

Bovine immunodeficiency virus: immunochemical characterization and serological survey

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Bovine immunodeficiency virus (BIV) was purified by isodensity centrifugation; viral activities were monitored in gradient fractions using the reverse transcriptase assay and a p26-specific monoclonal antibody ELISA. In the coincident peak fractions (density about 1·17 g/ml) proteins with M_r values of 26K, 17K, 53K, 14K and 100K (with decreasing intensity) were detect-

ed by Western blotting using serum of a calf after experimental BIV infection. When 957 randomly collected cattle sera from The Netherlands were tested by indirect immunofluorescence and confirmed using Western blot and/or radioimmunoprecipitation, 1·4% appeared seropositive. Thus BIV infection is not uncommon in one European cattle population.

Introduction

Some 20 years ago a virus was isolated from the leukocytes of cattle with persistent lymphocytosis, lymphadenopathy, lesions in the central nervous system, progressive weakness and emaciation (van der Maaten *et al.*, 1972). It was found to encode a reverse transcriptase (RT) with Mg^{2+} preference, to replicate and induce syncytia in a variety of embryonic bovine tissues *in vitro*, and to possess a morphology similar to that of the human immunodeficiency virus (HIV). Moreover, serological analyses have demonstrated conservation of epitopes between the major core protein of this bovine retrovirus and HIV. Sequence data and serological analyses have established that bovine immunodeficiency virus (BIV) is related to HIV and other lentiviruses (Gonda *et al.*, 1987).

The original BIV isolate (R29) has been molecularly cloned, and sequence analysis of two functional proviruses revealed a genome 8·4 kb in size. Besides the three large open reading frames (ORFs) encoding the *gag*, *pol* and *env* genes common to all replication-competent retroviruses, five additional small ORFs were found. The putative protein product of the first major ORF is a 53K protein (the *gag* precursor) which is probably processed into p26, p17 and p14. The non-glycosylated envelope precursor of BIV is predicted to be 102K, 145K in its glycosylated form; it is presumably cleaved into a surface protein of 62K and a transmembrane protein of 40K. The small ORFs probably encode non-structural proteins which may play a role in the regulation and pathogenesis

of lentivirus infections (Gonda *et al.*, 1987). Numerous point mutations and deletions were found, mostly in the *env* protein-encoding ORF. These data suggest that, within a single virus isolate, BIV displays extensive genomic variation (Garvey *et al.*, 1990).

So far, little is known of the pathogenic importance, host immune response and epidemiology of BIV in cattle. In this communication we present serological data which establish that BIV infections occur in cattle in Europe.

Methods

Virus and cells. BIV strain R29 was kindly provided by Dr M. van der Maaten. Both uninfected and BIV R29-infected cultures of Madin-Darby bovine kidney (MDBK) and Black bovine kidney (BBK) cells (J. Black, unpublished results) were used to demonstrate antibodies in cattle serum that react with BIV-specific antigens by indirect immunofluorescence assay (IFA). From the BBK-BIV R29 cells, a subpopulation designated BBK-BIV/clone 18 was isolated by limiting dilution. Clone 18 was selected on the basis of IFA reactions and by assaying the RT activity in the culture supernatants. Embryonic bovine spleen (BESP) cells were also used.

For large-scale virus production, monolayers of clone 18 cells were grown in Dulbecco's modification of Earle's MEM supplemented with 5% foetal calf serum, 100 units of penicillin per ml, and 100 µg of streptomycin per ml in roller flasks (850 cm²). Every week the culture fluids were collected and clarified by low-speed centrifugation (30 min, 10000 r.p.m.). The virus was concentrated by overnight sedimentation at 10000 g and the pellet material purified by isodensity centrifugation (17000 g, 16 h) in 10 to 50% (w/v) sucrose gradients prepared in TS buffer (50 mM-Tris-HCl, 150 mM-NaCl, pH 8·0). Gradient fractions of 0·5 ml were collected and assayed for the presence of RT activity; in addition, the distribution of p26 in the gradient was measured using an antigen-capture ELISA. The protein concentration in the fractions was determined using a standard method (Bio-Rad; Bradford, 1976).

Assay of RT activity. Virus preparations were assayed for the presence of RT activity. BIV was precipitated from 0.75 ml of culture supernatant by adding 0.5 ml 16% (w/v) polyethylene glycol 6000 (PEG) in 0.8 M-NaCl. After overnight incubation at 4 °C the virus was concentrated by centrifugation (10000 g, 30 min) and disintegrated by the addition of 37.5 µl lysis buffer (50 mM-Tris-HCl pH 8.0, 0.25% Triton X-100, 12 mM-DTT). The RT reaction mixture consisted of 50 mM-Tris-HCl pH 8.0, 150 mM-NaCl, 20 µM-MnCl₂, 12 mM-DTT, 0.025 units/ml poly(rA/dT) primer (Pharmacia LKB) and 25 µCi/ml [*Me*-³H]TTP (approx 40 Ci/mmol; Amersham). After 1 h of incubation at 37 °C the reactions were stopped by adding 10 µl 30% TCA/Napp (0.1 M-NaH₂PO₄, 0.1 M-Na₄P₂O₇, 0.4 M-HCl). Samples were spotted on Whatman filters, washed (TCA-ethanol-ether), dried, and [³H]TTP incorporation was measured by liquid scintillation counting. For measurements of RT activity in sucrose gradient fractions, PEG precipitation was omitted.

ELISA for antigen detection. The distribution of p26 in the fractions of sucrose density gradients was measured using an antigen-capture ELISA. Fraction material was incubated with 0.1% Triton X-100 for 15 min; after a 10-fold dilution, the samples (100 µl) were transferred to an ELISA plate which had been coated with 0.5 µg of a monoclonal antibody (MAb) directed against an epitope on p26. After 1 h, bound antigen was detected with a biotinylated second MAb. A streptavidin-peroxidase conjugate (Sigma) was used in the last step before the substrate and the chromogen (tetramethyl benzidine) was added.

IFA. Uninfected and persistently infected MDBK and BBK cells were seeded onto 10-well tissue culture glass slides (Nutacon), grown for 3 days at 37 °C, washed with PBS, fixed in acetone for 10 min at -20 °C and stored at -20 °C until use. Sera to be tested were diluted (1:50 and 1:100) in PBS supplemented with 5% (v/v) normal rabbit serum. After 1 h of incubation at 37 °C, the slides were washed (PBS) and incubated with rabbit anti-bovine immunoglobulin conjugated with fluorescein isothiocyanate (Dakopatts) for another hour at 37 °C. The slides were then rinsed with PBS, air-dried, mounted in glycerol-PBS and examined with a fluorescence microscope. Sera were considered positive when the reactivity in infected cultures revealed green foci against a dark background, since not all cells were infected; in addition, the intensity of fluorescence diminished with higher serum dilutions. A result was regarded as positive only if fluorescence was absent in the uninfected control cultures.

Western blot (WB) analysis. Gradient-purified BIV was analysed by electrophoresis in 15% SDS polyacrylamide gels (Rottier *et al.*, 1981). Transfer of the proteins to nitrocellulose filters was performed overnight at 0.25 A. After transfer, the filters were incubated with PBS containing 0.5% pig skin gelatin and 0.1% Triton X-100 (blot buffer) for 1 h at room temperature. The antisera and horseradish peroxidase-conjugated anti-IgG were diluted in blot buffer and incubations were performed for 1.5 h at room temperature. Immunoreactive protein bands were visualized using 4-chloro-1-naphthol as a chromogen.

Radioimmunoprecipitation (RIPA). BBK or BBK-BIV/clone 18 cells were labelled for 24 h with [³⁵S]methionine. After a 4 h chase with unlabelled methionine the supernatants were collected, TCA-precipitated and radioactivity was measured. Overnight incubation of 5 µl serum with lysed (0.5% Triton X-100) cell culture supernatant followed (6 × 10⁵ c.p.m.), and the immune complexes were adsorbed to Protein G-bearing streptococcal cells (Sigma). After three washes the sample was denatured in Laemmli buffer and run in a 15% SDS-polyacrylamide gel.

Experimental infection of calves. Two specific pathogen-free colostrum-deprived calves at 3 months of age were used for experimental infection with BIV. Peripheral blood lymphocytes from calf no. 1 were purified in a Ficoll-Hypaque gradient and stimulated with concanava-

lin A (5 µg/ml) and recombinant human interleukin-2 (100 units/ml). The lymphocytes were cocultivated with BIV-infected BESP cells for 1 week, after which time the cells were inoculated by the subcutaneous route back into the donor calf, into the prescapular lymph node area. At 4 weeks after inoculation, 100 ml of heparinized blood from calf no. 1 was transfused intravenously into calf no. 2. At intervals of 2 weeks, the animals were bled and the sera assayed for the presence of BIV-specific antibodies by IFA and WB. Peripheral blood mononuclear cells were purified and cultured *in vitro* for virus isolation.

MAbs. Four 10-week-old BALB/c mice (Broekman Institute) were injected subcutaneously with 50 µg of gradient-purified BIV antigen emulsified in 0.25 ml of PBS containing 0.5% (v/v) Triton X-100 and 0.25 ml of complete Freund's adjuvant. Three weeks later, the animals were given a booster injection of 50 µg purified BIV in incomplete Freund's adjuvant. Sera were collected 1 week after the booster immunization and tested for the presence of BIV-specific antibodies by WB analysis. Three days before the fusion, the animals were inoculated intraperitoneally with 50 µg virus in PBS containing 0.5% Triton X-100. The splenocytes of the immunized mice were then fused with myeloma cells according to standard procedures. Culture supernatants of growing cells were tested for antibodies against the BIV antigen by direct ELISA; culture medium from BBK cells served as a negative antigen control. Positive cultures were cloned by limiting dilution.

Ascites fluid was obtained after intraperitoneal injection of 5 × 10⁶ hybridoma cells into tetramethyl-pentadecane-primed mice. MAbs were characterized by WB and RIPA. The immunoglobulin subclass was determined by agar gel immunodiffusion (Ouchterlony test). Epitope specificities were determined with the aid of a competition ELISA using biotinylated antibodies.

Sera. The ovine sera had been obtained by Whetstone *et al.* (1991) after intravenous inoculation of sheep with BIV-infected BESP culture material. The 957 cattle sera used for this report were random samples from different provinces in the Netherlands; however, there was a sampling bias in that most animals were in the age group of between 3 and 5 years.

Results

Growth and purification of BIV

The presence of viral antigen in infected BBK cells was confirmed by IFA; a maximum of 10% of the cells in a monolayer was found to be infected. Released virus was monitored using RT determinations, after the optimum conditions for the assay had been determined. Maximum RT activity in BIV preparations from both BBK-BIV/clone 18 and BESP cells was encountered at 20 µM-Mn²⁺ in 150 mM-NaCl; at this cation molarity, incorporation in the presence of Mn²⁺ was superior to that in the presence of 20 µM (and higher concentrations of) Mg²⁺ (Fig. 1).

By using an ELISA with an MAb directed against p26, the distribution of antigen in a sucrose density gradient was compared with that of RT. As shown in Fig. 2, the peaks of both activities coincided. Analysis by WB of all sucrose gradient fractions revealed a distribution of virus-specific proteins over a wide density range, with a maximum at 1.166 g/ml (Fig. 3).

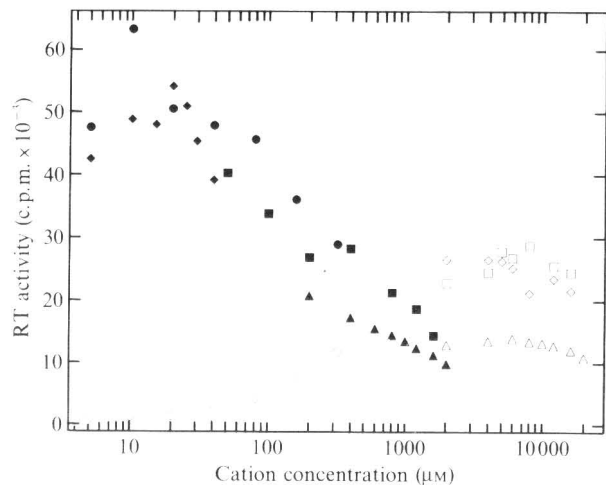


Fig. 1. Dependence of the RT activity of BIV upon the concentration of Mg^{2+} (open symbols) and Mn^{2+} (filled symbols) ions in the reaction mixture. The results of four separate experiments are combined in the graph.

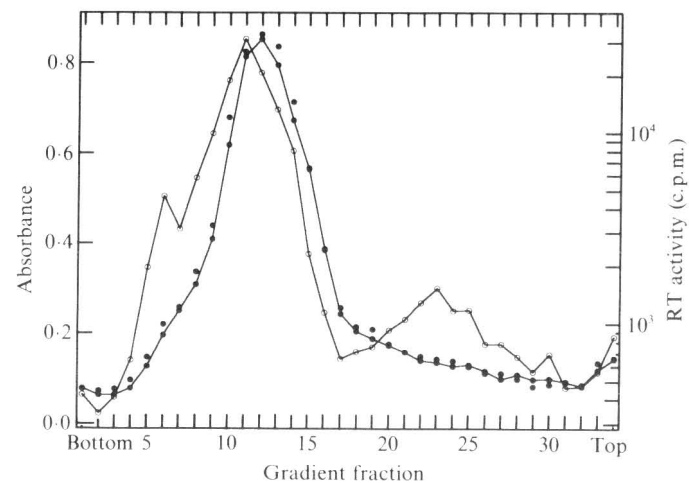


Fig. 2. Distribution of ELISA (filled circles) and RT activities (empty circles) in a 10 to 50% sucrose density gradient of BIV concentrated from supernatants of BBK-BIV/clone 18 cells.

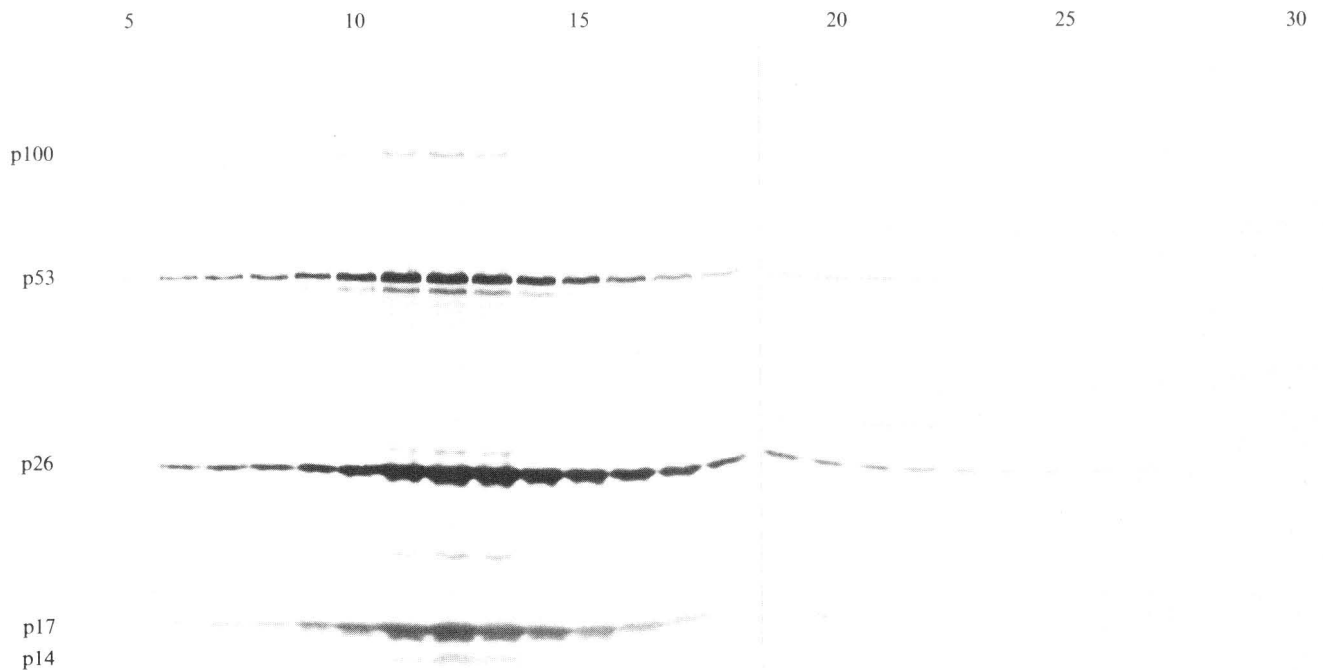


Fig. 3. Western blot analysis of fractions from the sucrose gradients depicted in Fig. 2; the density of fraction 12 was 1.166 g/ml.

Identification of BIV proteins

The identity of the polypeptides p53, p26, p17 and p14 found in the gradient analysis was confirmed by WB using sera from calves after experimental inoculation with BIV propagated in BESE cells. Serial bleedings were taken from two BIV-infected animals at 14-day

intervals. The immune serum from calf no. 1 recognized the above-mentioned proteins from week 4 onward (Fig. 4, lane 3); in calf no. 2, a weak anti-p26 reaction was seen as early as 14 days after infection (data not shown). Neither calf showed any symptoms during the observation period of 2 months.

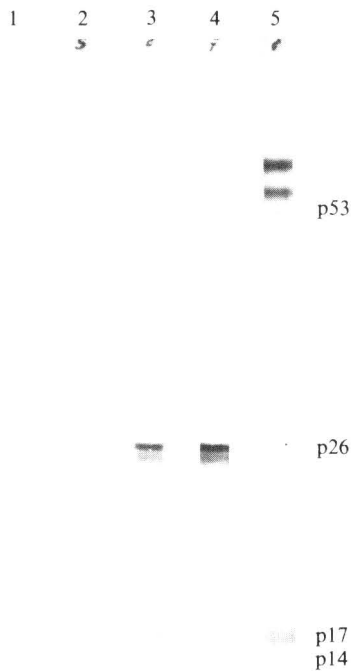


Fig. 4. Western blot analysis of immune sera using gradient-purified BIV. Lane 1, pre-inoculation serum of calf no. 1; lane 2, serum from experimentally infected cow 6618; lane 3, post-inoculation serum from calf no. 1; lane 4, sheep immune serum to BIV; lane 5, hyperimmune mouse serum (animal used for the preparation of MABs).

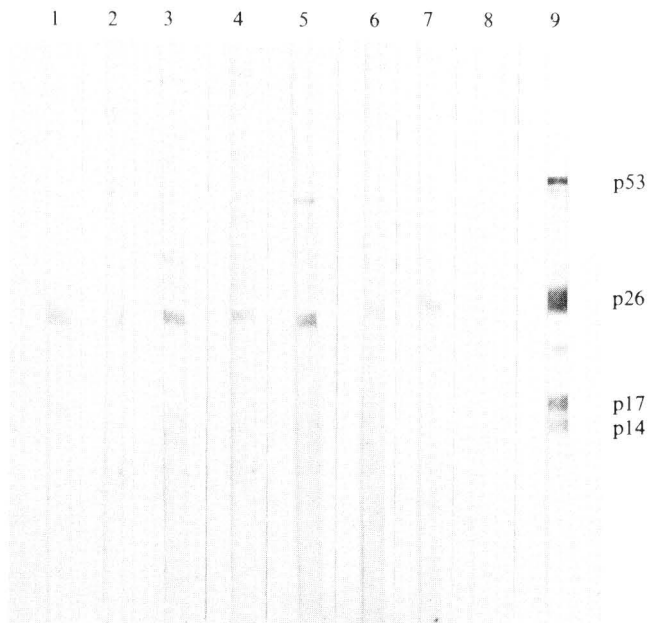


Fig. 5. Western blot analysis of bovine field sera using gradient-purified BIV. Lanes 1 to 7, sera from randomly selected cattle shown to react by IFA; lane 8, negative control; lane 9, post-inoculation serum from calf no. 1.

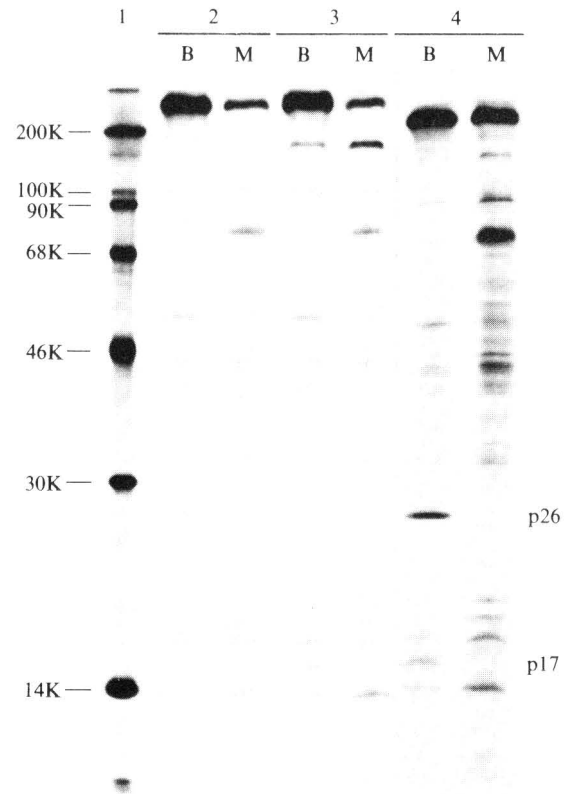


Fig. 6. RIPA using BIV-infected (B) and mock-infected (M) lysates of BBK cell supernatants. The pairs of lanes in 2 and 3 show the reactions obtained with two field sera, lanes 4 those with post-inoculation serum from calf no. 1. Lane 1 represents the M_r markers.

Using sera from sheep after an experimental BIV infection, bands in the same locations were found (Fig. 4, lane 4). Also the serum from a mouse which had been immunized for the preparation of MABs recognized p53, p26 and p17 (Fig. 4, lane 5); the conspicuous higher M_r proteins are not virus-specific.

Evidence for BIV infection in cattle in The Netherlands

When 957 randomly collected sera from cattle in The Netherlands were examined by indirect IFA, 64 (6.7%) were found distinctly positive in two independent assays. In nine cases, second bleedings were obtained from the same farms and positive or doubtful results were verified.

To eliminate the subjective element in assessing the intensity of immunofluorescence, confirmatory WB experiments were done. However, this technique did not resolve the ambiguity either: at the M_r positions where BIV proteins would be expected bands appeared in samples from sera negative by IFA; false positive

reactions occurred especially in the p26 range. Fig. 5 shows a series of WB reactions where p17/p14 and/or p53 appeared in addition to p26. Reactions with *env* gene products were not seen.

Another specificity criterion was introduced by testing those sera which were positive in both IFA and WB using the RIPA. In Fig. 6, lane 4 (B) shows the reactions obtained with serum from one of our experimentally BIV-infected calves; p26 is most prominent, and p53 and p17 can be distinguished. Faint but undeniable reactions have also been obtained with field sera (lanes 2 and 3). When our serum collection was re-examined and the rigorous criteria applied, 1.4% were positive in both IFA and WB, and 0.7% were positive in all three tests.

Since our preselection of sera to be tested by WB and RIPA was based on the results of the IFA, we asked the question whether IFA-negative sera would invariably be negative in the confirmatory tests. Of 70 randomly selected, IFA-negative samples from our collection, one showed a distinct p26 reaction in the WB. False negative IFA results may therefore be expected.

Discussion

During initial experiments to optimize growth and assay of BIV we found a higher activity of the RT at low concentrations of Mn^{2+} ions (20 μM) as compared to Mg^{2+} ions; at higher molarities inhibitory effects were seen, as also noted by Gonda *et al.* (1990a, b). This finding should be considered in view of the importance attributed to the cation requirement of retroviral RT for the construction of phylogenetic trees.

Antibodies against BIV can be detected in calves from 2 weeks after experimental inoculation onwards. Whetstone *et al.* (1990) were able to demonstrate them for 2.5 years by using both indirect IFA and WB; antibodies were above all directed against p26. These authors also found serological evidence of BIV infection in about 50% of the sera from a herd with a high incidence of lymphosarcoma and persistent health problems, but none in samples from cattle with other conditions or from healthy animals. The IFA technique was used in seroepidemiological surveys by Amborski *et al.* (1989) on 235 samples and by Black (1991, quoted from Whetstone *et al.*, 1990) on 1997 random serum specimens predominantly from dairy cattle; the authors report an incidence of about 4% seropositive animals in their collections from the U.S.A.

Had we relied exclusively upon IFA in the present study, 6.7% seropositive cattle would have been found in The Netherlands. When applying stringent criteria of confirmation by WB and RIPA, however, only 0.7% remained. Individual bands may appear in WB that,

from their position in the gel, must be considered BIV-specific; however, bands in the same positions were seen in IFA-negative samples. This observation is at variance with the finding by Whetstone *et al.* (1990) that WB-positive samples were always positive in IFA. False positive results can jeopardize both assays which are not really independent, being both based on immunoreactivity. It is the band pattern that should be arbitrated, and reactions with both *gag* and *env* gene products should be shown. While anti-*env* reactivity is seen in the gradient/WB analysis of BBK-BIV/clone 18 cell supernatant using post-inoculation serum from an experimental calf (Fig. 3), field sera did not show this reactivity, probably due to reasons of antigen quantity, perhaps also to antigenic differences. We have been able to clone BIV-specific sequences from this virus preparation (P. Sillekens, unpublished observations) which were virtually identical to those published (Gonda *et al.*, 1987). In view of the extensive genomic variation of BIV (Garvey *et al.*, 1990), which may affect epitopes on all proteins, antibodies in field sera may be of low affinity or even directed against BIV serotypes other than that used in our assays. Isolation of additional bovine lentivirus strains is mandatory for future progress in this field. Samples from seropositive cattle are presently being processed with this objective.

A recent serological study performed in Switzerland with the aid of recombinant DNA expression products of the *gag* gene of BIV (Rasmussen *et al.*, 1990) has independently confirmed the presence of this lentivirus in the cattle population in Europe (H. Lutz, personal communication).

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