Recombinant vaccinia virus expression of the bovine leukaemia virus envelope gene and protection of immunized sheep against infection

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The bovine leukaemia virus (BLV) envelope gene encoding extracellular glycoprotein gp51 and transmembrane glycoprotein gp30 was cloned into the HA locus of vaccinia virus (Copenhagen strain), downstream of the vaccinia virus early-late promoter, H6, or a triple promoter element consisting of the promoter for the vaccinia virus H6 gene, the promoter for the cowpox virus A-type inclusion (ATI) gene and the promoter for the vaccinia virus HA gene. Inoculation of rabbits or sheep with the recombinant vaccinia virus coding for gp51 and gp30 or an uncleaved env precursor induced neutralizing antibodies to BLV. These antibodies competed with monoclonal antibodies directed against gp51 epitopes F, G, and H previously shown to be of crucial importance for BLV infection. Seven out of eight sheep vaccinated with the vaccinia recombinants resisted a drastic challenge (1.5×10^3 sheep infectious doses) with BLV-infected lymphocytes. These results show that vaccination with BLV env vaccinia recombinants protects sheep against infection with extremely high doses of BLV-infected heterologous lymphocytes.

Keywords: Bovine leukaemia virus; env glycoprotein; recombinant vaccinia virus

INTRODUCTION

Enzootic bovine leucosis is a neoplastic disease with a worldwide distribution. The infectious agent, bovine leukaemia virus (BLV), is a trans-activating retrovirus found naturally in cattle. Nucleotide sequence, genome organization, absence of viraemia and lack of provirus expression in transformed cells emphasizes the relatedness between BLV and the human T-lymphotropic viruses type I and type II (HTLV-1-2) (for reviews, see Burny *et al.*[1,2]).

The virus induces lymphoid tumours in ruminants especially in sheep where all infected recipients succumb to the disease within a time course of a few months to 7 years[3].

BLV infection causes significant losses to the dairy cattle industry[4]. More and more data are emerging to indicate that chronic BLV infection by itself can cause reduction in productivity, reproductivity and shorter life span among highly qualified milk cows[5].

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Successful eradication campaigns of BLV infection have been engaged, essentially in Europe, by testing at intervals for antibodies to BLV proteins, especially the envelope glycoprotein gp51, and eliminating seropositive animals[6].

From a practical point of view, vaccination would be most indicated where BLV infection is endemic and where segregation and/or elimination of infected animals cause management problems and important financial losses.

Vaccination would also be an attractive choice from a medical and fundamental point of view, considering that BLV (as HTLV-1) does not cause viraemia, but is transmitted as a cell-associated agent. As few as 926 lymphocytes from a highly infectious animal have been shown to transmit infection to serologically negative sheep[7]. Prevention of BLV infection via vaccination thus implies that spread of cell-associated BLV can be efficiently controlled through immune defence mechanisms.

From a survey of published vaccination trials it is obvious that both very encouraging and desperately poor data have been obtained[8].

We remain, however optimistic about the design of an efficient BLV vaccine, considering that sheep, passively immunized with anti-BLV immunoglobulins successfully resist an infectious challenge provided they had sufficiently high anti-gp51 antibody titres[9,10]. Virus-neutralizing and syncytia-inhibiting antibodies interact

with the architectural configuration defined by three conformational epitopes named F, G, H, identified on gp51 by mouse monoclonal antibodies (mAbs)[11-13].

Since glycosylation, native and non-degraded architectural configuration have a major impact upon the immunogenicity of gp51[14-16], maximal expression of the cloned envelope gene (env) should be performed in a host–vector system allowing accurate addition of the saccharide moiety.

Vaccinia virus is a candidate system[17]. It has been used to produce envelope glycoproteins of HTLV-1[18] and to endow immunological memory without inducing anti-BLV gp51 response in rabbits[19].

We report here the cloning and expression of the BLV-envelope coding sequences in vaccinia virus vectors and the biological properties of these recombinants in elicting BLV-neutralizing antibodies in both rabbits and sheep and protective immunity in sheep against a severe cell-associated BLV challenge.

MATERIALS AND METHODS

Cells and viruses

Bovine leukaemia virus was propagated in a fetal lamb kidney cell line persistently infected by the virus (FLK-BLV); production and purification of BLV virions was essentially as described previously[11,14,16].

The Copenhagen strain of vaccinia virus and recombinants derived therefrom were propagated on either VERO (ATCC CCL81) or MRC5 (ATCC CCL171) cells in Eagle's minimal essential medium plus 10% fetal bovine serum as previously described[20]. The isolation and propagation of VTK$^-$79, a derivative of the WR strain of vaccinia virus, has been described[21].

Cloning of the BLV envelope genes into vaccinia donor plasmids

The BLV env coding sequence was obtained from bovine tumour T15-2 DNA as previously described[22]. A 1050 bp *Mst*II-*Sma*I (partial) fragment, containing the gene encoding the BLV gp51 glycoprotein was isolated from a plasmid containing a DNA copy of the BLV genome[22,23]. This fragment was blunt-ended with the Klenow fragment of *Escherichia coli* DNA polymerase I and cloned into the blunt-ended *Hin*dIII site of pMPIII (pMPIII was derived by replacing the polylinker region of pUC18 with the sequence 5'-AAGCTTTTAAATGGGAATTC-3'). This places an in-frame termination codon downstream from the gp51 sequence. The plasmid generated by this manipulation is called pBLV5.

The gene encoding gp51 was then cloned downstream of the early-late vaccinia promoter, H6[24,25]. This was accomplished by cloning the 580 bp *Hga*I-*Dra*I fragment of pBLV5 and the oligonucleotides BLVM1 5'-ATCC-GTTAAGTTTGTATCGTAATGCCTAAAGAAC-GTCGTTCTCGTAGACGTCCACA-3' and BLVM2 5'-TCGGTTGTGGACGTCTACGAGAACGACGTTC-TTTAGGCATTACGATACAAACTTAACGGAT-3' into the *Eco*RV site of pRW671 (pRW671 was constructed by cloning the 218 bp upstream from the H6 gene[26] into pUC8). This places the 5' end of the envelope gene (with no 5' noncoding sequence) next to the H6 promoter. The plasmid generated by this manipulation is called pBLV9.

The H6-promoted gp51 gene was then cloned between vaccinia (WR) flanking arms. This was accomplished by cloning the 750 bp *Hin*dIII-*Pvu*II fragment of pBLV9 (containing the 5' end of the gp51 gene) and the 370 bp *Pvu*II-*Eco*RI fragment of pBLV5 (containing the 3' end of the gp51 gene into the *Hin*dIII-*Eco*RI site of pRW692 (pRW692 was constructed by cloning the 2300 bp *Bgl*II fragment of the vaccinia virus (WR) *Hin*dIII F fragment[27] into the *Bam*HI site of pUC8. The *Hin*dIII F fragment contains a unique *Bam*HI site. In pRW692, the polylinker region of pUC18 has been cloned into this site). The plasmid generated by this manipulation is called pBLV10.

Unwanted DNA was then deleted from pBLV10. This was accomplished by cloning the oligonucleotides BLVM3 5'-GAAGCTT-3' and BLVM4 5'-AATTAAGC-TTCTGCA-3' into the 6100 bp *Eco*RI-*Pst*I (partial) fragment of pBLV10. The plasmid generated by this manipulation is called pBLV15.

The 3' end of the envelope gene, encoding gp30, was then cloned into pBLV15. This was accomplished by cloning the 1260 bp *Nco*I-*Mst*II (partial) fragment of pBR322BLV (containing the gene encoding gp30, as well as much of gp51) and the oligonucleotides BLVM5 5'-TCAGC-3' and BLVM6 5'-AGCTGC-3' into the 5500 bp *Hin*dIII-*Nco*I fragment of pBLV15. The plasmid generated by this manipulation is called pBLV16.

The envelope gene was then cloned into a different vaccinia virus donor plasmid. This was accomplished by cloning the 1580 bp *Eco*RV-*Pst*I fragment of pBLV16 (containing the entire envelope gene) into the 3700 bp *Eco*RV-*Pst*I fragment of pTP15[20]. This places the envelope gene under the transcriptional control of a modified H6 promoter[20,25] and between the vaccinia virus (Copenhagen) sequences flanking the HA gene[20]. The plasmid generated by this manipulation is called pBLVH14.

The promoter of the cowpox virus A-type inclusion gene[28,29] was then cloned upstream of the H6-promoted envelope gene. This was accomplished by cloning the oligonucleotides BLVM7 5'-GATCTACGATCTTATA-ATTACACGATTGTAGTTAAGTTTTGAATAAAAT TTTTTTATAATAAGAATTC-3' and BLVM8 5'-GATCGAATTCTTATTATAAAAAAATTTTATTCA-AAACTTAACTACAATCGTGTAATTATAAGATCG-TA-3' into the *Bgl*II site of pBLVH14. The plasmid generated by this manipulation is called pATI25.

The cleavage site of the envelope gene was then modified. This was accomplished by cloning the oligonucleotides BLVM9 5'-CCGGGTCAGACAAACTCC-CGTCGCAGCCCTGACCTTAGG-3' and BLVM10 5'-CCTAAGGTCAGGGCTGCGACGGGAGTTTGT-CTGAC-3' into the 5350 bp *Xma*I-*Stu*I (partial) fragment of pATI25. This changes the two amino acids flanking the cleavage site, at positions 268 and 269, from Arg-Ser to Gln-Thr. The plasmid generated by this manipulation is called pATI26.

Construction of vaccinia virus recombinants

The procedures used to construct vaccinia virus recombinants have been described previously[17,20,21].

The recombinant vaccinia virus vP391 was generated by transfecting pBLV15 into VTK$^-$79 infected cells. vP392 was generated by transfecting pBLV16 into VTK$^-$79 infected cells. vP459 was generated by transfecting pATI25 into vP425 infected cells[20]. vP482

was generated by transfecting pATI26 into vP425 infected cells. The virus, vP452, used in the challenge experiments as a negative control, is a variant of wild type vaccinia virus (Copenhagen) from which the TK and HA genes have been deleted[20].

ELISA tests

The presence of gp51 or the detection of antigenic variations in gp51 with respect to its reactivity toward anti-F, anti-G, and anti-H monoclonal antibodies were assessed by monitoring the reactivity of BLV or vaccinia recombinants. The extent of the reaction in a standardized multiple-site ELISA assay measures the presence and/or the avidity of a given mAb of the anti-gp51 battery for the corresponding epitope. It could identify FLK-BLV and T15-2-Recombinant as $F^+G^+H^+$ and $F^+G^-H^+$, respectively[16].

For anti-gp51 antibody titration, mAbs have been selected to develop an indirect enzyme-linked immunosorbent assay (iELISA) and a competitive ELISA assay (cELISA)[30].

Western blot (WB)

The proteins of the cell lysates of VERO cells infected with vaccinia virus recombinants or purified BLV were subjected to sodium dodecyl sulphate–15% polyacrylamide slab gel electrophoresis (SDS–PAGE) after SDS–mercaptoethanol treatment. Proteins were then blotted to Immobilon membranes and the membranes blocked essentially as described previously[16].

The membranes or strips were then treated with purified anti-gp51 and anti-p24 mAbs (each diluted 1:200) or with the bovine anti-BLV antibodies followed by purified anti-bovine IgG1 mAb (diluted 1:200), and alkaline phosphatase conjugate.

Pseudotype and early polykaryocytosis inhibition tests

Antibodies were evaluated for their ability to neutralize the plaque-forming activity of the vesicular stomatitis virus (VSV)-BLV pseudotypes on indicator cells or to neutralize syncytia induction between FLK cells and indicator cells[12,31,32].

Short-term cultures of peripheral blood lymphocytes (PBL)

PBL were isolated from whole blood by osmotic shock[15]. The cells were seeded at 4×10^6 cells ml^{-1} in Optimem (Gibco/BRL, Goithersburg, MD, USA) + 20% fetal calf serum. After 72 h culture, clarified supernatants were tested for the presence of BLV p24 and/or BLV gp51 in sandwich ELISA assays involving highly specific mAbs as binding antibody and as peroxidase conjugates.

Immunization of rabbits

Rabbits (6 months old) were inoculated intradermally (i.d.) at ten spots on both sides of the spinal cord with 1×10^7 $TCID_{50}$ recombinants in 1 ml phosphate buffered saline (PBS) (NaCl 0.15 M; $NaHPO_4$ 0.01 M; pH 7.4) or intravenously (i.v.) (5×10^6 $TCID_{50}$) and i.d. $(5 \times 10^6 TCID)_{50}$.

The animals were immunized two times at 6 week intervals. Blood was collected from the ear vein at 7 day intervals. Serum samples were stored at −20°C.

Immunization of sheep and challenge with BLV

Fourteen sheep (6 months to 1 year old, weighing between 40 and 80 kg) were used. Control sheep were maintained in contact with virus-injected animals. Group A (four animals) received two i.d. 0.1 ml inoculations of 10^8, 10^6, 10^4 and 10^2 $TCID_{50}$ of the wild type vaccinia virus vP452, on both sides of the spinal cord. Six weeks later, two subcutaneous 0.1 ml booster doses of 10^8 $TCID_{50}$ were used.

Group B and Group C were vaccinated in the same conditions with BLV env vaccinia recombinants vP459 and vP482, respectively.

Two sheep in Group D were left as contact and unvaccinated controls.

All sheep received, 6 weeks after the second immunization, a severe challenge (1.5×10^3 sheep infectious doses) of four i.d. 0.125 ml inoculations of a ten-fold citrate diluted blood from highly infectious cattle[7] ($\pm 31\,700$ leucocytes mm^{-3}; 86% lymphocytes; $F^+G^-H^+$ BLV variant, according to our phenotype nomenclature[16]).

Animal housing

All experimental animals (rabbits and sheep) were housed in isolated facilities.

RESULTS

Reactivity with monoclonal antibodies of recombinant env proteins synthesized from the vaccinia recombinants

Four recombinant vaccinia viruses containing either the entire BLV envelope gene, or the part of the gene encoding gp51 were constructed (*Figure 1*). These recombinant viruses were shown to express gp51 by indirect immunofluorescence using our panel of specific anti-gp51 mAbs. The gp51 antigen was detected on the surface of infected-cells with anti-F, and anti-H, but not with anti-G, confirming that T15-2-BLV is a G^- variant. Anti-F and H mAbs were maximally reactive toward vP392 expressing gp51 and gp30, vP459 expressing gp51 and gp30 and vP482 expressing the uncleaved precursor of gp51-gp30, whereas they showed poor affinities for their respective epitopes when produced from the vP391 recombinant expressing gp51 alone (data not shown).

The reactivity of our panel of anti-gp51 mAbs was also examined in ELISA in order to identify each recognized epitope (*Figure 2*). Negative results were obtained with anti-G antibody, again confirming that the env sequence of T15-2 proviral DNA encodes a G^- variant[16].

The reactivity of each mAb was decreased but not abolished when the env precursor was modified and uncleaved.

Taken together, the data confirm that vaccinia recombinants express the env glycoproteins in their native configuration in the target cells of rabbits and sheep and that cleavage of env precursor into gp51 and gp30 leads to the highest affinity of F and H epitopes for their respective mAbs.

Processing of env proteins

The molecular weight of expressed BLV antigens was estimated by Western blot analysis (*Figure 3*). As expected, relative molecular weights for gp51 and the uncleaved env precursor (pr72) were similar to those

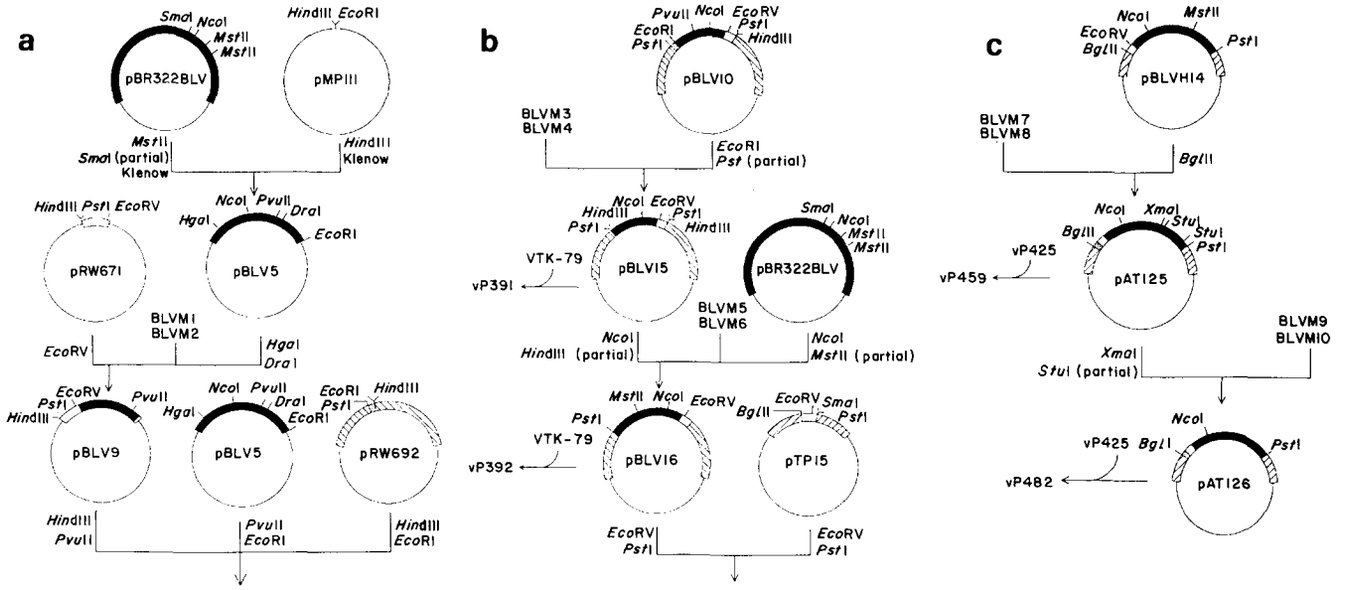

Figure 1 Generation of vP391, vP392, vP459 and vP482. Cloning is described in text. Symbols: ■, BLV *env* DNA; □, vaccinia virus H6 promoter; ▧, vaccinia virus (WR) sequences flanking the unique *Bam*HI site of the *Hin*dIII F fragment; ▨, vaccinia virus (Copenhagen) sequences flanking the HA gene; ⊞, cowpox virus A-type inclusion gene promoter

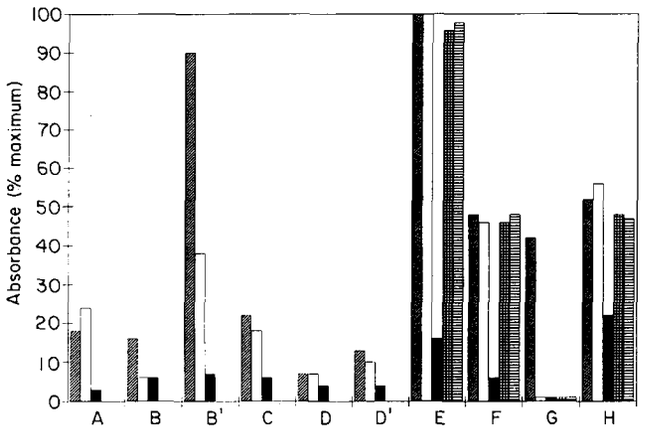

Figure 2 Relative antigenic reactivity of recombinant gp51 with a panel of anti-gp51 mAbs in a sandwich ELISA system. Each mAb (A, B, ... H) was first used as a binding mAb to select antigen; a mixture of mAbs against sequential epitopes (A, B, B', D, D', E) was then added as a peroxidase conjugate[16]. Results were expressed as relative values, where 100 was the maximum OD 492 nm observed for the different ELISA tests. Supernatant of BLV-producing cell line (FLK:BLV) was used as gp51 reference. Suspensions of recombinant infected-cell lysates were used to test the reactivity of recombinant gp51 products (10^7 $TCID_{50}$ per ml) (data not determined for A, B, B', C, D, D' epitopes from vP391 and vP392 preparations). ▨, FLK-BLV; □, vP459; ■, vP482; ⊞, vP391; ⊟, vP392

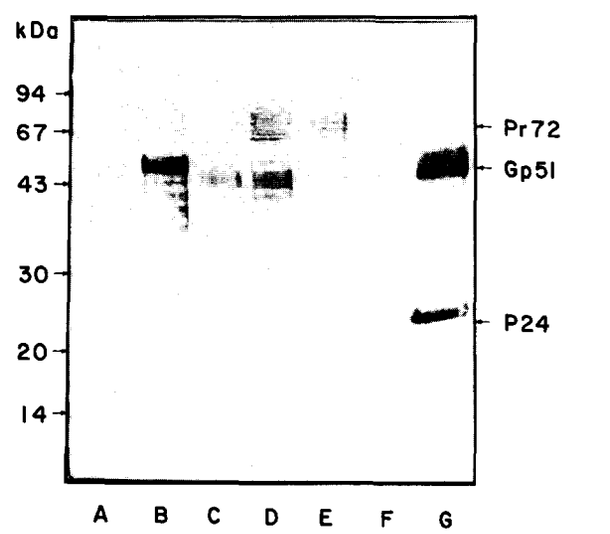

Figure 3 Western blot analysis of recombinant BLV env antigens with anti-gp51 mAbs. Confluent monolayers of VERO cells were infected with recombinant or wild type vaccinia viruses at a multiplicity of two. 24 h after infection, cells were collected, resuspended in sample buffer and boiled for three min. Proteins were subjected to SDS-PAGE (15%), electrophoretically transferred to Immobilon membranes and incubated with 1:200 dilution of a mixture of purified mAbs (anti-gp51 and anti-p24). The numbers at the left represent molecular masses (in kilodaltons) of standard markers. The notation in the right margin indicates the position of gp51, pr72 uncleaved env precursor and p24. Non-recombinant vaccinia: VTK⁻79 (A) and vP452 (F); vP391, gp51 (B), vP392, gp51 and gp30 (C); vP459, gp51 and gp30 (D); vP482, uncleaved precursor of gp51-gp30 (E); BLV virions from FLK cells (G)

observed for BLV products after immunoprecipitation analysis of different ^{3}H-amino acid labelled gp51-related antigens from FLK-BLV cells lysates[15].

Since the mAbs used for the analysis were monospecific to gp51 and pr72[15], gp30 was not detected with the antibodies. The presence of gp30 in the construction was assumed from the good reactivity in WB of sheep immunized with the recombinants (data not shown).

Immunogenicity of recombinant vaccinia viruses in rabbits

No significant virus-neutralizing antibody titre was found in sera collected from the two rabbits inoculated with vP391 (gp51 alone) or with a previously published[33] yeast-derived product. On the other hand, rabbit 2670, immunized with non-denatured purified gp51[16,32] and rabbits 7, 8, 10, 30, 32, 33, 34, experimentally inoculated with recombinant vaccinia viruses expressing gp51 and gp30 showed very high neutralizing titres in syncytia and pseudotype inhibition tests (*Table 1*).

The ability to raise high titre neutralizing antibody was previously shown to be linked to the presence of the native configuration of epitopes F, G and H[16,32,33].

These determinants[16] were thus in their native

Table 1 Neutralizing activities of anti-gp51 raised in rabbit

Rabbit no.	Vaccinia recombinant no.	Antigens	Mode of inoculation	Anti-gp51[c] 2 weeks after 2nd injection	Pseudotypes inhibition titre[d]	Syncytia inhibition titre[d]
2670		Purified gp51[a]	i.d./FCA	++++	7900	>16
9		Yeast-gp51[b]	i.d./FCA	++++	0	0
4	vP391	gp51	i.d.	+++	200	0
5	vP391	gp51	i.v.+i.d.	+++	0	0
7	vP392[e]	gp51+gp30	i.d.	++++	630	2
8	vP392	gp51+gp30	i.d.	++++	1250	8
10	vP392	gp51+gp30	i.d.+i.v.	++++(+)	12600	>16
32	vP459	gp51+gp30	i.d.	++	<200	2
30	vP459	gp51+gp30	i.d.+i.v.	++++	660	8
33	vP482	gp51−gp30 precursor	i.d.	++++	400	4
34	vP482	gp51−gp30 precursor	i.d.+i.v.	++++	700	8

FCA, Freund's complete adjuvant. [a] Purified BLV gp51; see Ref. 32
[b] Purified gp51 produced in yeast; see Ref. 33.
[c] Number of serum dilutions that competed at >50% in the cELISA test.
[d] Serum dilution harbouring 50% neutralization.
[e] Ovine kidney cell line (OVK) as target cell; Vero cells for other cases

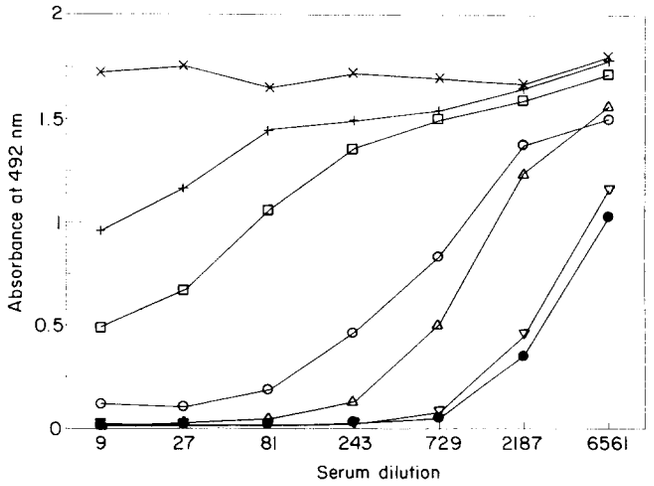

Figure 4 Titration curves of rabbit sera in competition ELISA against BLV gp51 where anti-gp51 mAb against site E was used as binding mAb and where anti-G was used as peroxidase–antibody conjugate. (×), negative rabbit serum; (+), anti-vP391 (Rab 4; i.d. injection); (□), anti-vP391 (Rab 5; i.d.+i.v. injections); (○), anti-vP392 (Rab 7; i.d. injection); (△), anti-vP392 (Rab 8; i.d. injection); (▽), anti-vP392 (Rab 10; i.d.+i.v. injection); (■), anti-purified gp51 (Rab 2670)

configuration when presented on the external gp51 coexpressed with the transmembrane gp30, or when gp51 was purified under mild extraction condition (Tween 80 instead of Triton X-100[34], rabbit 2670).

Anti-G mAbs were competed for by sera of immunized rabbits in cELISA for gp51 binding only when gp51 was coexpressed with gp30 (*Figure 4*) and even in the absence of G epitope on the T15-2 derived gp51 recombinant.

This competition, also seen with anti-F and anti-H mAbs (data not shown), reflected the situation observed with all sera from infected animals[30]: absence of one epitope, such as G, in a BLV-gp51 variant, represents a point modification in a small antigenic region which is differentially recognized by one mAb, but which reacts with polyclonal antibodies from infected animals; the region tentatively identified as containing F, G, and H epitopes (NH_2 part of the gp51 molecule[16]) is thus probably presented in its native configuration at the surface of expressing cells as well as BLV virions.

Protection of immunized sheep from challenge with BLV-infected lymphocytes

In a preliminary study, no neutralizing antibodies were observed in sheep after injection of recombinant vaccinia virus (vP391) containing the genetic information for gp51 alone (data not shown).

A vaccine trial based upon two different constructions harbouring the BLV env gene (gp51 and gp30 for vP459; uncleaved pr72 for vP482) has been performed in sheep (*Table 2*).

Animals that became infected after challenge (1.5×10^3 sheep infectious doses) with BLV-infected lymphocytes showed high anti-p24 antibody titres, increase of neutralizing antibody titres after challenge and no decrease of anti-gp51 titres. Moreover, BLV could be recovered from their lymphocytes after short term culture.

In contrast, absence of anti-p24 and decrease of anti-gp51 after challenge reflected protection of sheep inoculated with vaccinia virus recombinants. In any case, BLV could not be detected after short term culture of peripheral white cells from sheep immunized and challenged.

The persistence of anti-p24 antibodies and the presence of high neutralizing antibodies titre in sheep 29 suggested that only this animal was infected.

Some vaccinated animals were examined over a period of 4 months (68, 30, 31), or 7 months (41, 50) or were still alive 16 months after challenge (40, 43): in all cases, a decrease or a total disappearance of antibody was observed (data not shown). Blood of sheep 31 (2 ml) collected 3 months after challenge was unable to transfer BLV infection into a sheep inoculated at multiple spots i.d.[7].

DISCUSSION

Vaccinia virus has been used extensively as a live vaccine to control and eradicate smallpox. It has been developed as a cloning and expression vehicle for several antigens and in many cases protection was achieved by vaccination with appropriate recombinants.

We demontrate here that live vaccinia virus expressing BLV glycoproteins is able to confer protection against severe experimental BLV challenge. Furthermore, no

Table 2 Experimental protection of sheep after inoculation of recombinant vaccinia virus and challenge with BLV-infected bovine lymphocytes

Sheep No.	Recomb. No.	Antigens	Anti-gp51[a] 6 weeks after 1st inoc.	Anti-gp51[a] 6 weeks after 2nd inoc.	Anti-gp51[a] 3 months after challenge	Anti-p24[a] 6 weeks after 2nd inoc.	Anti-p24[a] 3 months after challenge	Recovery of BLV	Pseudotypes inhibition titres[b] 3 weeks after 1st inoc.	Pseudotypes inhibition titres[b] 3 weeks after 2nd inoc.	Pseudotypes inhibition titres[b] 6 weeks after challenge	Pseudotypes inhibition titres[b] 3 months after challenge	Conclusion
44	-	-	–	–	+ + + + + +	–	+ + + + +	+	0	0	1600	1800	Infected
99	-	-	–	–	+ + + + +	–	+ + +	+	NT[c]	NT	NT	NT	Infected
45	vP452	-	–	–	+ + + + +	–	+ + +	+	0	0	550	1500	Infected
74	vP452		–	–	+ + + + + + +	–	+ + + +	+	0	0	1000	6000	Infected
47	vP452	-	–	–	+ + + + + +	–	+ + + +	+	0	0	600	2600	Infected
90	vP452	-	–	–	+ + + + + +	–	+ +	+	0	0	390	630	Infected
40	vP459	gp51	–	+ + +	+	–	–	–	0	350	5	0	Protected
50	vP459	and	–	+ + + + +	+ + + +	–	–	–	0	6100	790	670	Protected
29	vP459	gp30	–	+	+ + + +	–	+ + + +	–	5	5	650	1000	Infected
68	vP459		+	+ + +	+ + +	–	–	–	0	90	40	20	Protected
41	vP482	Uncleaved	+ + +	+ + + + +	+ + + +	–	–	–	5	1860	190	110	Protected
43	vP482	Precursor	+ + +	+ + + + + +	+ + + + +	–	–	–	5	710	320	90	Protected
30	vP482	of gp51	+	+ + +	+ + + + +	–	+ +	–	10	980	4400	50	Prob. prot.
31	vP482	and gp30	+	+ + +	+ + +	–	–	–	0	360	390	60	Protected

[a] (+) Number of serum dilutions that competed at more than 50% in the cELISA test; + + + + + + strong competition; + moderate competition; – no competition
[b] Serum dilution harbouring 50% neutralization
[c] NT = not tested

transmission of recombinant virus to contact control animals was suggested (observation not published).

In field conditions, the recombinant preparations would certainly be presumed efficient against BLV spread since the infection is probably transmitted by infected cells in a lower amount than the heavy challenge used in this study and since even in the one immunized animal that did become infected (animal 29), the number of BLV-infected lymphocytes was reduced.

Several facts contribute to the efficacy of recombinants used in this study: (1) Expression of BLV glycoproteins was driven by the relatively strong H6 promoter of vaccinia virus, or the even stronger (data not shown) triple promoter consisting of the vaccinia virus H6 promoter, the cowpox virus ATI promoter and the vaccinia virus HA promoter. (2) Stability of the construct: no variability of the antigenicity of gp51 was observed after passage and growing of recombinant vaccinia viruses on cell lines from different origins (bovine, ovine, feline, equine, porcine, primate, rodent) (data not shown). (3) Presence of oligomeric structures at the surface of expressing cells similar to those observed on the surface of BLV virions: the architecture of the neutralizing epitopes depends upon the transmembrane glycoprotein gp30 linked to the extramembrane glycoprotein gp51. In the rabies system, a vaccinia virus recombinant carrying a rabies glycoprotein gene in which the transmembrane segment has been deleted is unable to elicit the formation of high titres of virus-neutralizing antibodies[35]. (4) High neutralizing titres protected animals against a BLV infection: only one vaccinated animal was infected after challenge with a high dose of BLV-infected cells; this situation clearly correlated with a weak titre of neutralizing antibodies before challenge. It could also be that the neutralizing titre is not the crucial parameter but reflects an overall weakness of the protective response.

In conclusion, our data stress the importance of presentation of glycoproteins in a native configuration at the surface of infected cells for the increased production of neutralizing and protective antibodies to retroviruses. Whether or not parameters other than neutralizing titre are of critical importance has not been addressed in this study but should not be underestimated at present.

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