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Protection of cattle against bovine leukemia virus (BLV) infection could be attained by DNA vaccination°

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The bovine leukemia virus (BLV) envelope gene encoding extracellular glycoprotein gp51 and transmembrane glycoprotein gp30 was cloned into a vehicle expression vector under the human cytomegalovirus (CMV) intermediate early promoter. The intramuscular injection of this plasmid vector generated a cellular immune response. Seven out of ten cows vaccinated with the DNA construct resisted a drastic challenge (500 BLV-infected lymphocytes as an infectious dose).

Enzootic bovine leukosis, the most frequent bovine neoplastic disease, has a worldwide distribution [1]. The infectious agent, bovine leukemia virus (BLV), is a trans-activating retrovirus found naturally in cattle. The virus principally infects B lymphocytes although it has been reported that T-lymphocytes also can be infected [2]. Proviral DNA, which is generated by reverse transcription of the viral genome, integrates at random in nuclear DNA and appears to be transcriptionally repressed [3, 4]. The virus is mainly transmitted horizontally by direct exposure to a biological fluid contaminated with infected lymphocytes (mainly blood, but also saliva, semen, and milk). However, cell-free virus, which apparently is shed into blood only during pregnancy, may account for transplacental vertical transmission [5, 6]. After viral infection 30–70% of cattle develop persistant lymphocytosis (PL), a polyclonal B-cell benign lymphoproliferative disorder. Only 0.1–10%

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Abbreviations: BLV, bovine leukemia virus; CMV, cytomegalovirus; PBS, phosphate-buffred saline; PL, persistant lymphocytosis.

of carriers develop lymphoid tumours, which also consist of B lymphocytes. In contrast to PL, however, they are of mono- or oligoclonal origin in terms of the integration site, which is characteristic for each tumour. Some cells contain one or more copies of the viral genome, chromosomal aberrations are common and, if deletions are present, they are invariably found in the 5'-half of the virus DNA sequence. In both types of affected cells transcription is repressed *in vivo*, but transient virus production can be induced *in vitro* and detected by means of syncytia induction or haemagglutination. Available data indicate that the external envelope glycoprotein gp51 (51 kDa) derived from a 72 kDa precursor Pr72env may be a critical viral target for the host immune system. The gp51 is not only responsible for the binding to a cell receptor and determining the tropism of BLV, but is the first antigen to be recognized by antibodies in the sera of newly infected hosts [7]. *In vivo* production of virus in some unknown cell is suggested by the presence of high antibody titres in infected animals, especially against the envelope glycoprotein gp51. This can be detected by various techniques such as immunodiffusion, radioimmune assay or ELISA. Although bovine leukosis is not of major economic importance, its eradication is desirable and feasible in countries with its relatively low incidence, by means of testing and elimination. For endemic situations (as in Poland) vaccination would be preferable, and certainly it would be possible to develop the gp51 based vaccines.

For generation of new vaccines still more sophisticated approaches are currently applied: recombinant protein subunits, viral vectors, and naked DNA that encodes immunogenic proteins. However, so far no DNA vaccines against BLV infection are known. Traditional vaccines are based either on a weakened or killed version of the disease-causing agent, whereas the new vaccine uses only the DNA coding for a particular segment of the disease-causing virus [8–10]. DNA-mediated immunization to a single antigen can provide protection against infection by a pathogen. DNA vaccination promises to be safer than the use of traditional vaccines because it eliminates the risks associated with possible contamination of vaccines. The immune response will be more long-lived, and vaccines for various diseases can be injected in a single shot. The new method will also be more cost-effective, since the vaccine development and production process becomes simplified with the progress in gene technology. Introduction of the DNA can be accomplished by simple intramuscular or intradermal injections using needles, as well as by propelling DNA-coated gold particles into various tissues [11], preferentially the dermis. Although very few cells can be transfected using these methods, the amount of protein produced as well as other parameters of the method (not all of which are understood) lead to surprisingly high Th_2 immune responses [12], in the absence of any further adjuvant apart perhaps from the DNA itself. The responses include not only antibody induction and T-cell activation with cytokine secretion, but also the production of cytotoxic T lymphocytes (CTL). Most importantly, it has been shown in several instances that DNA-media will provide protection against infection by a pathogen [12]. Although DNA vaccines share the same limitations as recombinant protein vaccines (i.e., the antigen conferring protection should in principle be known), this does not seem to be incompatible with achieving a fully protective immune response to at least some pathogens. Moreover, if combinations of antigens are required, then a simple mixture of plasmids could conceivably be used. Researchers are already working with DNA vaccines for a large number of diseases. A promising DNA vaccine candidate for malaria is currently being developed, as well as new vaccines for AIDS, herpes and tuberculosis [13].

In our preliminary studies we constructed and tested a DNA vaccine containing BLV *env* gene. The gene encoding gp51 and gp30 pro-

teins (*env*) was prepared by PCR amplification directly from BLV-infected cow genomic DNA, using primers designed to facilitate cloning (Fig. 1). The forward primer was: ENV1-*Hin*dIII: 5′ GCG AAG CTT **ATG CCT AAA GAA CGA CGG TCC** 3′ (30 nt, the underlined sequence contains *Hin*dIII restriction site, the bold part of the primer sequence is complementary to the nucleotide sequence of the 5′ end of *env* gene) and the reverse primer was: ENV1-*Eco*RI: 5′ GCG GAA TTC *TTA* **GTC AGG GCA GGG TCG** 3′ (27 nt, the underlined sequence contains *Eco*RI restriction site, the bold part of the primer sequence is complementary to the nucleotide sequence of the 3′ end of *env* gene, the stop codon is italicized).

The DNA of a BLV-infected cow was isolated from blood using Blood DNA Prep Plus Kit (A&A Biotechnology, Poland). The PCR reaction solution consisted of: 2 μg of the template DNA, 2 μl (10 μM) of each primer, 5 μl (200 μM) dNTP's, 5 μl 10 × PCR buffer (100 mM Tris/HCl, pH 8.9, 500 mM KCl, 20 mM $MgCl_2$, 1% Triton X-100), 2 units of Delta2 DNA polymerase (hyperthermostable *Pwo* DNA polymerase, from DNA-Gdańsk II s.c., Poland). The following thermal profile was applied: 40 s at 94°C, 60 s at 55°C, and 120 s at 72°C in a Hot-Shot12 thermal cycler (DNA-Gdańsk II s.c., Poland). Thirty five cycles were performed. A specific, 2026 bp, PCR product obtained was then digested with *Hin*dIII and *Eco*RI restriction enzymes, purified using DNA Clean Up Kit (A&A Biotechnology, Poland) and ligated into *Hin*dIII-*Eco*RI sites of pcDNA3 plasmid. The pcDNA3 vector is available from Invitrogen (U.S.A.) and is recommended as a general cloning and expression vector containing the CMV immediate-early promoter and the bovine growth hormone polyadenylation site. This plasmid also contains a neomycin resistance gene driven by the SV40 promoter. *Escherichia coli* DH5α cells were transformed with the ligation mixture and 7 colonies were obtained and assayed for the presence of the *env* gene by PCR amplification using ENV1-*Hin*dIII and ENV1-*Eco*RI primers and restriction analysis with *Hin*dIII and *Eco*RI restriction enzymes. Three out of

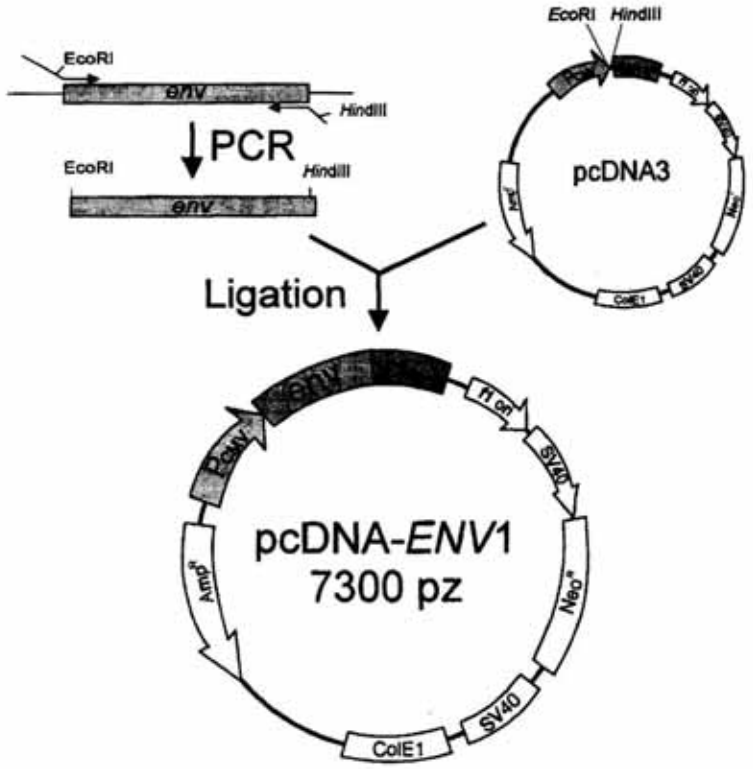

Figure 1. Construction of the plasmid pcDNA3-*ENV*1.

six clones were verified by sequencing using the dideoxy chain termination method. One of the isolated recombinant plasmids, pcDNA3-*ENV*1, was used for immunization procedures. The plasmid was purified using DNA Plasmid Prep Kit (A&A Biotechnology, Poland). The DNA concentration was estimated after agarose gel electrophoresis by comparison with known standards, and by spectrophotometry. For immunization, DNA was dissolved in pyrogen-free PBS, pH 7.6.

To verify the identity of gp51 and gp30 expression products of pcDNA3-ENV, Vero cells were transfected with 15 μg of plasmid DNA by calcium phosphate coprecipitation. Verification of the gp51 and gp30 expression products – Western blot analysis – was carried out according to a standard procedure (Fig. 2).

Ten, sero- and PCR-negative, three-month-old calves were obtained from a local farm, housed in isolation and fed on commercial feed. The animals were injected intramuscularly three times at 4-week intervals with a total of 2 ml plasmid DNA preparation at concentrations indicated in Table 1. A cow known to have been BLV-positive was used as a donor of BLV-infected peripheral blood leukocytes (PBLs) for challenge experiments. Each animal was infected with 500 PBL four weeks after the last immunization. Four weeks later white blood cells were isolated from each chal-

Table 1. Vaccination and challenge experiments

Animal number	pcDNA-ENV (in 2 ml dose)	Time after vaccination									4 weeks after challenge		
		0			4 weeks after the first vaccination		4 weeks after the second vaccination		4 weeks after the third vaccination		Syncytial induction assay	Cellular immune response[1]	PCR *U5 LTR5'*
		PCR *env*	ID	ELISA	ID	ELISA	ID	ELISA	ID	ELISA			
95532	0.06 mg	–	–	–	–	–	–	–	–	–	–	+	–
95503	0.06 mg	–	–	–	–	–	+	–	–	–	+	–	+
95546	0.125 mg	–	–	–	–	–	–	–	–	–	–	+	–
95977	0.125 mg	–	–	–	–	–	–	–	–	–	–	+	–
95974	0.25 mg	–	–	–	–	–	–	–	–	–	–	+	–
95971	0.25 mg	–	–	–	–	–	–	–	–	–	–	+	–
95505	0.5 mg	–	–	–	–	–	+	–	–	–	+	–	+
95545	0.5 mg	–	–	–	–	–	–	–	–	–	–	+	–
95504	1 mg	–	–	–	–	–	–	–	–	–	–	+	–
95550	1 mg	–	–	–	–	–	+	–	–	–	+	–	+

[1]Cellular immune response $CD8^+$ and $CD4^+$: "+" – 2-3 times higher over background level; "–" – background, before vaccination.

Figure 2. Expression of the BLV gp51 and gp30 from Vero cells.

Detection of protein by Western blot analysis. After electrophoresis, samples containing 30 μg of protein from Vero-transfected cells (lane 1), 1 μg of gp51 purified from NIH 3T3-transfected cells (lane 2) and 5 μg of purified BLV virus proteins (lane 3) were blotted onto a nitrocellulose membrane. A rabbit anti-BLV serum was applied to detect the gp51 and gp30 proteins.

lenged animal and tested for cellular immune response and BLV presence by syncytial induction assay and PCR. Titration of anti-BLV antibodies in the sera of immunized cattle was carried out using standard immunodiffusion and ELISA methods [14]. The sera from cattle with leukemia were used as a positive control. The cellular immune response was assayed according to Gatei *et al.* [15]. The PCR detection of BLV *env* gene was carried out as described by Kubiś *et al.* [16] and U5 LTR5′ sequence of BLV was determined as described by Kuźmak *et al.* [17].

Using immunodiffusion and ELISA methods no antibody response was dectected in any animal vaccinated with pcDNA3-*ENV*1 plasmid (Table 1). The syncytial induction assay is highly sensitive for detection of virus infection. Five thousand lymphocytes from vaccinated animals were seeded on indicator cells. No signs of infection were found in 7 vaccinated animals, while in 3 animals positive syncytial induction assay was observed (Table 1). The same 7 negative animals show that immunization with pcDNA3-*ENV*1 DNA induced cellular immune responses (Table 1).

These data are only of limited value but they clearly demonstrated the feasibility of anti-BLV DNA vaccination.

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