Ghysdael, J., Kettmann, R., Portetelle, D.: Comparative study of four diagnostic methods of enzootic bovine leukemia. In: Bovine leucosis: Various methods of molecular virology. Burny, A. (ed.), pp. 209–221. Luxembourg: Commission of the European Communities 1977
12. Mammerickx, M., Otte, J., Rase, F., Braibant, E., Portetelle, D., Burny, A., Dekegel, D.: Large scale serological detection in Belgium of enzootic bovine leukosis. Zbl. Vet. Med. B **25**, 416–424 (1978)
13. McDonald, H.C., Ferrer, J.F.: Detection, quantitation and characterization of the major internal virion antigen of the bovine leukemia virus by radioimmunoassay. J. Natl. Cancer Inst. **57**, 875–882 (1976)
14. Olson, C., Baumgartener, L.E.: Pathology of Lymphosarcoma in sheep induced with bovine leukemia virus. Cancer Res. **36**, 2365–2373 (1976)
15. Paul, P.S., Pomeroy, K.A., Castro, A.E., Johnson, D.W., Muscoplat, C.C., Sorensen, D.K.: Detection of bovine leukemia virus in B-lymphocytes by the syncytia induction assay. J. Natl. Cancer Inst. **59**, 1269–1272 (1977)
16. Portetelle, D., Bruck, C., Bex, F., Burny, A., Dekegel, D., Mammerickx, M.: Detection of complement dependent lytic antibodies in sera from bovine leukemia virus infected animals by the chromium-51 release assay. Arch. internat. Physiol. Biochim. **86**, 955–956 (1978)
17. Takashima, I., Olson, C., Driscoll, D.M., Baumgartener, L.E.: B lymphocytes and T lymphocytes in three types of bovine lymphosarcoma. J. Natl. Cancer Inst. **59**, 1205–1210 (1977)
18. Van der Maaten, M.J., Miller, J.M., Boothe, A.D.: Replicating type-C virus particles in monolayer cell cultures of tissues from cattle with lymphosarcoma. J. Natl. Cancer Inst. **52**, 491–497 (1974)
19. Wittmann, W., Urbaneck, D.: Leukosen des Rindes. In: Handbuch der Virus-Infektionen bei Tieren. Fischer, I. (ed.), Vol. V, pp. 41–174. 1969
20. Zavada, J., Dickson, C., Weiss, R.: Pseudotypes of vesicular stomatitis virus with envelope antigens provided by murine mammary tumor virus. Virology **82**, 221–231 (1977)