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Protection of Sheep against Bovine Leukemia Virus (BLV) Infection by Vaccination with Recombinant Vaccinia Viruses Expressing BLV Envelope Glycoproteins: Correlation of Protection with CD4 T-Cell Response to gp51 Peptide 51-70

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We have previously constructed vaccinia virus (VV) recombinants containing a complete or truncated envelope (*env*) gene of bovine leukemia virus (BLV). Only recombinants carrying the complete *env* gene (VV-BLV2 and VV-BLV3) expressed *env* glycoprotein on the surface of virus-infected cells and produced an antibody response in rabbits. In the present study, these VV recombinants were used to immunize sheep prior to challenge with BLV-infected peripheral blood mononuclear cells. Both humoral and cell-mediated immunity were monitored in infected animals. Sheep inoculated with recombinants containing the complete *env* gene showed a CD4 response to a defined epitope of gp51, but this response was absent 4 months postchallenge. Anti-gp51 antibodies appeared in animals inoculated with complete *env* 2 weeks after challenge, reached a peak at 4 weeks, and subsequently declined over 16 months. No CD4 response was recorded in animals inoculated with recombinants containing truncated *env* gene (VV-BLV1). BLV-infected control animals and those animals receiving VV-BLV1 were slower to develop antibodies postchallenge, and the titers of anti-gp51 antibodies continued to increase over 16 months. Proviral DNA was detected by the polymerase chain reaction in the four groups at 6 weeks after challenge. However, it could not be detected 4 months postinfection in the VV groups inoculated with complete *env*. Provirus was present in the VV-BLV1 and control groups over the 16-month trial period. These results demonstrate that vaccination with VV recombinants containing the complete *env* gene of BLV protects sheep against infection and that protection correlated with a CD4 T-cell response to a defined epitope.

Enzootic bovine leukosis is a neoplastic disease in adult cattle and is caused by the exogenous retrovirus bovine leukemia virus (BLV) (18). Infection is usually acquired by horizontal transmission of infected lymphocytes. Most infected animals (70%) develop lymphocytosis, and up to 10% go on to develop tumors (6). The majority of infected animals remain clinically healthy and act as carriers for the spread of the disease (6). On the other hand, sheep experimentally infected with BLV are highly susceptible to the oncogenic effects of the virus with a relatively short latency period (7, 16), making the sheep a good model for vaccine-related studies.

Antibody response to the BLV surface glycoprotein gp51 is detected shortly after infection, but despite the persistence of anti-gp51 antibodies, the disease is progressive (4, 24). Infected animals with lymphocytosis routinely have high titers of gp51-specific antibodies. Although these antibodies have neutralizing activity against BLV in vitro, this finding has raised the possibility that nonhumoral mechanisms of immunity may play a major role in protection. Furthermore, we have recently defined CD4 and CD8 T-cell epitopes on BLV gp51 (8) recognized by peripheral blood mononuclear cells (PBMCs) of most sheep and cattle. This finding raises the possibility that a defined immunogen designed to stimulate specific cellular immune responses may protect animals from BLV infection. Recently, a cell-derived vaccine producing BLV *env* gene glycoproteins and the core structural protein p24 was shown to protect cattle against BLV-induced leukemia (1).

We and others have produced vaccinia virus (VV) recombinants which express the BLV *env* glycoproteins on the surface of infected cells (15, 21, 22, 25). Seven from eight sheep vaccinated with these recombinants resisted a challenge with BLV-infected lymphocytes and possessed neutralizing antibodies to BLV (25). On the other hand, while Ohishi et al. (22) reported protective immunity in sheep vaccinated with VV recombinants expressing BLV *env*, they failed to detect binding or neutralizing antibodies to BLV. This result further highlights the possibility that cellular immunity may be the critical form of immunity and that specific antibodies measured in some studies may simply be a marker of an (unmeasured) T-cell response.

We report here a vaccine trial utilizing VV recombinants containing the complete or truncated form of the *env* gene of BLV and are able to show that protection, as monitored by polymerase chain reaction (PCR) detection of proviral DNA and blood passage from trial animals into naive recipients, correlates with induction of epitope-specific CD4 T-cell responses. However, clearance of viral antigen is followed by loss of specific CD4 responses in peripheral blood,

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suggesting that antigenic persistence may be necessary to maintain immunological memory.

MATERIALS AND METHODS

Cells and viruses. VV-WR-L929 and VV-ELSTREE strains were used for the construction of recombinant viruses, and CV-1 cells were used for growth and maintenance of these recombinants (3). Peripheral blood lymphocytes were separated from whole blood by Ficoll-Paque centrifugation.

Construction of recombinant viruses. The *env* gene of an Australian BLV provirus, isolated from a cow tumor, was released as a 2.33-kb *Xho*I fragment (nucleotides 4554 to 6886 [5]) and cloned into the *Sal*I site of pUC8. From this clone, three VV plasmid vectors, pSK26, pSK27, and pSK29, were constructed as described by Kumar et al. (15). Three recombinants (VV-BLV1, VV-BLV2, and VV-BLV3) were derived from pSK26, pSK27, and pSK29, respectively. VV-BLV1 (truncated form) contained the coding sequence for gp51 and part of gp30 (nucleotides 4813 to 6124). VV-BLV2 and VV-BLV3 contained the entire *env* gene of BLV under the control of promoters isolated from VV (P7.5) and fowlpox virus (PFE/L), respectively. The latter constructs are referred to as complete *env*.

Animals. Sheep (6 months old) were divided into four groups. Ten animals in group I were used for vaccination with VV-BLV1. Group II contained six sheep vaccinated with VV-BLV2. Group III (six animals) was vaccinated with VV-BLV3, and seven animals (group IV) were left unvaccinated as controls for challenge. Immunized groups were housed in fully screened, separate pens provided with isolated drainage, and the controls (unimmunized sheep) were kept isolated from vaccinated groups in a separate building.

Immunization and challenge of sheep. Groups I, II, and III were injected intradermally with 2×10^8 PFU of recombinant viruses VV-BLV1, VV-BLV2, and VV-BLV3, respectively. A booster dose of the same amount was administered once, 2 weeks after the initial vaccination. Vaccinated animals were challenged 5 weeks after inoculation with whole blood containing 4×10^4 PBMCs from a BLV-infected cow at the persistent lymphocytosis stage (80% lymphocytes). Blood samples were collected after the initial and booster inoculations, every 2 weeks for 8 weeks postchallenge, and at monthly intervals as indicated for DNA extraction, antibody measurement, cell-mediated response, and hematology.

DNA extraction. Chromosomal DNA was extracted from PBMCs according to the method of Miller et al. (19). Briefly, cells were lysed with nuclei lysis buffer (10 mM Tris-HCl, 400 mM NaCl, 2 mM EDTA [pH 8.2]). The cell lysates were digested overnight at 37°C with 0.2 ml of 10% sodium dodecyl sulfate (SDS) and 0.5 ml of a proteinase K solution (1 mg in 1% SDS–2 mM EDTA). After digestion, 1 ml of 6 M NaCl was added to remove protein, and DNA was precipitated with 2 volumes of ethanol and dissolved in 10 mM Tris-HCl–0.2 mM EDTA (pH 7.5). DNA concentration was estimated by measuring the A_{260}.

PCR. PCR was used to assay for the presence of proviral DNA in circulating blood of sheep immunized with VV recombinants, using oligonucleotide primers from the *env* gene as described previously (20). Another set of oligonucleotide primers for the *pol* gene was used for detection of BLV provirus in vaccinated animals every 2 weeks up to 8 weeks after challenge and at monthly intervals thereafter. These primers extended from sequence positions 2352 to 2937 (5), giving rise to a 586-bp fragment of DNA. The sequences of the oligonucleotides, the amplified fragment sizes, and the positions of the fragments within the BLV genome are shown in Table 1. Assay conditions were similar to those described by Naif et al. (20).

TABLE 1. Oligonucleotide primers used for amplification of BLV proviral DNA to discriminate between vaccinated and unvaccinated animals[a]

Primer no.	Primer sequence (5'-3')	Positions in BLV sequence	Amplified region and fragment size (bp)
1	CCCACAAGGGCGGCGCCGGTTT	5100–5121	*env*, 443
2	GCGAGGCCGGGTCCAGAGCTGG	5522–5543	
3	AGGCCCTTCAAGACCTGGTCCA	2352–2374	*pol*, 585
4	GGACACCAGAAGAGACTGGGAA	2775–2797	
5	AAAGGGACAGGCGAAGGCGTCT	2915–2937	

[a] Sequence and nucleotide numbers based on data from Coulston et al. (15).

DNA hybridization analysis. Southern blots were carried out by filter hybridization methods. PCR products were run on 1% agarose gels, stained with ethidium bromide, and photographed on a UV transilluminator. DNA samples were then transferred to Hybond-N by using a vacuum blotting system (LKB-VacuGene), and hybridization was carried out according to the method of Maniatis et al. (17). Filters were prehybridized at 65°C for 8 h in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, 0.1% SDS, 50 mM Na_2HPO_4 (pH 6.5), and 250 μg of denatured herring sperm DNA per ml. Hybridization was carried out at 65°C for 16 to 24 h with a 2.3-kb BLV *Xho*I fragment labeled with [^{32}P]dCTP, using the random priming method (Bresatec kit) under the same conditions as for prehybridization. Filters were subsequently washed twice with 2× SSC and finally with 0.1% SDS–1× SSC at 65°C for 10 to 30 min prior to exposure to Kodak X-Omat film at −80°C for several hours.

Agar gel immunodiffusion test. Serum samples from all sheep were tested for BLV gp51 antibodies by using the agar gel immunodiffusion test (Leukassy-B; Pitman-Moore, Washington Crossing, N.J.). The gp51 antibody titers in the sera were determined biweekly until 8 weeks after challenge and then at intervals as indicated.

Hematology. Venous blood samples for total and differential leukocyte counts were collected at the following times: before initial vaccination, at biweekly intervals after initial vaccination until 8 weeks after challenge, and subsequently at monthly intervals throughout the observation period.

Peptide synthesis. Peptides corresponding to the BLV gp51 protein sequences were synthesized manually by the multiple peptide procedure (13). A series of 26 peptides (20 amino acids in size and overlapping by 10 amino acids) was prepared. Mapping of proliferation and cytotoxic T-lymphocyte (CTL) epitopes on BLV gp51 molecules by using these peptides has been described elsewhere (8).

Lymphocyte proliferation assay. The proliferation assay was performed as described previously (10). Briefly, 2×10^5 PBMC per well were cultured in RPMI medium containing 10% fetal calf serum in flat-bottom microtiter plates (Nunc, Roskilde, Denmark) in the presence of 0.5, 1.0, 2.5, 5.0, and 10.0 μg of a synthetic peptide, NGYPKIYWPPPQGRRR FGAR (peptide 51-70 of gp51), per ml. Each concentration was prepared in triplicate. The final volume was 0.2 ml per well. Control cultures without peptide were included. After

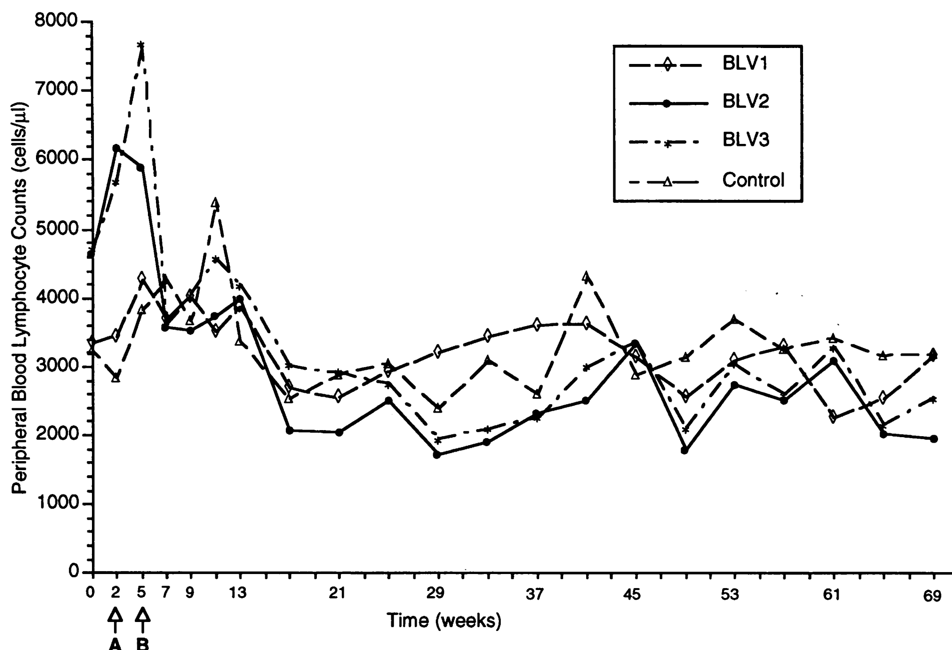

FIG. 1. Peripheral blood lymphocyte changes in sheep vaccinated with VV recombinants and challenged with PBMCs from a BLV-infected cow with persistent lymphocytosis. Group I, 10 sheep vaccinated with VV-BLV1; group II, 6 sheep vaccinated with VV-BLV2; group III, 6 sheep vaccinated with VV-BLV3; group IV, 7 sheep as controls. Animals were immunized at time 0, given booster immunizations after 2 weeks (A), and challenged after a further 3 weeks (B).

7 days of incubation at 37°C with 5% CO_2, the cells were pulsed with 0.5 µCi of [*methyl*-^{3}H]thymidine (Amersham) per well in a volume of 20 µl for 16 h. The cells were harvested, and proliferation was estimated by liquid scintillation counting. The proliferation was expressed as a stimulation index (SI = cpm in tested sample/cpm in control wells). The response to any concentration was considered positive if the SI was greater than 2.

Generation and assay of CTL. The assay used to measure the CD8 cytolytic activity was that described previously (12). Briefly, mononuclear cells (3×10^6) were cultured in the presence of 10 µg of synthetic peptide per ml for 8 to 10 days, with human interleukin-2 (50 U/ml) added on day 2. Next, 10^6 cells were restimulated in secondary culture with 9×10^6 irradiated (2.5 Gy) autologous mononuclear cells in the presence of peptide and interleukin-2 for 5 days. Autologous fresh target PBMCs (3×10^6), stimulated with phytohemagglutinin for 3 days, were incubated overnight with individual peptides or groups of peptides and then labeled with 100 µCi of $Na_2{}^{51}CrO_4$ (Amersham) for 90 min. The target cells were washed three times with culture medium and resuspended at a concentration of 10^4/100 µl. The cells were plated at 1:1, 5:1, 10:1, 20:1, and 30:1 ratios of effectors to targets in the presence of 10 µg of peptide per ml in a final volume of 200 µl per well and incubated for 6 h to determine chromium release activity. Percent specific lysis was determined as [(cpm experimental release − cpm spontaneous release)/(cpm total release − cpm spontaneous release)] × 100. The spontaneous release was always less than 30% of the total release.

RESULTS

Immunogen. We have previously constructed VV recombinants containing the complete *env* gene of BLV, VV-BLV2 (VV P7.5 promoter) and VV-BLV3 (PFE/L promoter), as well as VV-BLV1, which contains the open reading frame for gp51 (truncated). VV-BLV2 and VV-BLV3 expressed BLV *env* glycoprotein on the surface of virus-infected cells and elicited an antibody response in rabbits. VV-BLV1 showed expression of gp51 but not on the cell surface, and this construct did not give rise to an antibody response against *env* protein (15).

A vaccine trial was conducted in sheep inoculated twice intradermally with 2×10^8 PFU of the VV recombinants (VV-BLV1, VV-BLV2, and VV-BLV3). Five weeks after vaccination, all groups were challenged intravenously with 4×10^4 lymphocytes from a cow with persistent lymphocytosis. The challenge dose was based on previous data from this laboratory which showed that as few as 200 lymphocytes were effective in causing infection in sheep (9).

Hematological responses. After initial immunization, the mean lymphocyte counts for VV-BLV2 and VV-BLV3 groups were significantly higher than those of VV-BLV1 ($P < 0.05$) and control ($P < 0.01$) groups (Fig. 1). After booster inoculation, the mean lymphocyte counts of the VV-BLV2 and VV-BLV3 groups were still significantly higher ($P < 0.05$ and $P < 0.01$, respectively) than those in the VV-BLV1 and control groups. This increase occurred prior to BLV gp51 antibody appearance in the vaccinated sheep. The peripheral blood lymphocyte counts returned to low levels following challenge and remained so throughout the observation period of 16 months (Fig. 1).

Humoral response to BLV. Antibodies against gp51 antigen were not detected in vaccinated animals prior to challenge (Table 2). Two weeks after challenge, anti-gp51 antibodies were observed in the two groups inoculated with recombinants containing complete *env*, VV-BLV2 and VV-BLV3. Antibody levels reached a peak by 4 to 6 weeks postchallenge, declined, and remained at low levels over a period of

TABLE 2. Antibody titers to BLV gp51 antigen, using the agar gel immunodiffusion test on sera of control infected sheep and sheep immunized with VV vectors expressing BLV glycoproteins prior to challenge with PBMCs from an infected cow

Animal group	Antibody response[a]										
	5 wk postvaccination	Wk postchallenge									
		2	4	6	8	16	32	40	48	56	64
VV-BLV1											
69	–	+	4	4	4	4	4	8	8	8	8
70	–	+	+	4	4	4	4	8	16	16	16
71	–	+	4	4	4	4	4	4	8	16	16
72	–	+	4	4	4	4	8	8	16	16	32
73	–	+	+	4	4	16	16	16	16	16	32
74	–	+	4	4	4	4	4	4	8	8	16
75	–	+	+	4	4	4	4	8	16	16	16
76	–	+	4	4	4	16	16	16	16	16	32
77	–	+	+	4	4	4	4	4	16	16	16
78	–	–	+	4	4	4	4	4	8	16	32
VV-BLV2											
51	–	8	8	4	4	4	4	4	4	4	4
52	–	+	4	4	4	+	–	–	–	–	–
53	–	4	8	4	4	4	4	4	4	4	4
54	–	+	8	4	4	4	4	4	4	4	4
55	–	4	8	4	4	4	4	4	4	4	4
56	–	4	8	4	4	4	4	4	4	4	4
VV-BLV3											
63	–	+	8	4	4	4	4	–	–	–	–
64	–	+	4	4	4	4	4	4	4	4	4
65	–	+	8	4	4	4	4	4	+	+	+
66	–	4	16	8	4	4	4	4	4	4	4
67	–	4	4	8	16	8	4	4	4	4	4
68	–	4	8	4	4	4	4	4	4	4	4
BLV-infected group											
80	Unvaccinated	–	+	+	+	8	8	8	8	16	32
81	Unvaccinated	–	+	+	+	4	8	16	16	16	16
82	Unvaccinated	–	+	+	+	8	8	16	16	32	32
83	Unvaccinated	–	+	+	+	8	16	16	16	16	16
84	Unvaccinated	–	+	+	+	4	8	8	8	8	8
85	Unvaccinated	–	+	+	4	4	8	8	16	16	16
86	Unvaccinated	–	+	+	+	8	16	16	16	16	16

[a] –, negative to undiluted serum; +, positive to undiluted serum only. Numbers indicate reciprocal of titer.

16 months (Table 2). Antibodies were also present in the VV-BLV1 group by 2 weeks postinfection; levels stayed largely unchanged for up to 16 weeks and subsequently continued to increase. No antibodies were detected in the control infected group until 4 weeks postchallenge, and they too increased slowly over the period of the trial.

Detection of provirus. PCR was used to detect the presence of proviral DNA in lymphocytes before and after challenge. Oligonucleotide primers for both the BLV *env* and *pol* genes were used to discriminate between genetic material of the virus and that originating from the VV recombinants. No BLV proviral DNA was detected in any group in the period prior to challenge with use of the *env* set of primers. The presence of BLV proviral DNA was detected for the first time in all animals from all groups 6 weeks after infection as an amplified fragment of the predicted size (586 bp). Typical results for the VV-BLV2 and control infected groups appear in Fig. 2A and 3A, respectively. These results were confirmed by DNA hybridization using a BLV DNA probe (Fig. 2B and 3B). It was not possible to detect proviral DNA in the animals vaccinated with either VV-BLV2 or VV-BLV3 by 4 months postinfection or for the remainder of the trial. In contrast, an amplified fragment was detected throughout the trial period for the majority of animals in the VV-BLV1 and control infected groups. The results in Fig. 4 at 16 months postinfection demonstrate that 8 of 10 animals in the VV-BLV1 group were positive, as were all of the control infected group, whereas provirus was not detected in any of the animals in the VV-BLV2 and VV-BLV3 groups.

Detection of BLV infectivity in immunized sheep. To confirm the vaccine-mediated protection suggested by PCR, PBMCs (10^6 cells) from all animals in the four groups were used to inoculate recipient seronegative sheep at 2 years postimmunization. All sheep inoculated with PBMCs from the control infected group (7 of 7) and the VV-BLV1 group (10 of 10) had anti-gp51 antibodies 4 to 6 weeks following inoculation. Over an observation period of 10 weeks, none of six animals in the VV-BLV2 group seroconverted, and only two of six in the VV-BLV3 group seroconverted.

Cell-mediated response. We have recently used synthetic peptides to identify a proliferative (CD4) and a cytotoxic (CD8) epitope of the gp51 antigen of BLV (8). Peptide 51-70 was used in this study to assay for proliferative response, and peptide 131-150 was used to assay for CTL in sheep exposed to BLV.

(i) CD4 response. CD4 epitope-induced proliferation was observed in all of the sheep immunized with VV-BLV2 or VV-BLV3 immunogens and in only one of seven in the control group when the animals were tested at 3 weeks after booster inoculations (Table 3). The highest SIs were up to 11

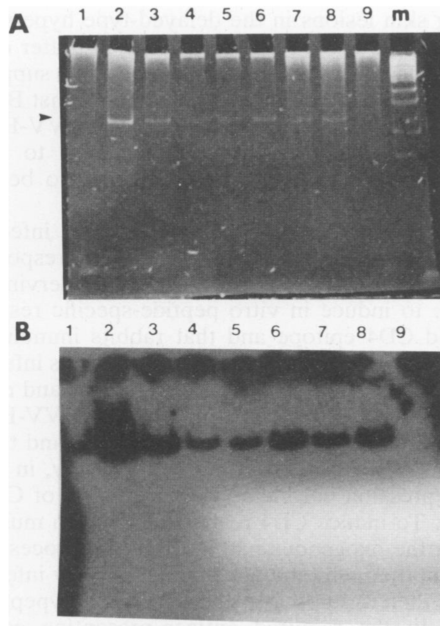

FIG. 2. PCR amplification of the BLV *pol* region from sheep (69 through 76) vaccinated with VV-BLV1 and challenged with PBMCs from a BLV-mediated cow with persistent lymphocytosis. (A) Polyacrylamide gel electrophoresis and ethidium bromide staining to detect PCR products; (B) hybridization analysis of PCR products of the same samples. Lanes: 1 to 8, sheep 69 to 76; 9, negative control; m, molecular weight DNA markers (bacteriophage SPP1).

at peptide concentrations of 2.5 and 5.0 μg/ml (Table 3). The appearance of the CD4 T-cell proliferation responses in the PBMCs of these sheep occurred before BLV gp51 antibodies could be detected by the agar gel immunodiffusion test. In contrast, individuals immunized with VV-BLV1 immunogen did not exhibit any specific proliferation following immunization. In these sheep, the SI values were usually less than 2 when tested with any peptide concentration (Table 3). None of the sheep in any of the groups responded to peptide (SI < 2) following challenge (Table 4).

(ii) CD8 response. We intended to assess the role of CD8 CTL in protection against BLV infection. However, in preliminary experiments, we observed that four separate sheep (immunized either with VV-BLV2 alone or with VV-BLV2 plus a VV containing the *gag* gene) did not develop CTL when tested at either 3 or 6 months postvaccination. Thus, although we believe that CD8 CTL may play an important role in protection, at this stage it is not possible for us to verify such hypothesis.

DISCUSSION

In this study, we have demonstrated that VV recombinants containing the complete *env* gene induce peptide 51-70-specific CD4 T-cell responses and antibody responses in sheep. Following challenge, the majority (10 of 12) of these animals were protected against infection, as determined by antibody response, PCR, and infectivity of PBMCs in recipient animals. The two positive animals in the VV-BLV3 group might be explained by the use of a fowlpox virus promoter (PFE/L) in that particular construct compared with the VV P7.5 promoter in the VV-BLV2 construct. The absence of antibodies in a previously successful vaccinia vaccination study together with the results of this study strongly suggest that cellular immunity is critical for protection. The correlation that we report here between protection and T-cell responses to peptide 51-70 is the most precise correlation of protection yet defined. This trial was more extensive and monitored for a considerably longer time period than was the case in two other recent trials with VV-BLV constructs (22, 25). Persistence of anti-p24 antibodies and a decrease in antibodies against gp51, accompanying protection, were reported for sheep inoculated with VV recombinants containing gp51 and gp30 (25). A similar pattern was observed in the present study; antibodies against gp51 reached a maximum in the first 4 weeks after challenge, decreased by 8 weeks, and remained at low levels for the duration of the trial. In contrast in uninoculated sheep and in those inoculated with truncated *env* (VV-BLV1), anti-gp51 antibodies were somewhat slower to appear after challenge and continued to rise for the duration of the 16-month trial. A gradual fall in antibody titers has also been observed to accompany protection in human immunodeficiency virus-infected chimpanzees immunized with recombinant human immunodeficiency virus *env* gp120 (2) and in macaques, in which antibodies to simian immunodeficiency virus increased postchallenge and subsequently declined (26). We did not observe antibodies in the prechallenge period in animals inoculated with complete *env*. Portetelle et al. (25) detected anti-gp51 antibodies after both immunizations with constructs containing *env*, whereas Ohishi et al. (22) did not observe a detectable antibody response prechallenge. Failure to observe an antibody response prechallenge may be due to less efficient expression of the particular VV constructs.

Ohishi et al. (22) reported a maximal antibody response more quickly in animals inoculated with VV recombinants

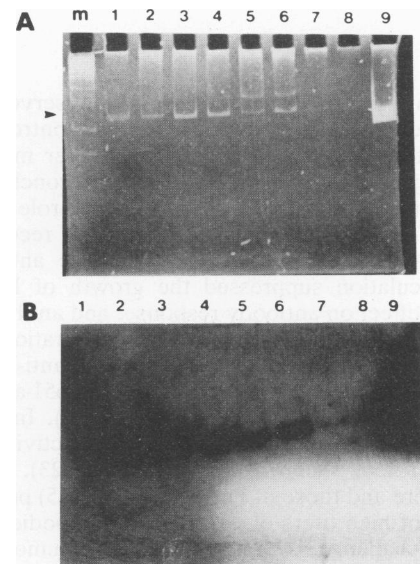

FIG. 3. Amplification of the BLV *pol* region from sheep (80 through 86) challenged with PBMCs from a BLV-infected cow with persistent lymphocytosis using PCR. (A) BLV DNA detection by polyacrylamide gel electrophoresis and ethidium bromide staining; (B) DNA hybridization analysis of PCR products from the same group. Lanes: m, molecular weight markers (bacteriophage SPP1); 1 to 7, sheep 80 to 86; 8, DNA from a BLV-infected sheep; 9, positive control (pBLV-A1).

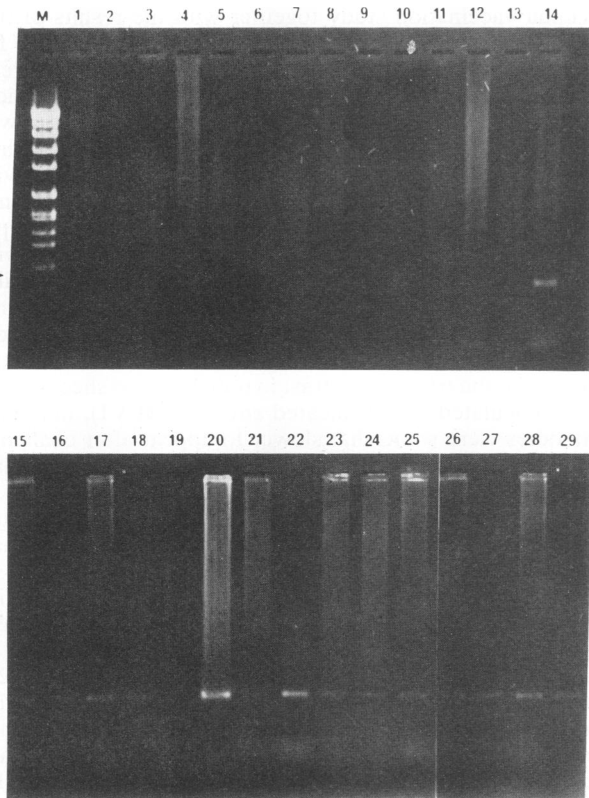

FIG. 4. Analysis by agarose gel electrophoresis of the PCR-amplified BLV *pol* region from the vaccinated animal groups (VV-BLV1, VV-BLV2, and VV-BLV3) and from the control animals 16 months after challenge. Lanes: M, molecular weight markers; 1 to 6, sheep 51 to 56 (VV-BLV2 group); 7 to 12, sheep 63 to 68 (VV-BLV3 group); 13 to 22, sheep 69 to 78 (VV-BLV1 group); 23 to 29, sheep 80 to 86 (control group). Arrowheads point to 586-bp amplified DNA fragments.

than in controls postchallenge, as was observed in this study. However, the antibody response in control and VV recombinant-inoculated animals did not differ much at 26 weeks postchallenge in their study. They concluded that humoral immunity is unlikely to play a major role in protection against BLV, since inoculation with VV recombinants containing *env* did not induce a detectable antibody response, inoculation suppressed the growth of BLV with virtually no effect on antibody response, and antibody titers did not correlate with extent of BLV proliferation. On the other hand, sheep passively immunized with anti-BLV antibodies, provided they had high titers of anti-gp51 antibodies, successfully resisted infection with virus (14). In addition, gp51 glycoprotein can induce neutralizing activity against BLV and protect sheep against infection (23). The data presented here and those of Portetelle et al. (25) point to the importance of high titers of neutralizing antibodies at early stages after challenge, with less reliance on humoral immunity over longer periods.

Ohishi et al. (22) suggested that cell-mediated immune response played a major role in the suppression of BLV proliferation. This contention is based on the observation that inoculation of sheep with VV recombinants induces a strong delayed-type hypersensitivity response and stimulates helper T cells, as deduced from the memory effect for antibody response. In that study, the composition of cells infiltrating skin lesions in the delayed-type hypersensitivity response was 25% CD4 and 65% CD8 48 h after challenge. The results described in the present study also support a role for cell-mediated immunity in protection against BLV infection. All of those animals inoculated with VV-BLV2 and VV-BLV3 showed a proliferative response to a peptide derived from gp51 which we have shown to be a CD4+ T-cell epitope (8).

It is interesting to observe that animals infected with VV-BLV1 do not generate helper CD4 T-cell responses. The evidence for this conclusion comes from observing that we are unable to induce in vitro peptide-specific responses to the defined CD4 epitope and that rabbits immunized with this construct do not generate antibodies. Cells infected with this construct express gp51 in the cytoplasm and not on the cell surface. By contrast, cells infected with VV-BLV2 and VV-BLV3 express gp51 on the cell surface, and these cells do induce CD4 responses. It is not clear why, in this case, surface expression correlates with induction of CD4 T-cell responses. To induce CD4 responses, antigen must be processed via the exogenous pathway. If this processing must occur in another cell (and not the one initially infected with the virus), then it is possible that the gp51 polypeptide is not presented to the required antigen-presenting cell in the appropriate form. For example, it may be destroyed by enzymes in the cytoplasm of the primary cell and not available for presentation.

TABLE 3. CD4 responses (3 weeks after booster inoculation) in PBMCs of sheep immunized with VV vectors expressing BLV glycoproteins

Animal no.	SI at peptide[a] concn (μg/ml) of:				
	0.5	1.0	2.5	5.0	10.0
VV-BLV1					
69	1.1	0.8	0.8	0.7	0.6
70	0.9	1.4	1.3	0.9	0.9
71	1.5	1.4	1.3	1.4	1.3
72	1.2	0.8	0.7	1.1	1.3
73	1.1	1.3	1.3	1.4	1.3
Mean	1.3	1.3	1.2	1.4	1.0
VV-BLV2					
51	2.1	2.3	4.8	3.7	1.6
52	4.6	7.5	11.1	11.3	1.9
53	4.1	6.9	6.9	7.4	1.2
54	2.1	6.7	10.4	2.8	1.5
55	4.1	6.9	7.0	10.0	1.9
56	5.0	6.4	10.0	11.0	2.1
Mean	3.3	5.7	5.7	6.7	1.7
VV-BLV3					
63	4.0	4.6	6.8	10.0	2.4
64	2.8	4.0	6.4	7.3	3.1
65	4.7	6.1	6.6	7.2	1.4
66	6.6	7.4	11.5	8.0	1.2
67	3.4	4.0	8.7	6.7	0.8
68	4.7	5.8	6.0	6.4	1.1
Mean	4.27	5.2	7.4	7.4	1.4
Unvaccinated					
80	3.1	3.7	4.5	6.0	0.9
81	0.9	1.2	0.9	1.5	0.9
82	1.5	0.8	0.7	1.8	0.9
83	1.5	1.1	1.4	1.6	0.9
84	1.4	1.5	1.1	1.4	1.3
85	1.2	1.1	1.7	1.7	0.6
Mean	1.7	1.6	1.8	2.4	1.0

[a] The peptide used corresponded to the amino acid sequence 51 to 70 of gp51.

TABLE 4. CD4 T-cell responses of PBMCs of sheep immunized with VV vectors expressing BLV glycoproteins[a]

Animal no.	SI at peptide concn (μg/ml) of:				
	0.5	1.0	2.5	5.0	10.0
VV-BLV1					
69	0.9	0.4	0.2	0.3	0.5
70	1.4	1.0	1.8	1.3	0.7
71	1.7	1.2	0.9	1.3	1.1
72	1.4	1.4	1.4	1.9	1.3
73	1.1	1.5	1.3	1.1	0.8
74	1.2	1.2	1.6	1.1	0.6
75	1.1	0.8	0.7	0.9	0.7
76	0.8	0.7	0.6	0.6	0.6
77	0.9	0.8	1.4	2.0	1.1
78	1.5	1.0	1.6	1.3	1.3
Mean	1.2	1.3	1.3	1.5	1.4
VV-BLV2					
51	0.4	0.9	0.5	1.4	1.1
52	0.7	1.6	0.9	1.9	0.9
53	0.4	1.5	0.8	1.0	1.2
54	0.5	1.4	0.5	1.3	0.9
55	0.4	1.5	0.9	1.8	1.6
56	0.2	0.5	0.3	1.1	0.8
Mean	0.4	1.2	0.6	1.4	1.1
VV-BLV3					
63	0.8	1.2	0.8	1.0	0.8
64	0.9	0.8	0.4	1.0	1.0
65	0.8	0.6	0.8	1.2	0.8
66	0.5	0.6	0.7	1.1	0.8
67	0.5	0.8	0.6	1.3	1.8
68	0.7	0.9	0.8	1.2	1.4
Mean	0.7	0.8	0.7	1.2	1.1
Unvaccinated					
80	0.9	0.9	0.8	0.8	1.1
81	1.3	1.2	1.5	0.6	0.6
82	0.8	0.9	0.9	0.9	0.7
83	0.8	0.9	0.7	0.8	0.7
84	0.8	0.9	0.8	1.5	0.8
85	0.8	0.7	0.3	0.8	0.9
86	1.1	1.2	1.2	1.2	1.4
Mean	0.9	0.8	0.9	0.9	0.8

[a] Proliferation was determined 4 months after challenge with mononuclear cells from an infected donor cow with persistent lymphocytosis. The peptide was the same as for Table 3.

The early appearance of anti-gp51 antibodies in sheep inoculated with VV-BLV2 and VV-BLV3 could be due to the presence of these helper T cells. On the other hand, only one of seven control sheep showed a response, and none of the animals inoculated with VV-BLV1 responded. The response to gp51 peptide was lost at 4 months after challenge and may indicate that loss of immunological memory occurs with loss of gp51 antigen. This would support the observation in mice that T-cell memory requires antigenic persistence (11).

In summary, we have described an extensive vaccine trial in sheep which demonstrates that presentation of the major surface BLV antigen gp51 in a configuration mimicking that of its native state provides protection against infection with this virus in sheep. While a humoral response may play some role in protection, it is likely that a cell-mediated response also contributes to protection. The CD4 T-cell response to peptide 51-70 correlates with protection, and a synthetic peptide vaccine based on this sequence may prevent BLV in animals at risk. Furthermore, BLV may provide a model for vaccine design for human leukemia mediated by the related virus human T-cell leukemia virus type I.

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