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Discordance between Bovine Leukemia Virus Tax Immortalization In Vitro and Oncogenicity In Vivo

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Bovine leukemia virus (BLV) Tax protein, a transcriptional activator of viral expression, is essential for viral replication in vivo. Tax is believed to be involved in leukemogenesis because of its second function, immortalization of primary cells in vitro. These activities of Tax can be dissociated on the basis of point mutations within specific regions of the protein. For example, mutation of the phosphorylation sites at serines 106 and 293 abrogates immortalization potential in vitro but maintains transcriptional activity. This type of mutant is thus particularly useful for unraveling the role of Tax immortalization activity during leukemogenesis independently of viral replication. In this report, we describe the biological properties of BLV recombinant proviruses mutated in the Tax phosphorylation sites (BLVTax106+293). Titration of the proviral loads by semiquantitative PCR revealed that the BLV mutants propagated at wild-type levels in vivo. Furthermore, two animals (sheep 480 and 296) infected with BLVTax106+293 developed leukemia or lymphosarcoma after 16 and 36 months, respectively. These periods of time are within the normal range of latencies preceding the onset of pathogenesis induced by wild-type viruses. The phenotype of the mutant-infected cells was characteristic of a B lymphocyte (immunoglobulin M positive) expressing CD11b and CD5 (except at the final stage for the latter marker), a pattern that is typical of wild-type virus-infected target cells. Interestingly, the transformed B lymphocytes from sheep 480 also coexpressed the CD8 marker, a phenotype rarely observed in tumor biopsies from chronic lymphocytic leukemia patients. Finally, direct sequencing of the *tax* gene demonstrated that the leukemic cells did not harbor revertant proviruses. We conclude that viruses expressing a Tax mutant unable to transform primary cells in culture are still pathogenic in the sheep animal model. Our data thus provide a clear example of the discordant conclusions that can be drawn from in vitro immortalization assays and in vivo experiments. These observations could be of interest for other systems, such as the related human T-cell leukemia virus type 1, which currently lack animal models allowing the study of the leukemogenic process.

Bovine leukemia virus (BLV) and human T-cell leukemia virus type 1 (HTLV-I) are members of the *Deltaretrovirus* genus in the *Retroviridae* family (6, 18, 28, 30, 54, 69, 78, 81, 82). In addition to the structural genes required for the synthesis of the viral particle (*gag*, *pol*, and *env*), these viruses also contain a region X located at the 3′ end of their genome. This region encodes a series of proteins involved in the regulation of viral expression (Tax, Rex, R3, and G4 for BLV). Among these, the Tax protein is a 34- to 38-kDa transcriptional activator which increases the synthesis of all viral mRNAs (15, 71). Transactivation by Tax requires 21-bp imperfect repeats located in the 5′ long terminal repeat (LTR). In fact, Tax does not bind directly to DNA but interacts with the CREB/ATF cellular proteins and increases their affinity for the 21-bp enhancer elements (1, 2, 8). Although some limited variation might be compatible with function, *tax* is an essential gene that is absolutely required for infectivity in vivo (77). Besides its transactivation activity, the Tax protein also exhibits another property in cell culture: its expression induces immortalization of primary rat embryo fibroblasts (REF) (74). In addition, coexpression of *tax* and the Ha-*ras* oncogene fully transforms REF cells yielding tumors in nude mice. These Tax activities can be dissociated by mutations within specific regions of the protein. For example, transcriptional activity requires an amino-terminal zinc finger structure and an internal leucine-rich activation domain (72, 76). Conversely, phosphorylation of Tax at serines 106 and 293 is required for in vitro transformation but not for transactivation (73). These phosphorylation-deficient Tax mutants should thus provide a unique opportunity to correlate in vitro transformation assays with pathogenicity in vivo.

During the last decades, several methods have been designed to unravel the oncogenic potential of selected viral proteins. One of the earliest-developed techniques, which was described in 1983 (36, 37), is based on the immortalization of primary REF. This method also allowed the characterization of two types of oncogenes: those that indefinitely prolong the cellular lifespan (like Myc), and others that induce transformation by altering cell morphology, impeding contact inhibition, and decreasing growth factor requirements (such as Ras). Both kinds of oncogenes are able to cooperate in order to yield fully transformed cells that induce tumors in nude mice. Similar studies in the BLV-HTLV field have shown that one of the viral regulatory proteins, called Tax, is able to functionally substitute for Myc in this type of assay (53, 74). A slightly modified version of this protocol utilizes murine cell lines (Rat-1 or -2) in which the *tax* gene provokes the formation of transformed foci upon transfection (19, 42, 63, 66, 79, 80). The main objection against these approaches concerns the cell type (fibroblast versus lymphocyte) and the origin of the species (murine instead of human, ovine, or bovine). Therefore, other cell culture systems utilizing T or B lymphocytes have been developed (3, 16, 22, 23, 47, 55–58, 70). Among these, protocols using recombinant proviruses and primary lymphocytes

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probably provide the most relevant information. Unfortunately, this type of technique has not been established for BLV. The conclusions drawn from these different studies have been a matter of dispute, in particular those concerning the pathways involved in transformation. For instance, the ability of HTLV-1 Tax to transform primary rat embryo fibroblasts requires its potential to activate the CArG element, whereas NFκB activity is essential in Rat-1 cells (42). The situation appears to be far more complex in cell culture systems based on T lymphocytes. Indeed, the NFκB function of Tax-I appears to be sufficient to promote growth response to interleukin-2, but clonal expansion of $CD4^+$ cells requires the CREB/ATF and SRF pathways (3).

To further understand the role of Tax during pathogenesis, extensive efforts have been made to establish animal models in mice, rats, rabbits, and monkeys (7, 12, 13, 17, 24–27, 29, 31, 33, 38, 48, 51, 55, 59, 60, 62, 65, 68, 83), and indeed, these systems yielded valuable information in various aspects of viral infectivity and pathogenesis. Despite this extensive progress, the main objection of these models is that the virus is not in the context of its natural host species environment and that none of them perfectly conciliates all the different phenomena occurring during leukemogenesis. In this context, an alternative approach based on viruses related to HTLV, like BLV, might provide very useful additional information.

MATERIALS AND METHODS

Animals. All sheep were maintained under controlled conditions at the Veterinary and Agrochemical Research Centre (Uccle, Belgium). At regular intervals of time, total leukocyte counts were determined by using a Coulter counter ZN, and the corresponding lymphocyte numbers were calculated using the blood formula after examination under the microscope. In parallel, the corresponding sera were analyzed for BLV seropositivity using immunodiffusion and enzyme-linked immunosorbent assay (ELISA) techniques. Sheep were infected with a wild-type strain (plasmid pBLV344 in animal 235), with viruses propagating with equivalent efficiencies and inducing pathogenesis after similar latency periods (plasmid pBLVIX in 8, 11, 247, 292, and 293 and pBLVgag150 in 175), or with the Tax mutant (pBLVTax106+293 in 103, 104, 296, and 480). The construction of the pBLV344, pBLVIX, pBLVgag150, and pBLVTax106+293 recombinant proviruses has been described elsewhere (73, 75, 77). Of note, the pBLVgag150 mutant, which was initially referred to as attenuated (75), appeared to induce pathogenesis at later times in sheep 175. Finally, three sheep (113, 114, and 115) were used as uninfected controls. The procedures used for infection have been described (77). Briefly, 100 μg of circular plasmid DNA was mixed with 200 μg of Dotap (Roche Diagnostics) and injected intradermally into the back of the sheep.

PCRs. Aliquots of peripheral blood were collected by jugular venipuncture at 4, 6, 15, and 30 months postinfection, and crude cell lysates were prepared as described (77). Briefly, 500 μl of blood sample was mixed with an equal volume of lysis buffer (0.32 M sucrose, 10 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 1% Triton X-100). The samples were centrifuged for 20 s, and the pellets were washed at least four times with 1 ml of the same buffer. The samples were then resuspended in 500 μl of PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl [pH 8.3]) and incubated with 6 μl of proteinase K (5 mg/ml) for 1 h at 50°C. Five microliters of these lysates was then amplified by PCR in the presence of 200 μM each of the four deoxynucleoside triphosphates, 200 ng of primers PCRTB (5′-CGGGGCGGTGGCGGCGCCTAGG-3′) and PCRTD (5′-TAACGACAA AATTAT-TTCTTGTC-3′), and 2 U of *Taq* DNA polymerase (Roche Diagnostics). Since PCRTD is located upstream of the splice acceptor site of the *tax* and *rex* sequences, the oligonucleotides used do not amplify DNA corresponding to reverse-transcribed double-spliced cDNA. The reaction mixtures were denatured for 5 min at 94°C and amplified by 22 cycles of PCR (30 s at 94°C, 30 s at 57°C, and 1 min at 72°C). After PCR, the amplicons were analyzed by Southern blotting hybridization using a *tax* probe (1-kb insert from plasmid pSGTax).

For sequencing, the *tax* amplicons were prepared as described above except for the number of cycles (36 cycles of PCR). The amplification products were purified with Gene Elute columns (Sigma) and sequenced by PCR with primers CAT3 (5′-CCTCAGGCCTTACAACGCTTC-3′) and CAT1C (5′-TCCGAGG ACAGGATGCGTTAC-3′) using the double-stranded DNA Cycle Sequencing system (Life Technologies).

Isolation of PBMCs. Peripheral blood mononuclear cells (PBMCs) were isolated by Percoll gradient centrifugation as described previously (14). Briefly, blood samples were collected by jugular venipuncture, and PBMCs were purified by Percoll density gradient centrifugation (Amersham-Pharmacia). Cells were then extensively washed with phosphate-buffered saline (PBS) supplemented with 0.075% EDTA and with PBS alone (three times each). Cell viability was next estimated by trypan blue dye exclusion.

Titration of the major capsid protein by ELISA. Purified PBMCs were cultivated for 24 h at 2×10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U of penicillin, and 100 ng of streptomycin (Life Technologies) per ml. The cell supernatants were recovered and analyzed for p24 protein expression using an ELISA procedure. Briefly, 96-well microtiter plates (Maxisorb immunoplate; Nunc) were coated for 4 h at room temperature with the 4′G9 monoclonal antibody (300 ng in PBS per well). After three washes with PBS-Tween 80 (0.2%), the cell culture supernatants were added and incubated overnight at 4°C in the presence of bovine serum albumin (0.67%) and Tween 80 (1.33%). After three washes, the presence of the p24 antigen was revealed by using two monoclonal antibodies (2′C1 and 4′F5) conjugated with horseradish peroxidase.

Flow cytometry analysis. After isolation, the PBMCs were labeled with different monoclonal antibodies specific for surface immunoglobulin M (IgM) (1H4 and PIg45), CD5 (CC17), CD11b (CC125), or CD8 (CC63). Optimal antibody concentrations were determined by serial dilutions of the ascites or the hybridoma cell culture supernatants. The cells were incubated in the presence of the monoclonals for 30 min at 4°C, washed with PBS containing 10% fetal calf serum, and labeled with isotype-specific secondary antibodies (Caltag Laboratories) conjugated with fluorescein isothiocyanate or phycoerythrin. Flow cytometry analyses were performed with a Becton Dickinson FACScan using the CELLQUEST software. Ten thousand events were collected, and the results were presented in dot plots.

A slightly different protocol was performed to determine the number of cells expressing the p24 major capsid protein. To trigger viral expression, the PBMCs first had to be cultivated for 1 day as described above. In addition, to label intracellular p24 antigen with the 4′G9 antibody, the cells had to be fixed in 1% paraformaldehyde (15 min at 4°C) and permeabilized with 70% ethanol for 1 h at −20°C.

RESULTS

Evolution of the proviral loads in sheep infected with the BLV Tax mutants. In a previous work, we reported the identification of the major phosphorylation sites of the BLV Tax protein at serine residues 106 and 293 (73). These two phosphoserines appear to be dispensable for transcriptional activation of the viral promoter and for infectivity in vivo. Indeed, their replacement by alanines still allows transactivation of LTR-based reporter plasmids during transient-transfection experiments. In addition, inhibition of the kinase which phosphorylates Tax does not alter transcriptional activation in cell culture (73). Finally, recombinant proviruses harboring serine-to-alanine mutations at positions 106 and 293 (BLVTax106+293) were infectious in the sheep animal model. Since *tax* is an essential gene, this observation is perhaps the best evidence for the dispensability of phosphoserines 106 and 293 during the viral life cycle.

In order to further characterize the role of these residues in vivo, we estimated the efficiency of viral propagation of the BLVTax106+293 mutant in four sheep (103, 104, 296, and 480). The proviral loads were measured at regular intervals after seroconversion by semiquantitative PCR of the *tax* gene. As a control for quantification, serial 10-fold dilutions of a positive control were analyzed in parallel (1×, 10×, 100×, and 1,000×; Fig. 1). At 4 months, the proviral loads in the animals infected with the Tax mutant or with the wild-type virus were similar. As a negative control, no *tax* sequences were amplified using lysates from an uninfected sheep (NI 113). It thus appears that the Tax mutants are infectious and propagate at wild-type levels, extending our previous observations performed at earlier times after seroconversion (73). At 6 and 15 months, the proviral loads rose gradually, indicating continuous viral spread within all the animals. Viral expansion appeared to be particularly fast in sheep 11 despite the low levels of virus determined at 4 months in this animal. A similar evolution also occurred in one of the sheep infected with the BLV Tax mutant (sheep 480). Three sheep (11, 103, and 480) died soon after this period and could not be analyzed at later times. In the remaining animals, the proviral loads were very

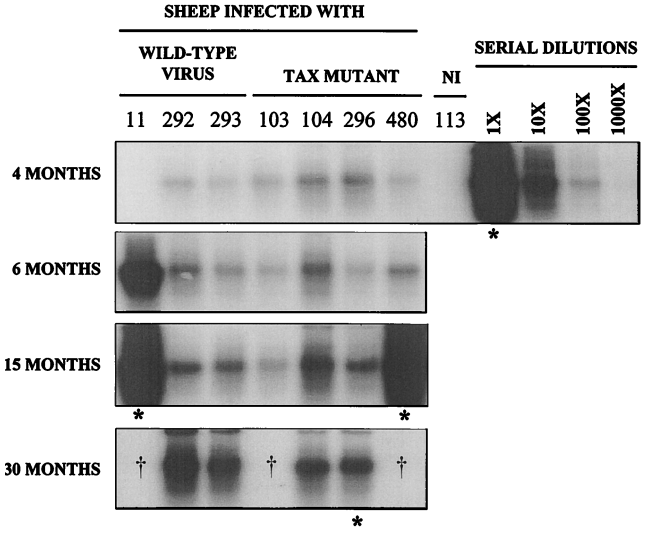

FIG. 1. Evolution of proviral loads in sheep infected with the BLV Tax mutants. Three sheep (11, 292, and 293) were injected with plasmid pBLVIX, which contains an infectious and pathogenic BLV provirus (clone 344). Four other animals (103, 104, 296, and 480) were infected with pBLVTax106+293, which is isogenic to pBLVIX except for two serine-to-alanine mutations in the *tax* gene. Blood was extracted by jugular venipuncture at regular times after seroconversion (4, 6, 15, and 30 months), and partially purified DNA was prepared from the corresponding lysates. A fraction corresponding to 5 μl of blood was amplified by 22 cycles of PCR using two primers flanking the *tax* gene, and the resulting DNAs were analyzed by Southern blotting using a *tax* probe. Under these conditions, the PCRs were semiquantitative, as shown by 10-fold dilutions (1×, 10×, 100×, and 1,000×) of lysate 480 at 15 months. In some lanes (*), the DNAs had to be isolated from smaller volumes of blood (50 μl instead of 500) because of the very high lymphocyte counts. Sheep 113 is an uninfected (NI) animal used as a negative control for PCR contaminations. Three samples are lacking at 30 months (†) because sheep 11, 103, and 480 died at about 19 to 20 months after seroconversion. Sheep 103 died because of enterotoxemia, whereas the other animals succumbed with leukemia or lymphosarcoma.

similar at 30 months independently of the type of virus (292, 293, 104, and 296).

We conclude that the BLVTax106+293 mutant is infectious in vivo and propagates at wild-type levels in four different sheep.

BLV Tax recombinant is leukemogenic in sheep. Among the three sheep that succumbed during the clinical survey, two of them (11 and 480) exhibited very high proviral loads at 15 months (Fig. 1). In contrast, sheep 103, which was infected with the BLV Tax mutant, only yielded low viral levels. At post mortem autopsy, this animal did not present any clinical sign that could be characteristic of leukemia (lymphocyte counts above 10,000/mm^3). In fact, this sheep died from an accidental cause linked to enterotoxemia. In contrast, sheep 480 and 11, infected with BLVTax106+293 and wild-type virus, respectively, harbored very high proviral loads. These animals also contained tremendous levels of lymphocytes within the peripheral blood (940,500 and 402,930 cells/mm^3) (Fig. 2). It thus appears that both sheep developed leukemia independently of the type of infecting virus. Another animal (296) infected with BLVTax106+293 also died with leukemia after a latency period of 3 years (Fig. 2). In contrast to sheep 480, which only exhibited an expansion of B lymphocytes in the blood stream (leukemia), animal 296 developed in addition a lymphoma-lymphosarcoma characterized by tumor infiltrations in the lymph nodes, the liver, and the kidney. Finally, sheep 104, infected with the Tax mutant, was still alive 44 months after seroconversion. We conclude that, during this period of time, two animals (480 and 296) that were injected with the BLVTax106+293 recombinant developed a pathology characteristic of BLV-associated leukemia.

Wild-type and Tax mutant viruses infect and transform similar cell types. Although BLV can infect many different cell types in vitro, the main cell target for BLV is the B lymphocyte in both sheep and cattle species. Elegant experiments based on single-cell PCR have indeed revealed that BLV infects only B cells in vivo (44). Besides surface IgM, BLV-infected B lymphocytes are characterized by the expression of several proteins at the cell membrane (5, 43, 45, 64). The most frequently encountered markers are CD5 and the CD11b integrin molecule (41, 61). Although the virus can also infect CD11b cells, the CD11b$^+$ lymphocytes preferentially expand during pathogenesis (10). In contrast, the CD5 marker is frequently lost at the final stages of BLV transformation in the sheep host (46).

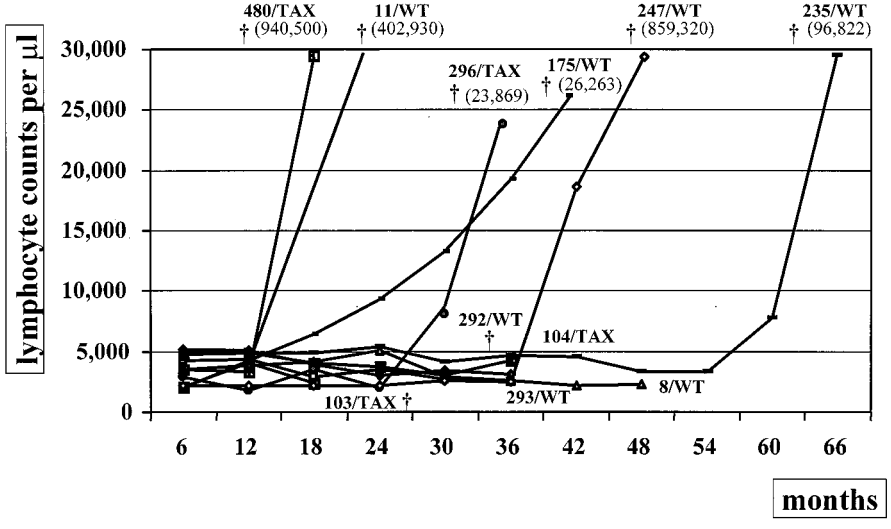

FIG. 2. Evolution of lymphocyte counts in BLV-infected sheep. Sheep were infected with the pBLVTax106+293 recombinant (TAX) (animals 103, 104, 296, and 480) or with viruses exhibiting wild-type (WT) behavior during pathogenesis (plasmid pBLVIX in sheep 8, 11, 247, 292, and 293; pBLV344 in 235; and pBLVgag150 in 175). Blood samples were extracted at regular intervals (routinely every month), and the number of leukocytes per microliter was determined by using a Coulter counter ZN. The lymphocyte counts (in parentheses) were deduced from these numbers after microscopic determination of the blood formula.

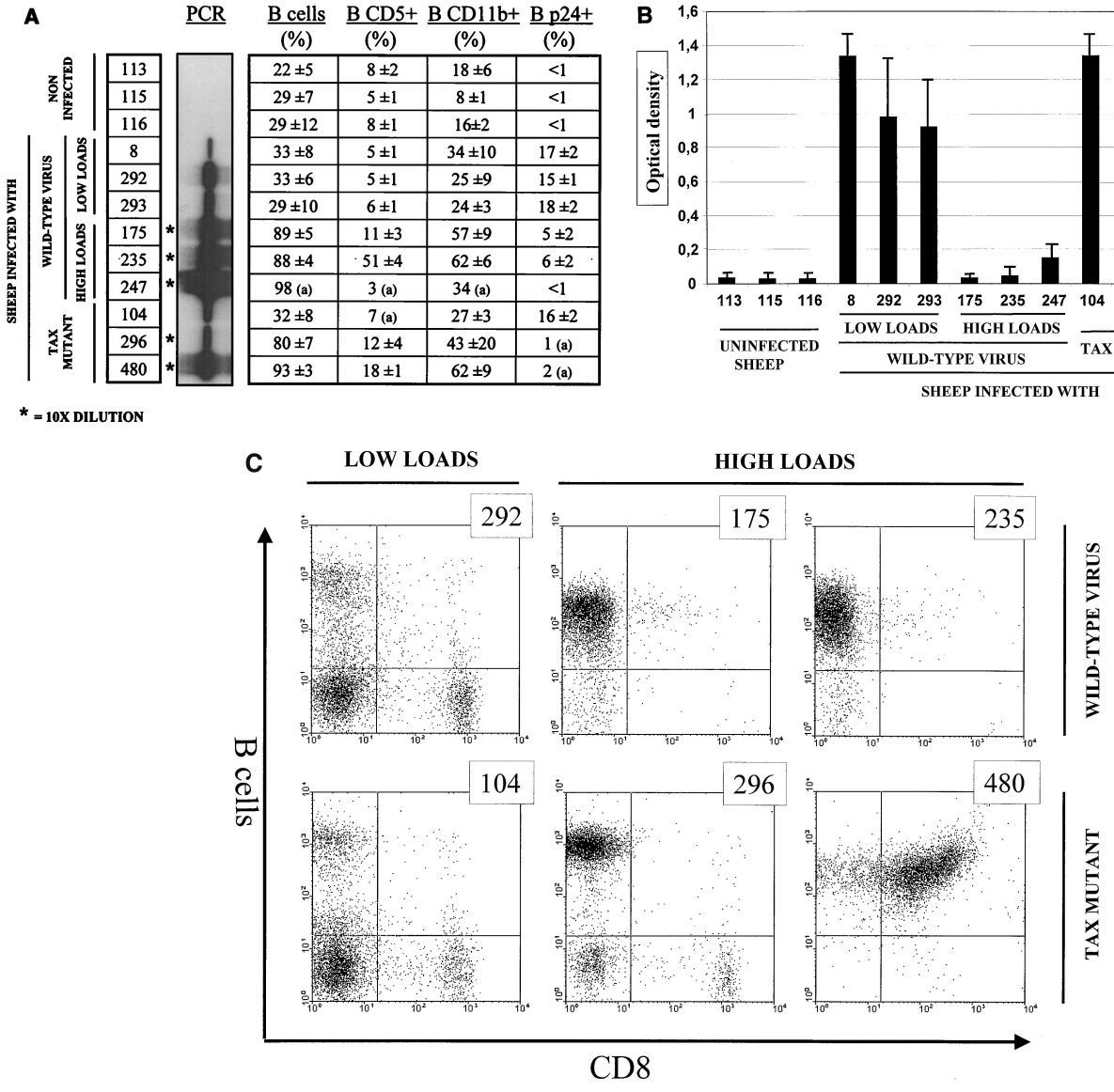

FIG. 3. (A) Phenotype of B cells in BLV-infected sheep. A series of 12 sheep were analyzed to determine and compare the phenotypes of the B-lymphocyte populations within the bloodstream of animals 104, 296, and 480 infected with pBLVTax106+293 (Tax mutant). Three sheep (113, 115, and 116) that were seronegative for BLV were used as controls, whereas six others were infected with viruses exhibiting wild-type behavior during pathogenesis (8, 292, 293, 247, 175, and 235). The different samples were classified in the figure on basis of the proviral loads as determined by semiquantitative PCR. In some lanes (*), the lysates were diluted 10-fold prior to PCR. PBMCs were isolated form the bloodstream and purified by Percoll gradient centrifugation. The cells were then labeled with monoclonal antibodies 1H4, CC17, and CC125, which recognize surface IgM, CD5, and CD11b, respectively. A similar protocol was applied for labeling the major capsid protein p24 with 4′G9 except that the cells were first cultivated for 24 h to trigger viral expression. Discrimination of the different cell populations was performed by two-color flow cytometry. The data, represented as percentage of the total PBMC population (± the corresponding standard deviation), were deduced from three independent experiments performed over a period of several weeks. When the standard deviation is not indicated (a), the results are the mean values of only two analyses. (B) Titration of the major capsid protein p24 after short-term culture. PBMCs were isolated from the sheep indicated and cultivated for 24 h. Then, the p24 antigen was titrated in the cell culture supernatants by using the ELISA procedure. The data, represented as optical densities, derive from three independent experiments. (C) Expression of CD8 marker on B lymphocytes from sheep 480. PBMCs from six representative sheep infected either with wild-type viruses (292, 175, and 235) or the Tax mutant (104, 296, and 480) were double-labeled with monoclonal antibodies 1H4 and CC63, specific for surface IgM B lymphocytes and CD8, respectively. The cells were then analyzed by two-color flow cytometry, and results from a representative experiment (out of three) are shown as dot plots.

It thus appears that the phenotype of the BLV target cell is a B lymphocyte potentially harboring CD5 and CD11b markers.

The presence of these molecules on cells infected by the BLV Tax mutant was assessed by flow cytometry. To this end, PBMCs were isolated from sheep 104, 296, and 480 by using the Percoll gradient centrifugation procedure. Labeling these cells with monoclonal antibody 1H4, which binds to surface IgM, and their subsequent analysis by flow cytometry revealed that the majority of the cells within the PBMC population from sheep 296 and 480 were B lymphocytes (respectively 80 and 93%; Fig. 3A). In contrast, sheep 104 exhibited normal B-cell counts (32% versus 22 to 29% in uninfected sheep 113, 115, and 116). The phenotypes of these lymphocytes were compared with those isolated from wild-type virus-infected sheep exhibiting either high (animals 175, 235, and 247) or low (animals 8, 292, and 293) B-cell counts within their peripheral blood. The B-lymphocyte concentrations paralleled the proviral loads, as determined by semiquantitative PCR (Fig. 3A). Of

note, the samples corresponding to sheep harboring very high viral loads (marked with an asterisk) were diluted 10-fold prior to amplification. Among the B-cell population from sheep 104 infected with the Tax mutant, a minority of lymphocytes harbored the CD5 marker (32% B versus 7% B $CD5^+$), but most of them were CD11b positive (32% B versus 27% B $CD11^+$). These values are within the normal range observed in wild-type virus-infected animals at similar viral loads (sheep 8, 292, and 293). At the leukemic stage, when the circulating blood contains almost pure populations of B cells (around 90% or more), expression of the CD5 molecule was only poorly associated with the transformed lymphocytes. Indeed, only one animal (235) contained high levels of B $CD5^+$ cells (51%; Fig. 3A). In contrast, CD11b appeared to be a far better marker for the transformed B lymphocytes both in Tax mutant- and in wild-type virus-infected sheep (between 34 and 62%). There was, however, no significant and systematic difference between these two categories of infected animals.

We next analyzed the ability of the wild-type and Tax mutant viruses to be expressed during ex vivo cell cultivation. In vivo, BLV is a hiding pathogen which is rarely expressed within the infected lymphocyte population, but isolation and cultivation of the infected PBMCs permits the evaluation of viral protein synthesis (34, 52). BLV expression was estimated by two complementary techniques, ELISA and flow cytometry, based on the synthesis of the major capsid protein p24. In the asymptomatic sheep, the B-cell population expressing the p24 antigen (i.e., double-positive B^+ $p24^+$ cells) accounted for 15 to 18% of the PBMCs independently of the type of infecting virus (Fig. 3A, compare 8, 292, 293, and 104). Among the animals harboring high viral loads, ex vivo p24 synthesis becomes inefficient, particularly at the final stages of leukemogenesis. Despite tremendous levels of B lymphocytes (around 90% of the PBMCs), less than 5% of the cells were p24 positive both in wild-type and in Tax mutant cell populations (Fig. 3A, sheep 175, 235, 247, 296, and 480). The total amount of p24 expressed in the culture supernatants, as measured by ELISA, generally paralleled nicely the percentages of cells revealed by flow cytometry (Fig. 3B). The sole exception was sheep 296, infected by the Tax mutant, whose PBMCs expressed significant levels of p24 protein in the culture medium despite low numbers of p24-positive cells as revealed by flow cytometry. It should be mentioned, however, that the total amounts of p24 corresponding to this particular animal also dropped just before death (data not shown). We conclude that the mean levels of p24 and their evolution at different stages of pathogenesis are similar in all the infected sheep, independently of the type of virus.

Interestingly, during the characterization of the cell phenotypes, we observed high numbers of CD8-positive cells in sheep 480, which was infected by the Tax mutant virus. In fact, most of the B-cell population harbored this marker, as revealed by double staining and flow cytometry (Fig. 3C, 480). The expression of the CD8 molecule was confirmed by using two independent antibodies (CC63 and ST8), and transcription of the corresponding gene was verified by RNA hybridization (data not shown). In addition, two independent antibodies (1H4 and PIg45) confirmed that the leukemic cells were B lymphocytes. Such a B/CD8 phenotype was not associated with cells from other animals harboring either high (175, 235, and 296) or low (104 and 292) viral loads. More specifically, the CD8 molecule was not expressed at the surface of the leukemic B lymphocytes from sheep 296 infected by the BLV Tax mutant.

To summarize, it appears that, with the exception of a peculiar B/CD8 phenotype in sheep 480, B lymphocytes from animals infected either by wild-type virus or by the Tax mutant

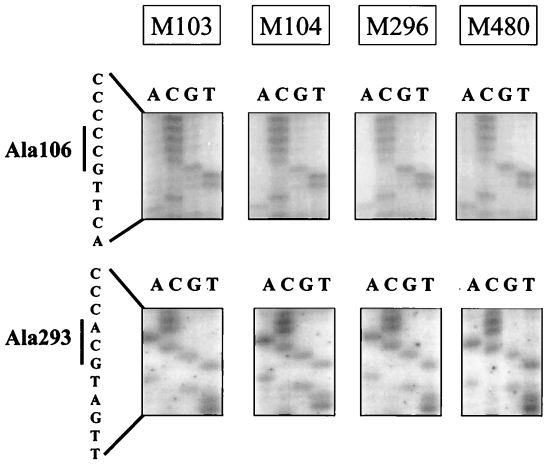

FIG. 4. Direct sequencing of codons surrounding alanines 106 and 293 of the *tax* gene in four sheep infected by the BLV Tax mutant. Blood was extracted by jugular venipuncture of sheep 103, 104, 296, and 480 infected with provirus pBLVTax106+293. After lysis, partially purified DNA was amplified by PCR using two primers flanking the *tax* gene. The resulting amplicons were then subjected to direct sequencing by PCR and migrated onto a denaturing polyacrylamide gel. The sequences surrounding alanines 106 and 293 are indicated.

are indistinguishable. In other words, both types of viruses can infect and transform similar cell types.

Tax mutant viruses in the transformed cells are not revertants. Since both the evolution of pathogenesis and the cellular phenotypes associated with the Tax mutant and wild-type virus were almost identical, our experiments needed an essential control demonstrating the lack of reversion in the tumor cells. It was indeed possible that the pathogeneses observed in sheep 296 and 480 were induced by viruses in which the two alanine mutations at positions 106 and 293 had reverted to a wild-type serine codon. Therefore, cell lysates were prepared from blood isolated by jugular venipuncture of sheep 103, 104, 296, and 480. The *tax* gene fragments were amplified by PCR, and the corresponding amplicons were subjected to direct sequencing. As illustrated in Fig. 4, the alanine codons 106 and 293 were perfectly conserved in all the lysates, demonstrating lack of reversion of the *tax* sequences. These analyses were performed at different time points, including at the terminal stage with fully transformed tumor cells. In addition, six independent amplicons were also completely sequenced over a region encompassing the entire *tax* gene. No mutation within all these samples could ever be identified (data not shown).

We conclude that the pathogeneses observed in sheep 296 and 480 infected by the BLV Tax mutant did not result from a reversion of the recombinant to a wild-type virus.

DISCUSSION

In this report, we have shown that mutations of the BLV *tax* gene that hamper immortalization of primary REFs still allow the occurrence of leukemogenesis in sheep. These observations cast light onto contradictory conclusions that might be drawn from transformation assays performed in cell culture and experiments in vivo.

A first critique to be answered concerns the lack of reversion of the recombinants in vivo. In fact, we have shown that the *tax* and *rex* sequences are not mutated after leukemogenesis in two different sheep (480 and 296; Fig. 4). However, it is possible that unidentified compensatory mutations occurred in other parts of the viral genome, for example, in the R3/G4 accessory genes. Although we have not formally ruled out this possibility

by sequencing the entire virus, we think that this is unlikely because deletion of R3 or G4 provokes a drastic reduction in the proviral loads (78).

A second point to be discussed concerns the immortalization assay itself. In our previous study on Tax phosphorylation, we reported that changing serine residues 106 and 293 into alanines abrogated its immortalization potential in primary REFs. It remains possible, however, that this type of experiment lacks sensitivity and reveals only the oncogenes harboring very high immortalization activity. Alternatively, the REF assay could be oversensitive, and slight differences in the oncogenic potential of Tax would generate large phenotypic variations during transformation in cell culture. However, in our previous studies (72; unpublished results), systematic screening of a series of mutants revealed that Tax oncogenic activity remains very frequently conserved, whereas its transactivation potential is destroyed. In fact, the Tax phosphorylation recombinant was the sole immortalization-deficient mutant that is still able to activate viral transcription. In other words, most Tax mutants are negative for transactivation and positive for immortalization, indicating that the REF assay is not sensitive to various modifications within Tax.

Another straightforward interpretation of the results would concern the differential pathways involved in transformation of REF cells or B lymphocytes. This has been illustrated in the HTLV system by specific Tax mutants that display various phenotypes depending on the cell type (3, 42, 57). In particular, the abilities of some mutants to activate transcription via the CRE, NFκB, or CArG enhancer elements were different in Rat-1, REF, and T cells. For example, clone 703 was competent for the NFκB pathway in the three cell types but displayed a mixed pattern for activation via the CRE: negative in T lymphocytes, intermediate in Rat-1 cells, and positive in REFs (3, 42). This mutant was unable to cooperate with the *ras* oncogene in REF fibroblasts but induced the formation of foci in soft agar in Rat-1 cells. Furthermore, the same mutant induced a higher proliferative response and allowed long-term expansion of CD8-positive T lymphocytes. Although the T-lymphocyte system appears to be the best mimic of the in vivo situation, this example illustrates the difficulties encountered during interpretation of the data. In this context, the availability of an animal model, such as the infection of sheep by BLV recombinants, might cast some light on our understanding of the leukemogenic process induced by these related viruses. In fact, there is an intriguing parallel between the phenotypes associated with HTLV mutant 703 and the BLV Tax recombinant, allowing us some speculation. Both clones are indeed unable to transform REF cells in cooperation with the *ras* oncogene but exhibit immortalization potential in cell culture and in vivo. In addition, a peculiar phenotype characterized by the expression of the CD8 marker occurred in 90% of the T cells transduced with Tax-I 703 and sheep 480 infected with the BLV Tax mutant. Although the CD8 marker was not present on the B lymphocytes from the other sheep (animal 296 infected with the same mutant) and was not identified at the early stages of infection (data not shown), the coincidence is appealing. Anyway, the modification of the target cell phenotype suggests that the metabolic pathways leading to full transformation are somehow perturbed. In fact, the emergence of $CD4^+$ $CD8^+$ double-positive cells has been reported in patients infected with HTLV-1 (11, 20, 39, 50). Similarly, $CD8^+$ T lymphocytes appear to be the main but not the sole target for the related HTLV-II virus (9, 21, 35, 40, 67). In contrast, the presence of a T-cell marker has only very rarely been observed on B lymphocytes from patients with large granular or chronic lymphocytic leukemia (4, 32).

The generation of B cells harboring T-specific markers in sheep 480 is a matter of speculation. First, it is possible that a rare IgM^+ $CD8^+$ subset already exists in seronegative animals and that this population is expanded after BLV infection. The two-color flow cytometry data presented in Fig. 3C would indeed suggest that such double-positive cells could be present among the PBMCs of other sheep. According to this hypothesis, the mutation of the Tax phosphorylation sites could somehow alter the viral target specificity and yield B lymphocytes with a CD8 marker. Modification of the cell preference has been illustrated in cultures of T lymphocytes infected by HTLV mutants (3, 55). An increased tropism for a given cellular phenotype might be due to better viral replication consecutive to, for instance, enhanced expression, receptor recognition, or escape from immune surveillance. In this context, the ability of Tax to activate the HTLV-1 LTR is greatly increased in $CD4^+$ cells compared to CD8-positive lymphocytes (49). From our data, it does not appear that the BLV Tax phosphorylation mutant mediates enhanced transcription during transient-transfection experiments (73) and ex vivo (Fig. 3B). In addition, based on the evolution of the proviral loads, viral replication of the Tax mutant also appears unaffected (Fig. 1). Another hypothesis underlining the generation of IgM^+ $CD8^+$ cells would be the ability of the Tax mutant to induce the expression of the CD8 protein. We think that this assumption is unlikely because (i) transient transfection of the pSG*tax106+293* vector does not augment CD8 RNA transcription in culture (data not shown) and (ii) the presence of the CD8 molecule is not always associated with the Tax mutant virus (in the case of sheep 296). We therefore favor the hypothesis based on alteration of cell target specificity.

A fact that merits some comment is the imperfect concordance between the presence of the CD8 marker and infection by the Tax mutant. The CD8 molecule indeed appeared to be expressed only at the final stage of leukemogenesis (data not shown), and this phenotype was not observed at any time in another animal (sheep 296; Fig. 3C). A similar situation holds true for T lymphocytes immortalized in cell culture by HTLV-1 Tax mutants, the cell lines generated in vitro being either $CD4^+$, $CD8^+$, or double positive (3, 55). In contrast, cells immortalized by the wild-type Tax-I protein were, in their great majority, pertaining to $CD4^+$ lymphocyte subtypes. Altogether, these observations indicate that the frequency of an altered phenotype is increased when the *tax* gene is mutated. Determining whether this is a general rule in sheep infected with the BLV Tax mutant is hampered by experimental restrictions, such as limited numbers of animals and long latency periods. It should, however, be mentioned that, in a series of eight tumor biopsies from sheep infected by wild-type viruses, none of them were CD8 positive (data not shown). In addition, this marker has never been associated with BLV-infected cells in naturally infected cattle (reviewed recently in reference 78). The presence of the CD8 molecule at the surface of the B lymphocytes of sheep 480 thus appears to be a very rare event, but the analogy with other viral systems like HTLV (3) or even other leukemias in humans (32) is striking (4).

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