JOURNAL OF VIROLOGY, Sept. 1991, p. 4959–4965
0022-538X/91/094959-07$02.00/0

Vol. 65, No. 9

Episodic Occurrence of Antibodies against the Bovine Leukemia Virus Rex Protein during the Course of Infection in Sheep

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Received 8 March 1991/Accepted 24 May 1991

Infection by bovine leukemia virus (BLV) is characterized by a long clinical latency after which some individuals develop B-cell tumors. The contributions of the viral regulatory proteins Tax and Rex during clinical latency and disease are incompletely understood. To learn about Rex expression in the host, we used a sensitive immunoprecipitation assay to detect Rex antibodies throughout the course of BLV infection in sheep. Sixty percent of the infected animals produced Rex antibodies in intermittent episodes. This pattern differed markedly from that of antibodies to virion structural proteins, which were maintained in all animals throughout infection. Only one of two animals that developed tumors had detectable Rex antibodies at the time, although the other had previously demonstrated an especially strong Rex antibody response. We examined the Rex response in the context of BLV infection by comparing it with the frequency of circulating mononuclear blood cells that could transcribe BLV RNA or produce infectious virus. Episodes of Rex antibody occurrence followed some but not all increases in the number of BLV-transcribing cells. Since the appearance of circulating antibodies requires that the intracellular Rex protein be available to serve as antigen, the episodic pattern of occurrence of Rex antibodies could result from intermittent killing by virus-specific cytotoxic cells. Fluctuations in titer that were observed during some episodes of Rex response could be due to antibody retention by antigen present in lymphoid tissue.

Bovine leukemia virus (BLV), human T-cell leukemia virus types I and II (HTLV-I and -II), and simian T-cell leukemia virus constitute a family of retroviruses that have similar genetic structures and modes of action. Infection by these viruses is characterized by long clinical latency followed, in only a few infected individuals, by terminal disease (1, 2). Virus expression is latent within most infected cells; circulating lymphocytes carry integrated proviral DNA but do not produce detectable virus (15). However, the continuing presence of antiviral antibodies throughout infection indicates that virus production does occur, most probably in lymphoid tissues. The specific cellular events that activate virus expression in the infected host are unknown, but in culture, polyclonal activators of lymphocytes stimulate virus production (11, 19, 29). This suggests that immune responses stimulate viral replication in vivo and may play important roles in the progression from clinical latency to disease.

Replication of these viruses is regulated by two virus-encoded proteins, Tax and Rex (10). Tax acts in concert with cellular factors to activate transcription from the viral long terminal repeat. Rex controls translational availability of different viral messages, although the mechanism is not understood. Since the Tax and Rex proteins regulate virus expression, they presumably mediate the exit of the virus from cellular latency. These regulatory proteins are expressed at low levels in the host cell, making their detection difficult. However, the patterns of antibody response to Tax and Rex might serve as indicators of prior increases in their production or availability in vivo.

Antibodies to virus-encoded regulatory proteins have been observed in both patients and asymptomatic carriers infected with either HTLV-I or human immunodeficiency virus (HIV). Titers of antibodies to some HIV regulatory proteins as well as to the virus capsid protein (CA; p24) change over the course of infection (17, 31, 32). In rare cases, all HIV antibodies can synchronously disappear for a time (8). Since studies most often have used only a few serum samples from each of many patients, it is difficult to interpret the results in the context of cellular activation and disease progression. Longitudinal studies with frequent sampling are required for such interpretation. Since little is known about antibodies to Rex during either HTLV or BLV infection, we assessed the presence of these antibodies throughout the course of BLV infection in sheep. Here we present the results and place them in the context of several cellular measures of infection.

MATERIALS AND METHODS

Plasmid construction. The 973-bp *ClaI*$_{7113}$-to-*RsaI*$_{8086}$ fragment (numbered according to reference 28), encompassing the majority of the second exon of the *rex* open reading frame, was isolated from the Belgian tumor clone of BLV (4). After digestion of the plasmid pSP6-XβM (16) with *BalI* and *Hin*cII, blunt-end ligation with the BLV fragment resulted in a construct (pSP6rex) which carries the SP6 bacteriophage promoter followed by the β-globin 5′ untranslated region and *rex* coding sequences. For in vitro transcription, the plasmid was linearized with *Sma*I and purified by using Geneclean (Bio101, La Jolla, Calif.).

In vitro transcription and translation. In vitro transcriptions were performed with SP6 polymerase in the Riboprobe system (Promega Corp., Madison, Wis.). A trace amount of [^{35}S]CTP was included in the 100-μl reaction mixture to allow quantitation of yield. Typically, 10 to 20 μg of uncapped RNA was transcribed from 2 to 3 μg of linearized plasmid. The reaction mixture was then treated with RNase-free DNase (RQ DNase; Promega), phenol extracted, and ethanol precipitated. To verify their sizes, ^{35}S-labeled transcription products were analyzed by electrophoresis through 1% agarose in the presence of 2.2 M formaldehyde (22). The

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electrophoresed RNA was transferred to nitrocellulose which was then saturated with 20% 2,5-diphenyloxazole (PPO) in toluene, air dried, and exposed to film.

In vitro translations were performed with amino acid-depleted rabbit reticulocyte lysates (Amersham Corp., Arlington Heights, Ill.) supplemented with 100 mM potassium acetate. RNA was denatured at 65°C for 10 min and quickly cooled on ice. Approximately 2 μg of RNA was used in 200 μl of translation reaction mixture. [^{35}S]methionine (167 μCi; 1,000 Ci/mmol; Amersham) was added along with amino acids lacking methionine, and the reaction was incubated at 30°C for 90 min. Synthesized protein was quantitated by trichloroacetic acid precipitation after RNA was removed by incubation with 20 μg of RNase A at 30°C for 10 min. Translation products were analyzed by electrophoresis through 12.5% polyacrylamide gels containing sodium dodecyl sulfate (SDS), as described below.

Immunoprecipitation. For use as antigen, ^{35}S-labeled Rex protein was diluted with RIPA buffer (10 mM Tris hydrochloride [pH 7.4], 0.15 M NaCl, 1.0% Nonidet P-40, 1.0% sodium deoxycholate, 0.1% SDS, 1% aprotinin). In each assay, 3 μl of sheep serum was mixed with 50 μl of antigen containing 80,000 cpm of Rex protein, an amount usually derived from 2 μl of the translation reaction mixture. After incubation at 4°C for 14 to 16 h, 50 μl of a 50% (vol/vol) suspension of protein G-Sepharose (Pharmacia, Piscataway, N.J.) in RIPA buffer was added, and the assay was further incubated for 2 h at 4°C on a rocking platform. The protein G-Sepharose and bound antigen-antibody complexes were pelleted at 4°C and washed three times with ice-cold buffer (10 mM Tris hydrochloride [pH 7.4], 0.1 M NaCl, 0.1% Nonidet P-40). Gel sample buffer (25 μl) containing 50 mM dithiothreitol was added to the pellet. After boiling, 10 μl of the resulting supernatant was electrophoresed through an SDS–12.5% polyacrylamide gel (18). Gels were fixed, treated with En^{3}Hance (New England Nuclear, Boston, Mass.), and then exposed to film at −80°C for 24 h unless otherwise indicated. Intensities of autoradiographic bands were quantitated by using a Visage 70 densitometer (BioImage, Ann Arbor, Mich.). A positive control was included in all assays.

Collection of serum samples from infected sheep. Sera were obtained from sheep that had been infected at 2 to 10 weeks of age with BLV derived from persistently infected bat lung cells, as previously described (20). Animals were bled by jugular venipuncture twice weekly for the first 3 months after infection and approximately once a month thereafter.

Detection of viral RNA and antiviral antibodies and determination of virus production. Viral RNA was detected by in situ hybridization using ^{35}S-labeled antisense RNA probes spanning the BLV genome, as previously described (19). Mononuclear blood cells were cultured for 2 to 5 h at room temperature in Iscove's medium containing 10% fetal bovine serum before they were deposited onto slides and fixed for in situ hybridization. An infectious-center assay was used as previously described (20) to measure mononuclear cells that produced BLV within 24 to 48 h of being cultured in minimal Eagle's medium supplemented with 5% fetal bovine serum. After day 642, infectious-center assays were also performed by suspending mononuclear cells in growth medium containing 1% methylcellulose (25). This improved procedure revealed increased numbers of infectious centers.

Immunoblots were performed as previously described (24) by using an alkaline phosphatase detection system. Antisera were tested at a 1/30 dilution. Signal intensities were assigned values of 0 to 4 after two independent visual evaluations. Neutralizing antibodies were measured as described previously (24) by the ability of heat-treated serum to inhibit formation of syncytia induced by cell-free virus. The percent inhibition was determined by comparison with average numbers of syncytia obtained from virus treated with culture medium containing fetal bovine serum.

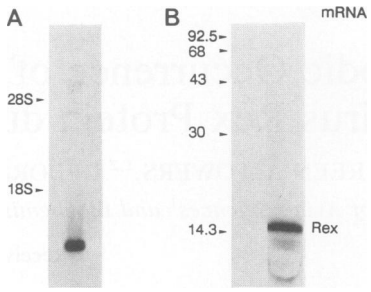

FIG. 1. In vitro transcription and translation of Rex. (A) ^{35}S-labeled *rex* RNA was transcribed, purified, and analyzed by 1% agarose-formaldehyde gel electrophoresis and fluorography. The positions of 18S and 28S rRNAs are indicated. (B) The products of translation of *rex* RNA were analyzed by 12.5% SDS-polyacrylamide gel electrophoresis and fluorography. The positions of molecular size markers (sizes in kilodaltons) are indicated.

Production of rabbit antipeptide antibodies. A C-terminal 12-amino-acid peptide (PSSGTAFFPGTT) of the Rex protein was synthesized with an Applied Biosystems (Foster City, Calif.) 430A peptide synthesizer. In the synthesis, a cysteine was added to the N terminus for cross-linking the peptide to keyhole limpet hemocyanin (KLH; Calbiochem, La Jolla, Calif.) through the bivalent agent *m*-maleimidobenzoyl *N*-hydroxysulfosuccinimide ester (Pierce, Rockford, Ill.) (9). The extent of cross-linkage was estimated by comparison of amino acid compositions of KLH and KLH-peptide conjugate.

Three New Zealand White rabbits were injected intradermally with 200 μg of KLH-peptide conjugate in Freund's complete adjuvant. Rabbits were boosted 7 months later and again after 5 additional months by intramuscular injection with 100 μg of peptide conjugate in Freund's incomplete adjuvant. Rabbits were exsanguinated 12 days after the final boost. All rabbits produced antibodies that recognized both the C-terminal Rex peptide and the product of in vitro translation.

RESULTS

In vitro transcription and translation. The expression vector pSP6rex was constructed by replacing the β-globin-coding and 3′ untranslated sequences of pSP6-XβM with those of the *rex* gene of BLV. Transcription of the linearized plasmid by SP6 polymerase produced primarily a single RNA species of approximately the expected size of 1,064 nucleotides when analyzed by denaturing gel electrophoresis (Fig. 1A). This RNA encodes 111 of the 154 amino acids of the native Rex protein, starting with Met-43. When the RNA was translated in a reticulocyte lysate, the major radiolabeled product was a polypeptide migrating in SDS-polyacrylamide gels at approximately 15,000 Da, somewhat larger than the calculated molecular size of 11,740 (Fig. 1B). Smaller bands were sometimes present. They probably correspond to products of internal initiation from downstream methionine codons, since these polypeptides could

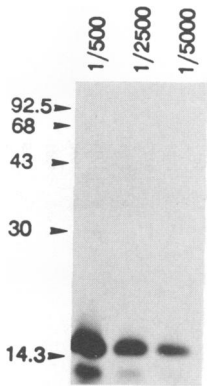

FIG. 2. Immunoprecipitation of Rex. Serial dilutions of a rabbit antipeptide serum were used to immunoprecipitate in vitro-translated Rex protein. Precipitated protein was analyzed on a 12.5% SDS–polyacrylamide gel. Positions of molecular size markers (sizes in kilodaltons) are shown. The gel was exposed to film for 3 h.

be immunoprecipitated with Rex C-terminal antiserum (Fig. 2).

Immunoprecipitation assay. To establish a sensitive immunoprecipitation assay for Rex antibodies, we tested several dilutions of a rabbit antipeptide serum which had been raised against the 12 C-terminal amino acids of the Rex protein (PS SGTAFFPGTT). With the standard amount of antigen that we chose to use (80,000 cpm), a strong positive signal was evident at a 1/5,000 dilution of the twice-boosted serum (Fig. 2). This signal was seen after the gel containing radiolabeled protein was exposed to film for only 3 h. The same antiserum exhibited a titer (50% of maximum signal) of 1/400 when tested in an enzyme-linked immunosorbent assay using the free peptide as antigen (data not shown).

Rex antibodies in BLV-infected sheep. We then used this immunoprecipitation assay to determine when, during the course of infection, BLV-infected sheep produced antibodies specific for the Rex protein. In previous immunoblot analyses, we had found that BLV structural protein antibodies have low titers early in infection (24). Since the regulatory proteins of BLV are produced in small amounts and are not incorporated into virions, we expected that titers of antibodies to Rex would also be low. Immunoblot analysis using in vitro-translated Rex as antigen yielded no convincing signals from sheep serum. Consequently, immunoprecipitations were performed with sheep serum at a dilution of 1/17, and gels were exposed to film for 24 h. Control experiments showed that signal intensity decreased with antibody concentration. No significant background signals arose from sera drawn prior to infection (Fig. 3A, day 0) or from uninfected animals. When sera drawn over periods of up to 4 years were tested, an immune response to Rex was detected in 6 of 10 infected animals.

In one especially striking case (animal 469), the response was strong and episodic (Fig. 3A). Antibodies to Rex first appeared by 21 days after infection and occurred in three discernible cycles. The first episode of strong antibody response diminished by day 91; it was followed almost immediately by a second episode which began by day 104 and lasted through day 250, encompassing a reproducible decrease in reactivity on day 131. Antibodies to Rex did not reappear until 2 years later, 1,007 days after infection. Titers, defined as the reciprocal of the highest dilution at which a positive signal was obtained, were determined for a strong positive serum from each cycle: these were 1,600 (day 36), 800 (day 166), and 400 (day 1035).

BLV infection in animal 469. We then asked whether animal 469's pattern of Rex reactivity could be explained by any other feature of BLV infection. The course of infection in these sheep has been characterized by several measures of viral gene expression and by their antibody responses to BLV structural proteins. The events of very early infection (0 to 225 days) have already been described (20, 24). Animal 469's profile of infection is presented in Fig. 3B. Two cellular measures of BLV infection were made: counts of peripheral blood mononuclear cells (PBMCs) that transcribed BLV when stimulated briefly in culture and counts of PBMCs that produced infectious virus. (Later during infection, the use of methylcellulose to immobilize PBMCs in the infectious-center assay significantly increased counts of virus-producing PBMCs.) Neutralizing antibodies and antibodies binding to the structural proteins, capsid (CA) and surface glycoprotein (SU), were also measured. For comparison, the results of the Rex antibody assays are presented as a histogram indicating relative strength of the response.

Rex antibodies first appeared together with viral RNA-transcribing and virus-producing PBMCs. The timing of the Rex antibody response differed in several respects from the response to the structural proteins. A rapid initial response to Rex, CA, and SU (neutralizing antibodies) coincided with the first increase in transcriptionally competent cells. However, Rex antibodies reached maximum strength rapidly, while structural-protein antibodies developed more slowly. Following the major increase in BLV-transcribing cells, there was a second cycle of circulating antibodies which began much earlier for CA and SU (days 42 and 60, respectively) than for Rex (day 104). For each protein, this response persisted for approximately 150 days.

During the next 2 years, Rex antibodies were not detectable, but antibodies binding to the structural proteins reappeared and were maintained, along with the neutralizing-antibody titer. Nearly 3 years after infection, Rex antibodies reappeared for approximately 100 days after a long period of increases in both BLV-transcribing cells and infectious centers. Thus, each of the three episodes of anti-Rex activity occurred along with or subsequent to a peak in numbers of transcriptionally competent cells, although not all such increases were followed by an antibody response to Rex.

One year following the final episode of Rex antibody occurrence, the animal developed B-cell leukemia and eosinophilia. Cultured PBMCs did not produce virus. At autopsy, lymph nodes were highly enlarged and a large lymphoproliferative focus was present in one kidney. Despite the progression to disease, Rex antibodies were not detected again.

Rex antibodies in other infected animals. Five other animals exhibited measurable but weaker immune responses to Rex during BLV infection (Fig. 4). Animals 407 and 468 both had a transient response to Rex that appeared approximately 200 days after infection and lasted for 200 to 250 days. In each case, Rex antibodies appeared after a peak in numbers of BLV-transcribing cells, although for neither animal was this the only episode of increased viral transcription (data not shown). Antibodies to Rex reappeared in both sheep at 1,500 days, the most recent time assessed. Animal 167, too, exhibited a very brief Rex antibody response more than 1,800 days after infection. In all three animals, these late antibodies appeared following long and gradual increases in the numbers of BLV-producing PBMCs. None of these sheep has yet shown clinical signs of disease.

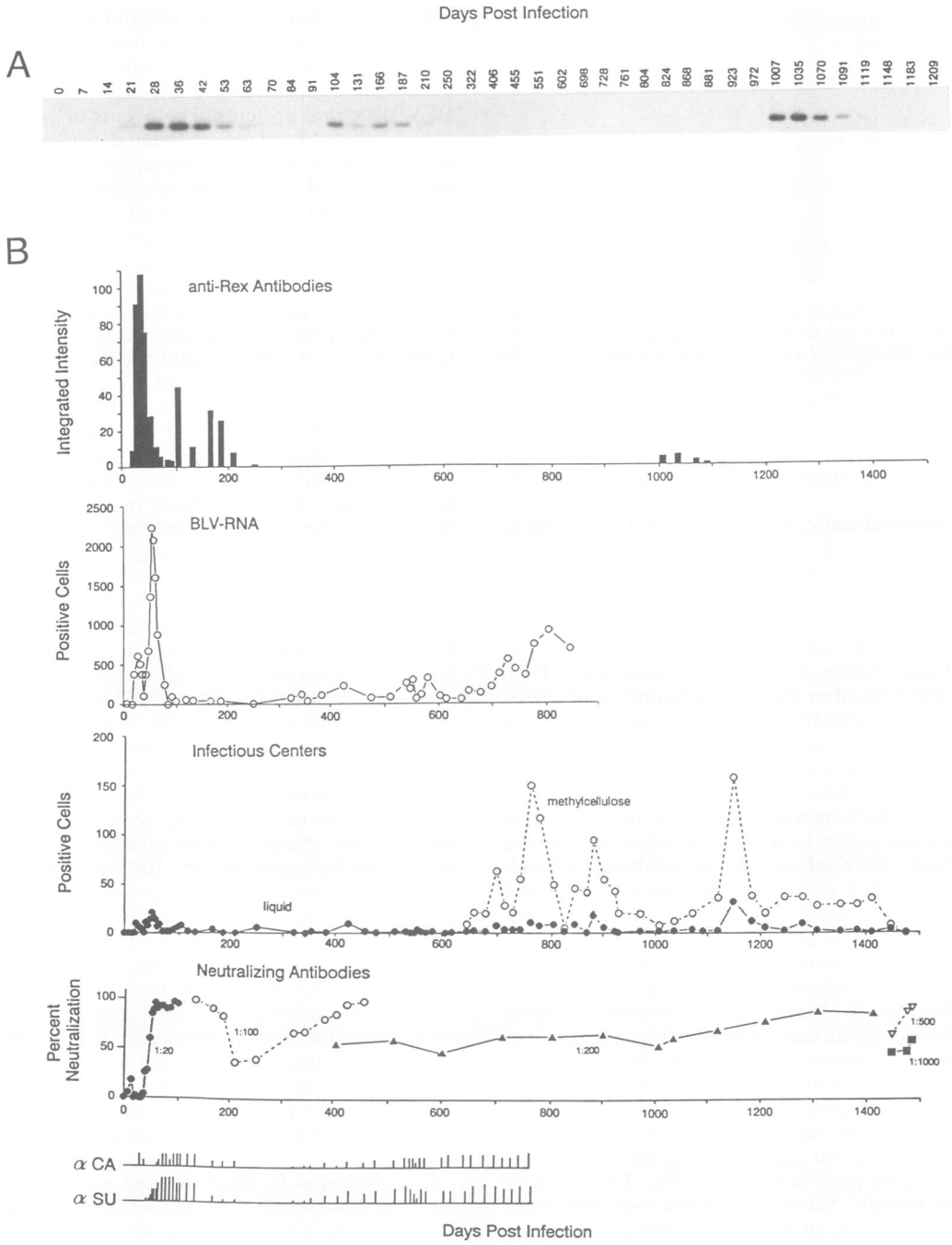

FIG. 3. Episodic Rex antibody occurrence and BLV infection in sheep 469. (A) Autoradiograms of the immunoprecipitation assay for Rex antibodies. The signals for days 0 to 698 result from an 8-h exposure of the gels to film, and those for days 728 to 1209 are from a 24-h exposure. (B) Quantitative representation of Rex antibodies aligned with other measures of BLV infection. Rex antibodies were quantitated by densitometric scans of autoradiograms exposed for either 8 or 24 h. Results were normalized by using a sample (day 91) which could be quantitated at both exposures. BLV transcription in PBMCs was determined by in situ hybridization of ^{35}S-labeled probes to cells briefly cultured after isolation. Production of infectious BLV was measured as syncytia induced after PBMCs were cocultivated with an indicator cell line. Neutralizing antibodies were measured by the ability of serum to inhibit infection by cell-free BLV. As titer rose over the course of infection, different antibody dilutions were used as indicated to avoid saturation of the assay. Structural protein antibodies (αCA and αSU) were determined by immunoblot analysis using 1:30 serum dilutions. Results are presented on a relative scale of 0 to 4, with 4 being the strongest signal seen. Except for data on Rex antibodies, data for days 0 to 225 are replotted from references 20 and 24.

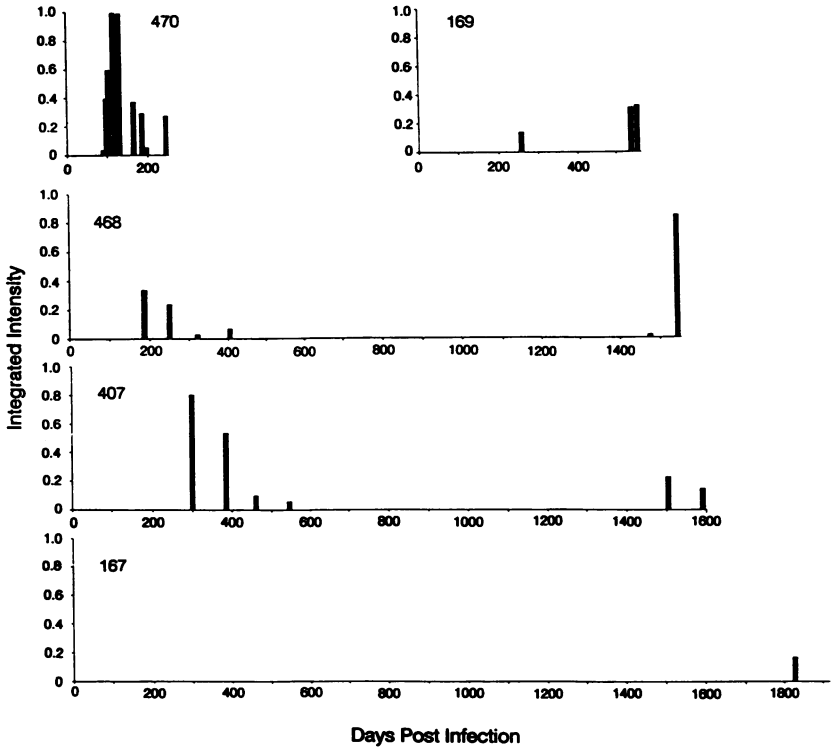

FIG. 4. Rex antibodies in other infected sheep. Quantitative results of immunoprecipitation assays performed on sera drawn at least every 100 days over the course of infection. The *x* axis extends to the final day tested, even when antibodies were not detected (animals 468, 407, and 167), or to the day of the animal's death (animals 470 and 169). Note that the scale for intensity is only 1/100 of that used in Fig. 3. All data were taken from 24-h exposures.

Animals 169 and 470 exhibited quite different Rex responses. Antibodies to Rex appeared in animal 169 only during the terminal 2 weeks of a B-cell leukemia which developed relatively soon after infection. PBMCs produced substantial amounts of BLV in culture. Multicentric lymphoproliferation and a solid tumor on the omasum were revealed upon autopsy. In contrast, animal 470 developed antibodies to Rex by day 98 after infection, at the end of its third early peak of transcriptionally active cells (20). When this animal was euthanized on day 246 because it had failed to thrive, lymphoproliferation was evident but no tumors were found. Although Rex antibodies reached maximum strength on days 117 and 138, detectable levels persisted until the animal's death.

The remaining 4 of the 10 infected animals had no detectable Rex antibodies. This was not due to lack of virus production, as evidenced by the ability of their PBMCs to produce BLV in culture and by their neutralizing-antibody titers, which reflect both virus production in vivo and capacity for immunological response (Table 1). Rex-responsive and nonresponsive animals showed similar ranges in these measures of infection.

DISCUSSION

We delineated the pattern of antibodies against a retroviral regulatory protein to learn about the antigenic availability of the protein in vivo and potentially about its contribution to disease progression. Using a sensitive immunoprecipitation assay, we found that 6 of 10 BLV-infected sheep produced antibodies to Rex in an episodic fashion over the course of infection and disease. In one animal, the response was especially strong. Both of the sheep that developed tumors produced Rex antibodies, although only one exhibited a detectable titer at the time of disease. This is consistent with the results of Yoshinaka and Oroszlan (36), who found that only 2 of 11 cows with BLV-induced tumors had antibodies to Rex.

Regulatory protein antibodies have been studied in other retroviral infections. HIV encodes multiple regulatory proteins, including Tat and Rev, which are functionally analogous to Tax and Rex, as well as Nef, Vif, Vpu, and Vpr, whose functions are less well defined (34). Nef elicits an immune response more frequently than does Tat, Rev, or Vif, but correlations between these antibodies and disease progression have not been consistent (5, 17, 27, 32). In the sole longitudinal study of Rev antibodies (26), 30% of asymptomatic HIV carriers seroconverted with antibodies to Rev that persisted for the duration of the 42-month study,

TABLE 1. Similar ranges for measures of infection in Rex-responsive and nonresponsive animals

Rex antibody	Animal	Day postinfection	Infectious centers/ 500,000 cells[a]	Neutralizing titer
−	380	1498	21,900	2,200
−	409	1652	3,070	680
+	407	1561	4,075	1,600
+	468	1567	428	400

[a] PBMCs were cultured in methylcellulose medium containing 50 μg of lipopolysaccharide per ml.

while 10% showed a transient or intermittent antibody response.

During infection by HTLV, which is closely related to BLV, Tax antibodies have been found in 50 to 60% of carriers (13, 14, 35). In one study of intravenous drug abusers, 50% of those seropositive for HTLV recognized only Tax among the viral proteins (6). The presence of Tax antibodies may correlate with increased likelihood of virus transmission (3, 13). None of the HTLV studies have reported episodic Tax antibody responses, and Rex antibodies were not investigated. Antibodies to the BLV Tax protein have been detected in a single serum from each of six carrier sheep, from 25% of infected cows, and from 90% of tumor-bearing animals (33). We attempted to analyze Tax antibodies throughout BLV infection but found that Tax translated in vitro bound nonspecifically to sheep immunoglobulins. Despite extensive modification of the assay, we could not prevent this binding.

The Rex antibody response differed strikingly from the response to the BLV structural proteins CA and SU. All infected animals produced antibodies to both structural proteins, but only some responded to Rex. The Rex response was episodic, while CA and SU antibodies were continuously maintained. Antibodies to both types of proteins showed early fluctuations in animal 469. For the structural-protein antibodies, this could result when immunoglobulins are sequestered by binding to antigen, for example, in lymphoid tissues, where virus-producing cells might be concentrated. Such immune complexes have been found circulating in the blood during HIV infection (21) and are thought to reduce the titers of free antibodies to structural proteins. Sequestering of immunoglobulins by antigen probably contributed to the early fluctuations in animal 469's Rex titer at a time when virus production was extensive. However, sequestering is less likely to explain the prolonged absence of Rex antibodies, as this would require that Rex protein be available for extended periods without eliciting a detectable antibody response.

Although not yet prognostic, regulatory protein antibodies may yield information about virus replication in the host. When comparing the results of Rex immunoprecipitations with measures of viral transcription and infectivity, we found that Rex antibodies appeared following some but not all peaks in number of virus-transcribing PBMCs. Increases in circulating BLV transcription-competent PBMCs most probably reflect preceding bursts of viral replication within lymphoid tissues along with recruitment of new host cells. Some periods of virus spread must make Rex available as an antigen.

The fact that retroviral regulatory proteins elicit antibodies raises the interesting question of how these intracellular proteins become available to the immune system as antigens. Virus-induced cytopathicity and cell lysis release HIV regulatory proteins into the extracellular environment. However, for the noncytopathic HTLVs and BLV, the route is less apparent. Episodes of antibody production may follow bursts in killing of virus-producing cells by cytotoxic T lymphocytes (CTLs) or natural killer cells. High levels of these lytic cells have been observed in seropositive HIV carriers, where they represent an early antiviral response which decreases prior to clinical deterioration (30). In contrast, high levels of Tax-specific CTLs have been detected in HTLV-infected patients with tropical spastic paraparesis (12). No CTLs specific for HTLV proteins were detected among fresh PBMCs from three asymptomatic carriers (12), but this does not preclude their intermittent presence throughout the many years of infection. Episodes of CTL activity could release Rex from infected cells, producing the transient surges in Rex antibodies seen in all six animals. It is noteworthy that our sheep with the strongest response to Rex showed the largest increases in numbers of virus-producing cells in the infectious-center assays performed in the presence of methylcellulose. This viscous medium reduces cell-to-cell contact and hence may block cell-mediated killing in the assay.

Another way in which Rex could become extracellular is by secretion from infected cells. Neither Tax nor Rex contains a conventional secretory signal sequence; both of these proteins, in fact, contain sequences which direct them to the nucleus. However, recent work by Marriott and coworkers (23) indicates that Tax is found in the medium of HTLV-I-infected T-cells and stimulates proliferation of primary lymphocytes in culture. A similar growth factor-like role has been proposed for HIV Tat in Kaposi's sarcoma (7). Likewise, Rex could have a second, extracellular activity, but to our knowledge, the presence of Rex in supernatants from either HTLV- or BLV-producing cultures has not been explored.

In summary, we find that Rex antibodies are produced during brief episodes over the course of BLV infection. This is not representative of the humoral antiviral response, as animals maintain almost continuous reactivity to other viral antigens. Intermittent CTL killing could explain the episodic nature of the Rex response. Characterization of the cell-mediated immune response in BLV infection should prove important in our understanding of latency and disease.

ACKNOWLEDGMENTS

We thank Lourdes Adamson for excellent assistance with blood collection and data analysis; Maria Lau for help with the immunoblotting experiments; Donna Lagarias for in situ hybridizations; Frank Masiarz of Chiron Corp. for peptide synthesis and amino acid analysis; Larry Hjelmeland for advice on antibody production; Harold Burger for valuable discussions; and Martin Privalsky and Kathelyn Steimer for comments on the manuscript.

This investigation was supported by Public Health Service grant CA-46374 from the National Institutes of Health. M.A.P. was a Regents Graduate Fellow of the University of California. L.C.K. was a National Institutes of Health predoctoral trainee supported by Public Health Service grant GM-07377.

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