Long-term protection against bovine leukaemia virus replication in cattle and sheep

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In this report, we have evaluated the ability of two different types of live attenuated bovine leukaemia virus (BLV) variants (BLV DX and BLV 6073) to protect cattle and sheep against a heterologous wild-type BLV challenge. Four months after challenge, the protection of the vaccinated animals was effective in contrast to unvaccinated controls. However, long-term protection (18 months after challenge) was observed only in six out of seven animals, one of the vaccinated cattle being infected 12 months after challenge. A second prospective approach investigated the injection of naked plasmid DNA. Two sheep were injected with plasmid DNA encoding the BLV envelope proteins; the challenge virus infection was delayed but could not be completely abrogated. Our results demonstrate that vaccines based on live attenuated viruses and naked DNA injections are able to delay BLV infection, although complete protection cannot be achieved. In addition, our data cast light onto the need to perform long-term vaccination trials because challenge superinfection can occur even after apparent protection for 12 months.

Introduction

Bovine leukaemia virus (BLV), a retrovirus closely related to human T cell leukaemia viruses types I and II (HTLV-I and -II), is the causative agent of enzootic bovine leukosis (EBL), a neoplastic disease of cattle (Miller et al., 1969). Approximately, one-third of BLV-infected cattle develop a persistent lymphocytosis, and only a minor fraction of these cattle develop lymphosarcoma (Burny et al., 1988). The majority of infected cattle remain clinically healthy and act as carriers for the spread of EBL. After experimental infection, BLV induces lymphosarcoma in sheep at a higher incidence and within a shorter latency period (Mammerickx et al., 1981). This infection model is of the greatest importance in the study of the pathogenesis of oncoviruses like HTLV-I and -II and may provide useful information for the development of a vaccine against retroviruses. 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 (Mammerickx et al., 1981).

The results of published vaccination trials have been reviewed by Miller (1986), Portetelle et al. (1993), Kettmann et al. (1994) and Willems et al. (2000). BLV vaccinations were first performed using inactivated virus, fixed BLV-infected cells or purified viral antigens. A significant breakthrough in vaccinology arose from the use of recombinant DNA technology, allowing the expression of large quantities of viral proteins or the development of synthetic vaccines. Among the trials based on these technologies, the use of recombinant vaccinia viruses producing the gp51 antigen in its native configuration was quite encouraging (Gatei et al., 1993a, b; Ohishi et al., 1990, 1991, 1992; Portetelle et al., 1991). Besides inactivated immunogens, attenuated vaccines have proven their efficacy against several virus agents. In the context of retrovirus infections, the simian immunodeficiency virus model has been extensively investigated. In this model, strongly attenuated viruses were obtained by the deletion of viral genes, i.e. the nef gene (Yasutomi et al., 1996). The results obtained with these attenuated vaccine candidates are considered to be very promising.

In the BLV system, we have demonstrated that the G4 gene is required for virus propagation and pathogenicity in vivo (Kerkhofs et al., 1998). Indeed, the pathogenic potential is
strongly decreased, if not completely abrogated, by the deletion of G4. A virus deleted in this gene could thus be used for the design of a live vaccine.

In addition to these conventional vaccines, recombinant vectors and subunit vaccines, one of the more surprising entrants to the world of vaccines has been the use of naked DNA. Different studies have raised the possibility that DNA-based immunization can induce a virus-specific immune response and protective immunity (Cox et al., 1993; Robinson et al., 1993; Wang et al., 1993; Yasutomi et al., 1996). This novel method of vaccination elicits both cellular and humoral immunity. Furthermore, the DNA-mediated immune response has been shown to present a long-lived effector activity (Michel et al., 1995; Robinson et al., 1997; Tang et al., 1992; Yasutomi et al., 1996). For DNA immunization against retroviruses, the envelope gene products have been used preferentially (Agadjanyan, 1994; Wang et al., 1993; Yasutomi et al., 1996).

In the present study we have undertaken an evaluation of the ability of two attenuated BLV variants or DNA-expressed envelope glycoproteins to protect cattle and sheep against a heterologous BLV virus challenge.

**Methods**

**BLV recombinant and plasmid used in this study.** The BLV DX recombinant virus was constructed by deletion of the R3 and G4 genes from a wild-type proviral clone, pBLV344 (Fig. 1a; Kerkhofs et al., 1998; Willems et al., 1993, 1994).

The BLV 6073 virus has a mutation in the transmembrane gp30 gene. The tyrosine residue of the first YXXL motif in the gp30 cytoplasmic tail was mutated to aspartic acid, leading to an infectious virus greatly impaired in propagation within the host (Fig. 1b; Willems et al., 1995).

The plasmid DNA that encodes the BLV envelope gene under the control of the cytomegalovirus promoter (pCMVenv) was constructed by insertion of the env gene from plasmid pBLV344 in the pcDNA plasmid (Invitrogen).

**Experimental design.** In the first experimental group, two sheep (animals 278 and 279) and two cattle (animals 171 and 152) were vaccinated by subcutaneous injection of 2 ml of blood from a sheep infected with the attenuated BLV DX variant.

In the second experimental group, two sheep (animals 284 and 285) and one cow (animal 269) were vaccinated by subcutaneous injection of 2 ml of blood from a sheep infected by the second attenuated virus (BLV 6073).

In the third experimental group, two sheep (288 and 289) were vaccinated by intramuscular injection of 3.7 mg of the recombinant plasmid pCMVenv in 50 ml PBS in the quadriceps muscle. The injection was repeated twice after 32 and 74 days as booster immunizations.

As challenge controls, one sheep (animal 290) and one cow (animal 205) remained uninfected until challenge. The negative controls were sheep 291 and cow 605, which also remained uninfected during the whole period of investigation. The design of this vaccination trial is summarized in Table 1.

One hundred and forty days after inoculation of the attenuated viruses, the seven animals and the two controls were challenged by subcutaneous injection of the heterologous FLK-BLV (10⁸ FLK-BLV-producing cells) (Van der Maaten & Miller, 1976).

**Serological analysis.** Antibodies against BLV gp51 and p24 were detected by ELISA as described by Portetelle et al. (1989).

**PCR analysis.** Whole blood was used as a sample for PCR analysis. Semi-quantified PCRs of the sequences corresponding to the tax gene were performed as described by Willems et al. (1998) using the oligonucleotides 5′ CTCTTCGGGATCCATTACCTGA 3′ and 5′ CTCTTCGGGATCCATTACCTGA 3′.

Fig. 1. The BLV variants used for vaccination are virologically attenuated. (a) Schematic representation of the BLV variants used for vaccination and challenge. Based on their ability to propagate efficiently in the animal model, the FLK-BLV virus and the recombinant proviruses were separated into two groups. The mutant virus pBLVIX (which contains a small deletion between two XbaI restriction sites at positions 6614 and 6732) behaves similarly to the wild-type FLK-BLV. Two types of attenuated viruses were used in this study: pBLV DX (which is deleted in both the R3 and G4 genes) and pBLV 6073 (in which the tyrosine residue of the first YXXL motif in the gp30 cytoplasmic tail was mutated to aspartic acid). (b) Virus propagation of the variants used for vaccination. To quantify the efficiency of virus propagation of the variants used in this study, semi-quantified PCRs were performed on whole blood samples collected from vaccinated animals. Sequences corresponding to the tax gene were amplified. As a control for semi-quantification, undiluted and 1:10 dilutions of samples from the two challenged control animals were incorporated in this experiment. Southern blot hybridization was performed with the PCR products using a tax probe.
Table 1. Summary of the vaccination experiment

The number of animals seropositive for p24 or gp51 antibodies after vaccination is indicated (post-vaccine serology). After challenge, wild-type superinfection was detected by PCR at 4, 12 and 18 months post-inoculation.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Animal no.</th>
<th>Post-vaccine serology</th>
<th>Challenge virus detection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>p24 gp51</td>
<td>4 months</td>
</tr>
<tr>
<td>BLV DX</td>
<td>Sheep 278, 279</td>
<td>2/2 2/2</td>
<td>0/2 0/2</td>
</tr>
<tr>
<td></td>
<td>Cattle 171, 152</td>
<td>2/2 2/2</td>
<td>0/2 1/2</td>
</tr>
<tr>
<td>BLV 6073</td>
<td>Sheep 284, 285</td>
<td>2/2 2/2</td>
<td>0/2 0/2</td>
</tr>
<tr>
<td></td>
<td>Cow 269</td>
<td>1/1 1/1</td>
<td>0/1 0/1</td>
</tr>
<tr>
<td>pCMVenv</td>
<td>Sheep 288, 289</td>
<td>0/2 2/2</td>
<td>0/2 0/2</td>
</tr>
<tr>
<td>Challenge control</td>
<td>Sheep 290</td>
<td>– –</td>
<td>1/1 1/1</td>
</tr>
<tr>
<td></td>
<td>Cow 205</td>
<td>– –</td>
<td>1/1 1/1</td>
</tr>
<tr>
<td>Negative control</td>
<td>Sheep 291</td>
<td>– –</td>
<td>0/1 0/1</td>
</tr>
<tr>
<td></td>
<td>Cow 605</td>
<td>– –</td>
<td>0/1 0/1</td>
</tr>
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CCTGCATGATCTTTCATACCAAAAT 3’. As a control for semi-quantification, undiluted and 1:10 dilutions of DNA samples from two challenged control animals were amplified in parallel.

PCR amplification of the R3–G4 region of the BLV genome was performed using the oligonucleotides 5’TGGAAAGAACTAACGGAGACGG 3’ and 5’ACGGTGGGTCTCGCAGGTGAGCGTGGTGCAGATCC 3’, as described by Dequiedt et al. (1997) (see Fig. 3a).

The amplification products were analysed by Southern blot hybridization using a BLV probe (SacI insert from plasmid pBLV344), as previously described (Willems et al., 1993).

Results

Vaccination using attenuated viruses

Two different types of attenuated recombinant viruses were used in this study to immunize sheep and cattle against BLV.

To quantify the efficiency of virus propagation of these variants, semi-quantified PCRs of the tax gene were performed on blood samples collected in vaccinated sheep and cattle just prior to challenge (4–5 months after vaccination). As a control for semi-quantification, undiluted and 1:10 dilutions of DNA samples from two challenged control animals (sheep 290 and cow 205) were amplified in parallel (Fig. 1b). Compared to the control animals, in which the challenge virus (FLK-BLV) was detected 4 months after infection, after the same time the two BLV mutants (BLV DX, BLV 6073) used as vaccine candidates remained undetectable in the semi-quantified PCR conditions used.

These results are in agreement with a previous report of the virological attenuation of the BLV variants used in this study (Kerkhofs et al., 1998; Willems et al., 1995). This attenuation phenotype is a mandatory requirement for their possible use as...
Fig. 3. PCR amplification of viral sequences. (a) Schematic representation of the 3' end of the BLV provirus. The primers used for PCR amplification are indicated by arrows. Mutants BLV 6073 and BLV DX harbour a deletion within their genome (shaded box). PCR amplification generates fragments of 370, 640 and 750 bp for proviruses BLV DX, BLV 6073 and FLK-BLV (wild-type), respectively. (b) Blood samples were collected at regular intervals of time after challenge (4, 12 and 18 months). Viral sequences were amplified by PCR and analysed by Southern blot hybridization using a BLV probe (SacI insert from plasmid pBLV344).

These data also extend to cattle our previous observations of virus attenuation in sheep (Willems et al., 1994, 1995).

After vaccination, humoral responses against BLV were detected by ELISA against gp51 and p24 (Portetelle et al., 1989) in all vaccinated animals prior to challenge (Table 1 and Fig. 2, day 140). These data indicated that the humoral responses directed towards the two attenuated viruses were quite different. The BLV DX mutant was more effective at inducing higher anti-gp51 and anti-p24 antibody levels than BLV 6073. This difference in humoral response mainly concerned the anti-p24 antibodies and was even amplified 20 months after challenge (Fig. 2, day 760). Indeed, at this time the four animals vaccinated with BLV DX presented the highest anti-p24 titres.

To evaluate the protective effect conferred by the attenuated viruses, PCR amplification of the R3–G4 region of the BLV genome was used to assay for the presence of FLK-BLV proviral DNA in the circulating leucocytes of animals immunized with the attenuated viruses. Therefore, blood samples were collected at regular intervals over an 18 month period after challenge. After cell lysis, these samples were submitted to PCR amplification of the R3–G4 region and the products were analysed by Southern blot hybridization. Four months after challenge, the use of this pair of oligonucleotides allowed the amplification of a 370 bp fragment in sheep 278 and 279 and cattle 171 and 152 infected by attenuated BLV DX and a 640 bp fragment in sheep 284 and 285 and cow 269 infected by attenuated BLV 6073. A 750 bp fragment corresponding to the challenge BLV was only detected within control animals 290 and 205 infected with FLK-BLV (Fig. 3 b).

These observations led us to conclude that 4 months after challenge, protection against infection by a heterologous BLV strain was achieved in seven out of seven animals infected with the attenuated DX (sheep 278, 279 and cattle 171, 152) or the attenuated 6073 (sheep 284, 285 and cow 269) virus. Irrespective of the BLV DX or BLV 6073 attenuated viruses used for vaccination, long-term protection, as measured 12 and 18 months after challenge, was observed for the four sheep (278, 279, 284 and 285) but only for two out of the three cattle (171 and 269). The infection of BLV DX-vaccinated cow 152 with FLK-BLV was detected 12 months after challenge by the amplification of a 750 bp insert generated by the FLK-BLV virus in addition to the 370 bp fragment corresponding to the attenuated virus BLV DX. The immune response directed against one of the attenuated viruses (DX or 6073) was thus effective in suppressing the challenge infection in four out of four sheep and in two out of three cattle. It should be mentioned that, after vaccination and prior to the challenge, cow 152 presented a lower antibody response against BLV, especially against the capsid protein and that the breakthrough of challenge virus was accompanied by an increase in that antibody response. Sheep vaccinated using one of the two live attenuated BLV variants appeared to be completely protected against the challenge by subcutaneous inoculation of a heterologous BLV strain. Indeed, we were unable to detect the presence of the FLK provirus in their PBMCs by PCR–Southern blot analysis. This result indicates that the challenge virus very poorly, if at all, replicated in the host. However, the attenuated BLV strains (DX and 6073) used as vaccines were detectable during the post-challenge period.

DNA immunization against BLV

To reduce the potential pathological risk linked to the use of live attenuated viruses, we developed another strategy to immunize sheep against BLV. This strategy is based on vaccination with plasmid DNA that encodes the BLV envelope gene placed under the control of the cytomegalovirus promoter (pCMVenv). Therefore, in a third experimental group, two sheep were vaccinated by intramuscular injection of this recombinant DNA construct (Table 1). This nucleic acid vaccine. These data also extend to cattle our previous observations of virus attenuation in sheep (Willems et al., 1994, 1995).
immunization induced in sheep 288 and 289 an effective but rather late humoral response against BLV gp51 as shown by the presence of specific antibodies in the sera of these animals 4.5 months after the first injection (Table 1 and Fig. 2, day 140). One hundred and forty days after the first injection, the animals were challenged by subcutaneous injection of $10^5$ FLK-BLV-producing cells. The onset of low titres of p24 antibodies 5 to 12 months after challenge suggested that the FLK-BLV virus replicated at a lower rate compared to the control unvaccinated challenged animal (data not shown). The presence of the 750 bp fragment in the PCR–Southern blot analysis performed 18 months after challenge confirmed the serological data. Therefore, it seems likely that our DNA immunization protocol was effective in delaying challenge virus replication but long-term protection was not achieved.

Discussion

Previous experimental infection of sheep with mutated BLV proviruses revealed that the humoral responses directed towards wild-type and attenuated viruses were similar. Since vaccination is able to protect against BLV infection, it was reasonable to hypothesize that variants such as the R3–G4 deletion mutant or BLV 6073 could be of potential use for vaccination. Therefore, we designed a vaccination trial including sheep and cattle. This present report is the first publication which describes the use of life attenuated recombinant viruses for vaccination against BLV infection. Our results indicate that the immune response directed towards the two attenuated viruses was able to protect sheep against a challenge infection. Similar protection results were also obtained by Reichert et al. (2000, accompanying paper) after vaccination of sheep with the attenuated BLV DX mutant. However, the protection conferred in cattle, which is the natural host for BLV, was only partial.

Of note, the challenge conditions that we used were estimated to be of approximately 100 BLV infectious doses (Portetelle et al., 1993) and were thus particularly severe. Severe challenge conditions were also reported by Reichert et al. (2000, accompanying paper). Ideally a candidate vaccine should completely block the establishment of infection in vivo; it is now recognized that even very effective live attenuated lentiviral vaccines cannot completely block infection in all exposed individuals. However, virus loads may be significantly reduced and the onset of the disease may be significantly delayed (Putkonen et al., 1995; Shibata et al., 1997). This might be true in the case of cow 152, in which virus replication was significantly delayed.

Nevertheless, a similar situation holds true for vaccinia virus-based vectors, which were shown to be quite effective in sheep but not in cattle (Ohishi et al., 1990, 1991, 1992; Portetelle et al., 1993).

Although we still cannot explain the reasons for these discrepancies, this observation implies that different strategies or protocols have to be used in cattle to achieve full protection. On the other hand, since most of the successful vaccination trials were performed in sheep (reviewed by Kettmann et al., 1994 and Portetelle et al., 1993), it will also be of great interest to perform them in cattle. Nevertheless, under the experimental protocol used here it appears that the replication of the challenge virus was significantly delayed, if not abrogated, compared to the situation in unvaccinated control animals.

Direct injection of DNA into animals has been shown to induce both humoral and cellular immune responses against several infectious agents, such as influenza virus, rabies virus, human immunodeficiency virus and bovine herpes virus (Cox et al., 1993; Robinson et al., 1993; Wang et al., 1993; Yasutomi et al., 1996). Plasmid DNA is highly stable, easily administered and is inexpensive to produce in large quantities. The lack of protein components represents a major advantage, since it makes the potential vaccine unlikely to be affected by a pre-existing immune response. In addition, the apparent long-term persistence of antigen expression from DNA vaccines may mean that they will be effective in the induction of T cell response as well as the establishment of immunological memory (reviewed in Manickan et al., 1997). The present vaccination trial using two sheep demonstrated that the injection of a recombinant plasmid encoding the envelope genes of BLV was able to elicit an immune response effective in delaying for more than a year the infection by a challenge virus. Even if complete protection was not observed during this experiment, these results are encouraging for the development of a plasmid-based vaccine in cattle.

Our results demonstrate that attenuated BLV variants might be useful for vaccination of sheep, conferring long-term protection against heterologous BLV virus infection. Plasmid-based immunization of sheep was effective in delaying the challenge virus infection for several months. Further experiments are required to optimize this vaccination strategy against BLV in its natural host, cattle. Nevertheless, these results obtained in the BLV system give significant evidence that attenuated viruses and DNA-based immunization are potential tools for the protection against infection by a member of the BLV/HTLV-I and -II family.

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References


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