Virus Genes 22:2, 187–199, 2001

Large Envelope Glycoprotein and Nucleocapsid Protein of Equine Arteritis Virus (EAV) Induce an Immune Response in Balb/c Mice by DNA Vaccination; Strategy for Developing a DNA-Vaccine Against EAV-Infection

E. TOBIASCH,[1] R. KEHM,[1] U. BAHR, C.A. TIDONA,[1] N. J. JAKOB,[1] M. HANDERMANN,[1] G. DARAI,[1] & M. GIESE[2*]

[1]*Institut für Medizinische Virologie der Universität Heidelberg, Im Neuenheimer Feld 324, 69120 Heidelberg, Federal Republic of Germany*

[2]*Boehringer Ingelheim Vetmedica GmbH, Binger Straße 173, 55216 Ingelheim am Rhein, Federal Republic of Germany*

Received June 11, 2000; Revised/Accepted August 15, 2000

Abstract. Equine arteritis virus (EAV) is a member of the *Arteriviridae* family, that includes lactate dehydrogenase-elevating virus (LDV), porcine reproductive and respiratory syndrome virus (PRRSV), and simian haemorrhagic fever virus (SHFV). Equine arteritis is a contagious disease of horses and is spread via respiratory or reproductive tract. The objective of the present study is to evaluate the possibility for developing a model system for prevention horses against an EAV infection by DNA vaccination. A cDNA bank from the RNA of EAV was established. This gene library contains the translation unit of the EAV open reading frames (ORF) 1 to 7. The identity of the cDNA was confirmed by nucleotide sequence analysis. Using this defined EAV cDNA gene library the cDNA sequence of the viral ORFs were molecularly cloned into the corresponding sites of well characterized and powerful expression vectors (pCR3.1, pDisplay, and/or pcDNA3.1/HisC).

The capability of these recombinant plasmids expressing the gene products of the individual viral ORFs 3 to 5, and 7 in induction of an immune response in mouse system was investigated. The Balb/c mice (ten mice per assay) were inoculated with the DNA of the constructed expression vectors harboring and expressing the EAV cDNA of the viral ORFs. The Balb/c mice were injected with about 100 µg DNA diluted in 100 µl PBS. The DNA was injected subcutaneously and into the *tibialis cranialis* muscle (*Musculus gastrocnemius*). The mice were boosted 3 to 5 times with the same quantities of DNA and under the same conditions at about two week intervals. Control mice received the same amount of parental expression vectors via an identical route and frequency.

The pre- and post-vaccinated sera of the individual animals were screened by neutralization tests (NT). Neutralizing antibodies against EAV were detected when the animals were inoculated with the DNA of the expression vectors harboring cDNA of the EAV ORFs 5 and 7. Highest NT-titers were observed when the animals were administered with the cDNA of ORF 5 and/or with the cDNA of the neutralization determinants of EAV that is located on the N-terminal ectodomain of the gene product of ORF 5 between the amino acid positions 1–121. These results obtained from these studies justified proofing the capability of the EAV cDNA sequences of the viral genes including ORFs 5 and 7 in the autologous animal system horse.

Key words: equine arteritis virus (EAV), arteriviridae, RT-PCR, nucleotide sequence analysis, neutralizing antibodies, recombinant plasmids, expression vectors pCR3.1, pDisplay, pcDNA3.1/HisA-C

Introduction

Equine arteritis is a contagious disease of horses and is spread via respiratory or reproductive tract and caused by equine arteritis virus (EAV) which is a member of the *Arteriviridae* family, that includes lactate dehydrogenase-elevating virus (LDV), porcine reproductive and respiratory syndrome virus (PRRSV), and simian haemorrhagic fever virus (SHFV).

*To whom correspondence should be addressed.

EAV is well investigated and its biological and biophysical properties together with the data on viral pathogenesis and cell virus interactions had been documented. The EAV virions are 60–65 nm in diameter and possess a 49S RNA genome that is a single-stranded, nonsegmented, capped and polyadenylated message-sense RNA (12687 nucleotides, accession number: X53459 (1)). The EAV genome is infectious and contains at least eight open reading frames (ORF) 1a, 1b, 2, 3, 4, 5, 6, and 7. The two largest viral ORFs (ORF 1a and ORF 1b) encode the viral replicase (1) and are located at the 5′-end of the viral genome between the nucleotide positions 1 and 9807. The ORFs 2 to 7 are overlapping and located at the 3′-end of EAV genome. These ORFs encode four known structural viral proteins and two proteins of unknown function (gene products of ORFs 3 and 4). The EAV virion consists of a phosphorylated nucleocapsid protein (N, 14 kDa, gene product of ORF 7), a N-glycosylated major membrane protein (G_L, 30–44 kDa, gene product of ORF 5), a N-glycosylated minor membrane protein (G_S, 25 kDa, gene product of ORF 2), and an unglycosylated membrane protein (M, 17 kDa, gene product of ORF 6).

The EAV infection is maintained in horse population, because chronic carrier animals shed EAV in their semen and transmit it venereally to mares during the mating season. PRRSV is also characterized by reproductive failure, e.g. late-term abortions in sows and causing respiratory illness and mortality in young pigs.

The analysis of the genetic stability of EAV during horizontal and vertical transmission in an outbreak of equine viral arteritis revealed that the carrier stallion is the source of genetic diversity of EAV (2). It is known that the infected carrier stallion is the critical natural reservoir of EAV. The outbreak of an EAV infection can be initiated by the horizontal aerosol transmission of specific viral variants that occur in the semen of carrier stallions. However, Patton and coworkers show that not only the carrier stallion is the critical natural reservoir of EAV, but also genetic diversity of the virus is generated in the course of persistent infection of carrier stallions (3).

Consequently, PRRSV and EAV cause economically important infectious diseases in swine and horse farms worldwide. The development of an efficient vaccine is of particular importance, since it focuses attention on the prevention of the diseases. An immune response was demonstrated in mice and pigs that were vaccinated with plasmid DNA encoding ORF5 of PRRSV harboring a viral glycoprotein associated with neutralizing epitopes (4). Furthermore Balasuriya and co-workers have reported that equine arteritis virus derived from an infectious cDNA clone is attenuated and genetically stable in infected stallions (2).

The objective of our study is to evaluate the possibility of developing a model system for prevention of horses against an EAV infection by cDNA vaccination. The aspects and perspective of the DNA vaccine against viral diseases in past and future had been summarized and reviewed by M. Giese, 1998 (5).

Materials and Methods

Viruses and Cells

The equine arteritis virus (EAV) used in this study was kindly provided by Professor H. Ludwig, Berlin and propagated on rabbit kidney cells (RK13, ATCC number CCL-37). The cell cultures were obtained from the American Type Culture Collection and propagated in Basal Medium Eagle (BME) supplemented with 10% fetal calf serum, 100 IE/ penicillin G, 100 IE /ml streptomycin. Medium and serum were purchased from GibcoBRL (Eggenstein, Germany).

Production of EAV-Specific Antisera

Antiserum against EAV was induced in New Zealand white rabbit. The animal was inoculated subcutaneously with 0.5 ml purified EAV. Inoculation was repeated for four times. The sensitivity of rabbit antiserum was determined by Western blot analysis. It was found that the rabbit antiserum raised against EAV is able to recognize viral specific protein at a dilution of about 1:2000 and higher.

Preparation of Viral RNA

Virion RNA and total infected cell RNA were prepared from EAV-infected RK13 cell cultures at 12, 24, 36, and 48 h p.i. using a guanidinium isothiocyanate/cesiumchloride procedure (6). Infected cells or virions from clarified infected cell culture supernatants were dissolved in a 4.0 M guanidinium

thiocyanate (GTC) solution. Cellular DNA in the infected cell preparation was sheared by repeatedly passing the solution through a 23-gauge needle. CsCl and sarcosyl (30% aqueous solution) were added to the GTC preparation to final concentrations of 0.15 g/ml and 3.0%, respectively. In volumes of 8 ml the preparation was transferred onto a 3 ml 5.7 M CsCl cushion and centrifuged at 29,000 rpm in a Beckman SW41 rotor for 24 h at 20°C. The supernatant was discarded and the RNA pellet was dissolved in RNase free H_2O to a final concentration of about 10µg/ml. RNA preparations were stored at −20°C in 80% ethanol containing 100 mM sodium acetate. As an alternative total RNA of EAV-infected cells was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer.

First-Strand cDNA Synthesis

For each first-strand cDNA synthesis reaction approximately 0.5 µg of purified RNA were pelleted and dissolved in 10 µl RNase free H_2O containing 20 U RNase inhibitor (Takara Shuzo Co., Ltd., Shiga, Japan). The reaction was prepared in 20 µl volumes using enzymes and reagents from the RNA LA PCR Kit Ver.1.1 (Takara Shuzo Co., Ltd., Shiga, Japan) according to the instructions of the manufacturer. The reaction included 5 mM $MgCl_2$, 1 mM of each dNTP, 10 pmol of a specific reverse oligonucleotide primer, and 5 U AMV reverse transcriptase XL. The reaction was incubated in an automated temperature cycling reactor (Genius, Techne, Cambridge, UK) for 2 min at 60°C followed by 15 min at 50°C. Then the temperature was gradually lowered to 42°C at a speed of 1°C/min. As a final step the reaction was incubated for 2 min at 80°C and rapidly cooled to 4°C. RNase free H_2O was added to the reaction products to obtain a final volume of 100 µl. The first-strand cDNA stocks were stored at −20°C.

Oligonucleotides and Polymerase Chain Reaction (PCR)

Specific oligonucleotides were synthesized with an Oligo 1000 M DNA Synthesizer (Beckman Instruments GmbH, München, Germany). The properties of the individual oligonucleotides primers are summarized in Table 1. Polymerase chain reaction (PCR) was performed in 100 µl volumes using TaKaRa LA *Taq* DNA polymerase (supplied with reaction buffer, Takara Shuzo Co., Ltd., Shiga, Japan). Each reaction contained 1.5–2.5 mM $MgCl_2$, 12.5 nmol of each dNTP (Roche Diagnostics Biochemica, Mannheim, Germany), 50 pmol of each oligonucleotide primer, and 1 µl of a first-strand cDNA stock solution (see above). An improved PCR protocol was developed based on a combination of commonly used hot-start and touch-down procedures. Briefly, before adding the dNTP mixture and the DNA polymerase the samples were preheated for 5 min at 94°C and rapidly cooled to 4°C. Then dNTPs and DNA polymerase were added at 4°C and the reaction tubes were directly transferred to a preheated temperature cycling reactor (Genius, Techne, Cambridge, U.K.) at 94°C. PCR reactions were incubated for 35 cycles under cycling conditions of 94°C for 30 sec, 70–56°C for 1 min (starting at 70°C and decreasing by 0.4°C per cycle), and 72°C for 1–5 min, depending on the size of the expected PCR product. As a final step the reaction mixture was incubated for 7 min at 72°C. Reaction products were analyzed by polyacrylamide slab gel electrophoresis and ethidium bromide staining.

Molecular Cloning of Viral cDNA and Preparation of Plasmid DNA

PCR products representing EAV-specific cDNA sequences were subjected to restriction endonuclease treatment and restriction fragments were purified using preparative low melting point agarose gel electrophoresis. Specific DNA bands were extracted from the gel by a hot phenol procedure followed by gel filtration. Restricted and purified EAV cDNA was inserted into one of the following mammalian expression vectors: pCR3.1, pcDNA3.1, pcDNA3.1/His, pDisplay. The pCR3.1 (5044 nucleotide (nt)) is a mammalian vector for high-level constitutive expression (Invitrogen Cat. no. K3001-01) containing a putative transcriptional start site (620–625 nt), a T7 promoter/priming site (638–657 nt), a multiple cloning site (670–785 nt), and a reverse priming site (796–815 nt). The cytomegalovirus enhancer-promoter (1–596 nt) permits high-level expression. The bovine growth hormone polyadenylation signal (796–1010 nt) allows efficient transcription, termination, and polyadenylation of mRNA to enhance mRNA stability. The SV40 promoter and origin (3178–3516 nt), as well as the thymidine kinase polyadenyla-

Table 1. Properties of the constructed mammalian expression vectors harboring EAV specific cDNA of the individual translations units

Plasmid and Genomic Position of EAV cDNA (nt)	Cloning Vector and Insertion Sites	Sequence of Oligonucleotide Primers Used
pCR3.1-EAV-03-BX (ORF 3, 10289–10780)	**pCR3.1** (*Bam*HI / *Xba*I)	**P-BamHI-EAV-03-F1n (10271–10295)**[a] 5′-*GGGGGGATCC***TTTGACCGGACCGGCCACATGGGTC**-3′ **P-XbaI-EAV-03-R1n (10790-10764)** 5′-*GGGGTCTAGA***GTAAGTAAAATTACGAGCCTCTGCAGC**-3′
pCR3.1-EAV-04-BX (ORF 4, 10683–11141)	**pCR3.1** (*Bam*HI / *Xba*I)	**P-BamHI-EAV-04-F1n (10674–10697)**[a] 5′-*GGGGGGATCC***CCTTTGTAGATGAAGATCTACGGC**-3′ **P-XbaI-EAV-04-R1n (11150–11119)** 5′-*GGGGGTCTAGA***GCAATACAATCATAGATAACATCGTTGAGCCC**-3′
pCR3.1-EAV-05-BX (ORF 5, 11129–11896) **large glycoprotein**	**pCR3.1** (*Bam*HI / *Xba*I)	**P-BamHI-EAV-05-F1n (11109–111131)**[a] 5′-*GGGGGGATC***CTGGTACGTTGGGCTCAACGATG**-3′ **P-XbaI-EAV-05-R1n (11912–11189)** 5′-*GGGGGTCTAGAG***TCACCACAAAATGAATCTATGGC**-3′
pDP-EAV-05-Bgs (ORF 5, 11129–11896) **large glycoprotein**	**pDisplay** (*Bgl*II / *Sal*I)	**P-BgIII-EAV-05-F1n (11129–11155)**[a] 5′-*GGGGGGAGATCT***ATGTTATCTATGATTGTATTGCTATTC**-3′ **P-SalI-EAV-05-R1n (111893–11873)** 5′-*GGGGGGGTCGAC***TGGCTCCCATACCTCAGCTGC**-3′
pCR3.1-HIS-EAV-05-del-1-121-BX (Part of ORF 5, 11129–11492) **C-terminal ectodomain of large glycoprotein**	**pcDNA3.1/HIS C** (*Bam*HI / *Xba*I)	**P-EAV-GL-ECTO-F (11129–11155)**[a] 5′-GGGGGGATCC**ATGTTATCTATGATTGTATTGCTATTC**-3′ **P-EAV-GL-ECTO-R (11191–11469)**[a] 5′-*GGGGGGTCTAGA***CAACACAACTATGCCGAATTCAC**-3′
pCR3.1-EAV-07-BX (ORF 7, 12296–12628) **nucleocapsid protein**	**pCR3.1** (*Bam*HI / *Xba*I)	**P-BamHI-EAV-07-F1n (12272–12295)**[a] 5′-*GGGGGGATC***CCGCAGTTGGTAACAAGCTTGTCG**-3′ **P-XbaI-EAV-07-R1n (12701–12629)**[a] 5′-*GGGGGTCTAGA***CCACACAGGAGAATATCCACGTC**-3′
pDP-EAV-07-Bgs (ORF 7, 12296–12628) **nucleocapsid protein**	**pDisplay** (*Bgl*II / *Sal*I)	**P-BgIII-EAV-07-F1n (12296–12319)**[a] 5′-*GGGGGGAGATCT***ATGGCGTCAAGACGATCACGTCCG**-3′ **P-SalI-EAV-07-R1n (12605–12624)**[a] 5′-*GGGGGGGTCGAC***CGGCCCTGCTGGAGGCGCAAC**-3′

[a]The real positions of the viral specific cDNA are indicated in parenthesis and marked with bold letters on the primer sequences. The remaining nucleotides of the individual primers that include the particular recognition sites for restriction endonucleases are shown in italic letters. Note: Primer sets used for evaluation of the specificity of the EAV mRNA:
P-EAV-N-F1: 12307(5′-ACGATCACGTTCGCAGGCCG-3′)12326
P-EAV-N-F2: 12357(5′-CTACAAGTTACAATGACCTACTG-3′)12379
P-ORF7-R1: 12651(5′-CCACACAGGAGAATATCCACGTC-3′)12529
P-EAV-5-F1: 9(5′-TGTGTATGGTGCCATATACGGC-3′)30 within the 5′ leader sequence of EAV (GenBank Acc. No. U65726)
P-EAV-5-F2: 48(5′-GCAAGAATTACTATTCTTGTGGGC-3′)71 within the 5′ leader sequence of EAV (GenBank Acc. No. U65726)
P-ORF-5-R1: 11913(5′-GTCACCACAAAATGAATCTATGGC-3′)11890

tion site (1910–2180 nt) allows efficient high-level expression and termination of the neomycin resistance gene (2355–3143 nt) that served as a selection marker. The ampicillin resistance gene (3595–4455 nt) and the ColE1 origin (1100–1773 nt) permit selection and maintenance in *E. coli*. The fl origin (4586–5042) allows rescue of single-stranded sense strand. The properties of the expression vectors and pDisplay (5325 nt, Invitrogen Cat. no. V660-20) and pcDNA3.1/His (5515 nt, Invitrogen Cat. no. V385-20) are identical to the features of pCR 3.1 vector with few exeptions. The expression vector pDisplay possesses a different multiple cloning site (827–873 nt) that is flanked with murine Ig kappa-chain V-12-C signal peptide (737–799 nt), hemagglutinin A epitope (800–826 nt), and *myc* epitope (874–903 nt). The multiple cloning site of pcDNA3.1/His A,B,C (1012–1085 nt) is different from the vectors described above. A poly histidine region (932–949 nt) and an anti-Xpress antibody epitope (989–1012 nt) con-

taining enterokinase recognition site (998–1012 nt) are located between the T7 promoter/priming site and multiple cloning site.

Vector plasmids were prepared using restriction endonucleases and purified as described above. In addition, restricted vector DNA was dephosphorylated using calf intestine phosphatase (CIP). Ligation of specific EAV cDNA fragments with expression vector DNA was performed as described previously (7). The resulting recombinant plasmid constructs are listed in Table 1. The specificity of the reaction products was confirmed by nucleotide sequence analysis of the insert and flanking vector regions.

Nucleotide Sequence Analysis

PCR products were treated with phenol:chloroform (5:1) and precipitated with 95% ethanol containing 100 mM sodium acetate. The DNA was then washed with 70% ethanol and dissolved in bidestilled water to a final concentration of 20 ng/µl. Plasmid DNA was prepared using the Qiagen tip100 Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Purified DNA was adjusted in H_2O to a final concentration of 1µg/µl. Purified DNA was automatically sequenced with a 373A ''Extended'' DNA sequencer using the BigDye Terminator-*Taq* cycle sequencing technique (Applied Biosystems GmbH, Weiterstadt, Germany). Each sequencing reaction was performed in a volume of 20 µl containing 100 ng of a PCR product or 0.5 µg of plasmid DNA, 50 pmol of the sequencing primer, and 5 µl of the BigDye Terminator reaction mixture. The cycle sequencing reaction was incubated for 28 cycles in an automated temperature cycling reactor (GeneE, Techne, Cambridge, U.K.) under cycling conditions of 96°C for 30 sec and 60°C for 4 min per cycle. The samples were prepared for electrophoresis as described by the manufacturer. The electrophoresis of the samples was carried out on a 36-well 48-cm WTR (well to read) polyacrylamide gel. The nucleotide sequences obtained from individual sequencing reactions were assembled using the Sequence Navigator software (version 2.1, Applied Biosystems GmbH, Weiterstadt, Germany). Nucleotide and amino acid sequences were compared to current GenBank, EMBL, and SwissProt database sequence entries using the BLAST service of the National Center for Biotechnology Information (National Library of Medicine, Bethesda, MD, U.S.A.). Physico-chemical properties of proteins were determined and conserved sequence motifs were identified with the PHYSCHEM and PROSITE programs included in the PC/Gene software (release 6.85, A. Bairoch, University of Geneva, Switzerland). The ClustalX program (version 1.64b) (8) was used to generate multiple sequence alignments.

Preparation of Viral RNA and Northern Blot Analysis

Total cellular RNA was isolated at different times after infection using the guanidium/cesium chloride method as described previously (6,7,10). The northern blot analyzes of these RNAs were carried out using formaldehyde agarose gel (1%) electrophoresis as described elsewhere (6,7,10).

DNA Vaccination of Animals

The immunogenic potential of the EAV translation units were investigated *in vivo*. The BALB/c mice were administered with DNA of the constructed expression vectors harboring and expressing the cDNA of the individual ORFs of EAV genome. The BALB/c mice were injected with about 100 µg/DNA diluted in 100 µl PBS. The DNA was injected subcutaneously and into the *tibialis cranialis* muscle (*Musculus gastrocnemius*) and with a 27 gauge needle. The mice were boosted 3 to 5 times with the same quantities of DNA and under the same conditions at about two week intervals. Control mice received the same amount of parental expression vectors via an identical route and frequency.

Neutralization Test

Neutralization tests were carried out by diluting EAV-specific mouse or rabbit sera with PBS (1:2 to 1:1024) in a 96 well microtiter plate. Serum dilutions (50 µl) were mixed with 100 PFU of EAV (50 µl). The serum-virus mixture was incubated in a 5% CO_2-air atmosphere at 37°C for 2 h. Subsequently, 5×10^3 RK13 cells in suspension were added to each sample of the serum-virus mixture. After 12 h the infected cultures were overlayed with BME containing 10% FCS and 0.5% carboxymethylcellulose. Then the cultures were incubated for 3–4 days at 37°C in a 5% CO_2-air atmosphere. Titers of infectious units were determined after staining with 1% crystal violet.

Immunoblot Analysis

Confluent monolayers of cells were harvested by scraping the cells from the culture well, petri dishes, and/or flasks after being washed three times with PBS (pH 7.2). The final cell pellet was resuspended in distilled water. Protein concentration was measured under the standard method (11). Samples were dissolved in an equal volume of lysis buffer (0.01 M Tris HCl, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.1% (w/v) bromophenol blue, pH 8), heated for five minutes at 95°C, and subjected to SDS-PAGE according to the method of Laemmli (12). Proteins derived from infected and transfectant cells, as well as recombinant N protein were separated by SDS-PAGE and electroblotted onto nitrocellulose filters using semi-dry electroblotting chambers (Renner, Dannstadt, Germany). Transfer efficiency was monitored by Ponceau staining (Sigma, Munich, Germany). Filters were blocked for 1 h and incubated with a 1 : 1000 and 1 : 2000 dilution of the rabbit antisera mentioned above. Alkaline phosphatase conjugated antibodies (anti rabbit or mouse Ig-AP, Roche Diagnostics, Germany) were used to detect interaction of the rabbit or mouse antiserum with EAV protein.

Immunofluorescence Assay

Indirect Immunofluorescence assay was performed essentially as described earlier (13). Briefly, RK13 cell lines were seeded on tissue chamber slides (Nunc Inc., Naperville, USA). The monolayer cell culture were infected with EAV at the MOI of 2 PFU/cell and 48 h after infection the cells were fixed with acetone-methanol and the slides were stored at $-20°C$. Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit or mice IgG (F(ab′)2 fragment immunoglobulin (Roche Diagnostics, Germany) was used as second antibody. In addition, rhodamine B-isothiocyanate (Merck, Darmstadt, Germany) was used at a final concentration of 10 ng/µl for counterstaining of the cells, together with the second antibody.

Enzyme Linked Immunosorbent Assay (ELISA)

Polysorp F8 Microtiter Plates (Nunc, Wiesbaden, Deutschland) were coated with 50 µl EAV Protein (EAV + Host (RK13)) at a concentration of $2\mu g \times ml^{-1}$ in PBS (+ 0.05% N_3Na) over night at room temperature, followed by three cycles of washing with H_2O and then postcoated with 300 µl Blocking Buffer (0.017 M $Na_2B_4O_7 \times 10H_2O$, 0.12 M NaCl, 0.05% Tween 20, 1 mM EDTA, 5% BSA, 0.05% NaN_3) for 3 h at 28°C followed by three cycles of washing with H_2O.

For the assay, the following reagents were successively used: rabbit anti EAV serum at a reciprocal dilution up to 16,000 or mouse anti EAV serum at a reciprocal dilution up to 800. The dilutions were made in Sample buffer POD (DADE Behring, Marburg, Deutschland). After three cycles of washing with 300 µl/well, horseradish peroxidase labeled rabbit-anti IgG second antibody or the horseradish peroxidase labeled mouse-anti IgG second antibody (Roche Diagnostics, Germany) at a predetermined optimum dilution of 1 : 3000 each was added. The dilutions were made in blocking buffer. Incubation steps were done for 1 h at 28°C, each followed by three cycles of washing with 300 µl/well Washing Buffer (DADE Behring, Marburg, Deutschland). Color was developed by adding 200 µl/well of freshly prepared Buffer/Substrate TMB and Chromogen TMB (10 : 1) (DADE Behring, Marburg, Deutschland). The assay was stopped after 30 min by the addition of 50 µl/well Stopping Solution POD (DADE Behring, Marburg, Deutschland) and read according to standard procedures at 450 nm on an automatic ELISA reader (MR5000, DYNATECH, Denkendorf, Deutschland).

Computer-Assisted Sequence Analysis

Nucleotide sequences were compiled using the ABI sequence navigator software version 1.2. Nucleotide and amino acid sequences were analyzed using the PC/GENE program release 6.85 (Intelligenetics Inc. Mountain View, California, U.S.A.) and OMIGA program release 11.3 (Oxford Molecular Group Ltd., Oxford, U.K.).

Results

Amplification of Viral Genes by RT-PCR and Molecular Cloning of Viral Genes in Expression Vectors

The EAV genome consists of about 12.287 nucleotides with a short 3′poly- (A) tail. The genomic

organization and transcriptional strategy of arteriviruses are shown in Fig. 1. For molecular cloning of the viral genome specific oligonucleotides were synthesized (Table 1) and purified viral RNA was used in 3′-RACE experiments to generate a cDNA bank from the genomic RNA including cDNA of viral mRNA transcripts. The specificity of the viral mRNA was tested using RT-PCR and four sets of primers (Table 1). An example of the results of RT-PCR in which the cDNA of the EAV ORFs 5 and 7 were amplified is shown in Fig. 2. The complete cDNA sequence of the individual viral genes were amplified using specific primers that were flanked with recognition sites for different restriction endonucleases (Table 1). The RT-PCR products were molecularly cloned into a suitable expression vector (*pCR3.1, pDisplay, and pcDNA3.1/His A, B, C; Invitrogen*). The identity of the cDNA of the individual viral ORFs was confirmed by nucleotide sequence analysis. The properties of the individual expression vectors constructed are summarized in Table 1.

DNA Vaccination of Mice with Vector Construct Expressing Viral ORFs 5 and 7

Balb/c mice were used as a model system for the evaluation of the immune responses against individual gene products of EAV raised by administration of recombinant plasmid DNA. The capability of the expression vectors harboring and expressing EAV ORF5 and ORF7 that encode the viral nucleocapsid protein and the viral major glycoprotein to induce immune response in the mouse system was investigated. The animals were inoculated subcutaneously and intramuscularly five times with about 100 µg of

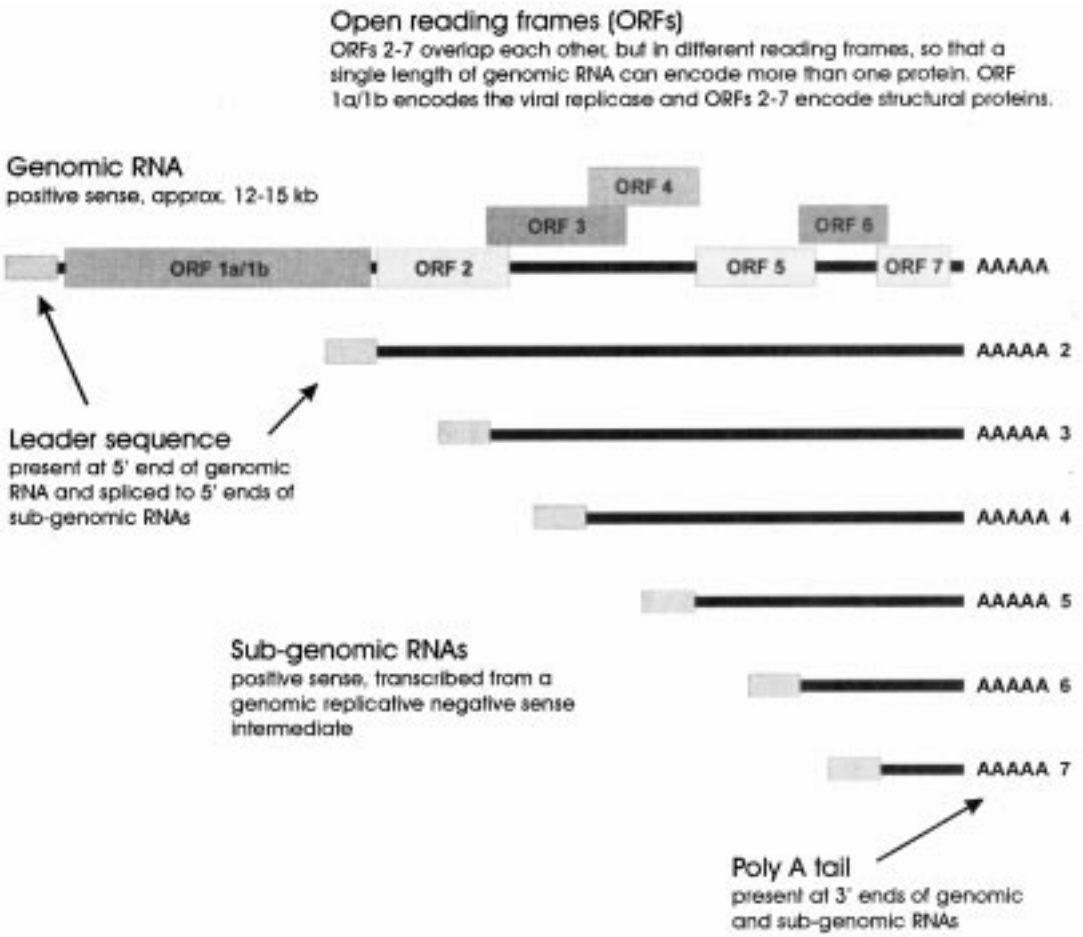


Fig. 1. Schematic diagram of the genomic organization and transcriptional strategy of the family *Arteriviridae*.

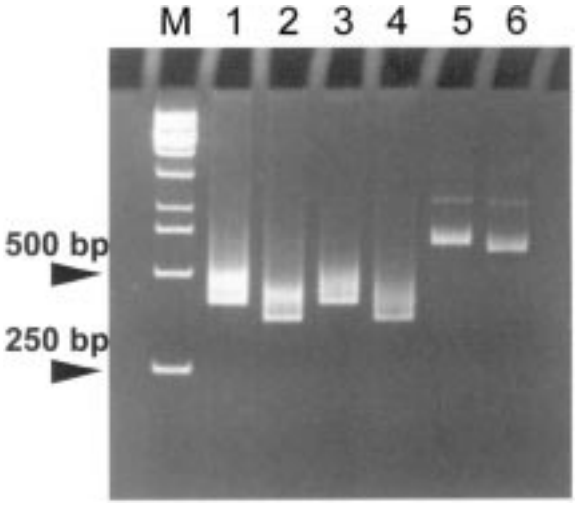


Fig. 2. Microphotograph of a ethidium bromide-stained agarose gel (1.5%) electrophoresis in which the specificity of the viral mRNA was determined by RT-PCR. The viral ORF 7 (lanes 1 to 4) and ORF 5 (lanes 5 and 6) encoding viral nucleocapsid and large envelope glycoprotein, respectively. In lanes 1 and 2 purified virion RNA was used while in the other lanes (3 to 6) total infected cell RNA served as an RT-PCR template. Bands represent specific amplification products of ORF 7 that was amplified by RT-PCR using two different primer sets as shown in lanes 1 and 3 (primers P-EAV-N-F1 and P-ORF7-R1, see note of Table 1) and lanes 2 and 4 (primers P-EAV-N-F2 and P-ORF7-R1, see note of Table 1). Lanes 5 and 6 represent the RT-PCR products of the cDNA of EAV OFR 5 mRNA obtained using two different primer sets (lane 5: primers P-EAV-5-F1 and P-ORF5-R1; lane 6: primers P-EAV-5-F2 and P-ORF5-R1; see note of Table 1). M represents a molecular weight marker (sizes of selected bands are shown on the left side of the panel).

the particular DNA in 14-day intervals. Analysis of the individual mouse sera revealed that the administered DNAs of the vectors expressing both viral gene products are able to induce significant immune response in mice as tested by neutralization test (NT). The results of these studies are summarized in Table 2 and are shown in Figs. 3 to 6 for ORFs 7 and 5, respectively. A significant immune response was detected when the animals were immunized with the recombinant plasmids pCR3.1-EAV-O7-BX-C3 and pCR3.1-EAV-O5-C14 harboring ORFs 7 and 5 coding for viral nucleocapsid protein and large envelope glycoprotein (G_L), respectively. As shown in Table 2 and Fig. 3 it was found that the recombinant plasmids pCR3.1-EAV-O7-BX-C3 are able to raise antibodies against EAV in mice (80% immune response) as detected by neutralization test. Similar results (50–70% immune response) were obtained in two independent experiments when the animals were inoculated with the recombinant plasmids pCR3.1-EAV-O5-BX-C14 (Table 2 and Fig. 4a and b).

In the next step of this investigation the animals were administered with the DNAs of the recombinant plasmids pCR3.1-EAV-O7-BX-C3 and pCR3.1-EAV-O5-C14 simultaneously under the same conditions described above. The results of this study that is in agreement with the corresponding results obtained from the analysis of the gene products of the EAV ORFs 7 and 5 (Table 2 and Figs. 3 and 4) are summarized in Table 2 and shown in Fig. 5. These data indicate that the native DNA of recombinant plasmids pCR3.1-EAV-O7-BX-C3 (harboring ORF 7) and pCR3.1-EAV-O5-C14 (harboring ORF 5) is able to express the corresponding gene products (viral nucleocapsid protein and large envelope glycoprotein (G_L)) *in vivo*. The results of these studies unambiguously underline that the gene products of the EAV ORFs 7 and 5 are suitable candidates for development of a DNA vaccine protecting an EAV infection.

DNA Vaccination of Mice with Vector Construct Expressing EAV N-Terminal Hydrophilic Ectodomain of Large Envelope Glycoprotein (G_L)

Balasuriya and coworkers (14) found that the neutralization determinants of EAV are located on the gene product of ORF 5 (large envelope glycoprotein) within the N-terminal ectodomain (amino acid positions 1–121 (15)). Accordingly, a novel expression vector based on pcDNA3.1/His system (Invitrogen) was constructed. This vector is similar to pCR3.1, but allows the specific detection and purification of the expressed fusion proteins using N-terminal amino acid sequence tags. A recombinant plasmid was constructed and termed pC31-HIS-EAV-O5-del-121. The insert of this expression vector possesses the coding region of EAV ORF 5 corresponding to amino acids 1–121 of the viral large envelope glycoprotein, which was inserted into the corresponding sites of the parental vector pcDNA3.1-HIS-C. Balb/c mice were inoculated with recombinant plasmid pC31-HIS-EAV-O5-del-121 under the conditions described above. The sera

Table 2. Summary of the results obtained by DNA immunization of mice using different gene products of equine arteritis virus

Experiment (No. of Animals)	Expression Vector (ORF/Gene product)	Average of NT-AB[a] Titer Minimum/Maximum		% of Immun-Response[b]
		PIS	PVS	
MV-00-01(10) 07/27/1998 to 10/19/1998	pCR3.1	< 1 : 10	< 1 : 10	0
MV-O7-01(10) 07/27/1998 to 10/19/1998	**pCR3.1-EAV-O7-BX-C3[c] (ORF 7/nucleocapsid protein = NP)**	**< 1 : 10/1 : 10**	**1 : 20/1 : 640**	**80**
MV-O5-01(10) 08/29/1998 to 11/11/1998	**pCR3.1-EAV-O5-BX-C14[c] (ORF 5/large envelope glycoprotein = G_L)**	**< 1 : 10**	**1 : 10/1 : 160**	**70**
MV-O5-02(10) 12/07/1998 to 03/20/1999	**pCR3.1-EAV-O5-BX-C14[c] (ORF 5/ G_L)**	**< 1 : 10**	**1 : 10/1 : 160**	**50**
MV-57-01(10) 09/11/1998 to 11/23/1999	**pCR3.1-EAV-O5-BX-C14[c] (ORF 5/G_L) pCR3.1-EAV-O7-BX-C3[c] (ORF 7/NP)**	**< 1 : 10**	**1 : 10/1 : 80**	**30/70[d]**
MV-O3-01(10) 12/21/1998 to 03/22/1999	pCR3.1-EAV-O3-BX-C1[c] (ORF 3/?)	< 1 : 10	< 1 : 10/1 : 10	0
MV-O4-01(10) 12/22/1998 to 03/22/1999	pCR3.1-EAV-O4-BX-C3[c] (ORF 4/?)	< 1 : 10	< 1 : 10/1 : 20	0
MV-O5d-01(10) 12/29/1998 to 03/22/1999	**pC3.1-HIS-EAV-O5-del-121 (amino terminus of large envelope glycoprotein; the first 121 amino acids)**	**< 1 : 10**	**1 : 20/1 : 160**	**90/100[d]**

[a]Neutralizing antibodies.
[b]Neutralizing titer 1 : 20 excluded.
[c]The C3, C14, etc. indicate the clone number used.
[d]Neutralizing titer 1 : 20 included.
PIS: Preimmune serum; PVS: Post vaccination serum.
? Protein of unknown function.

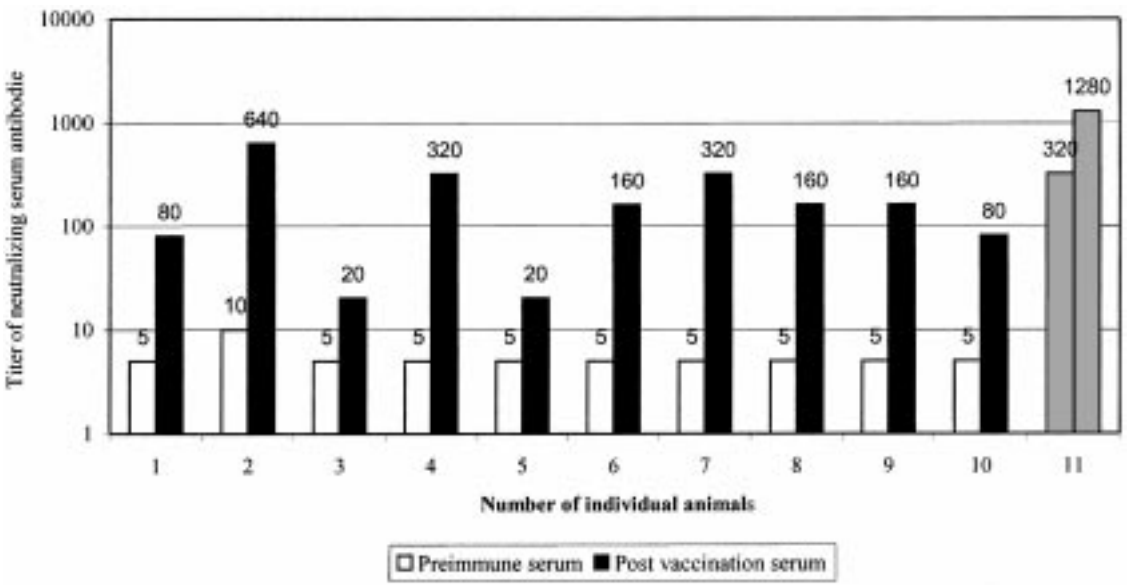
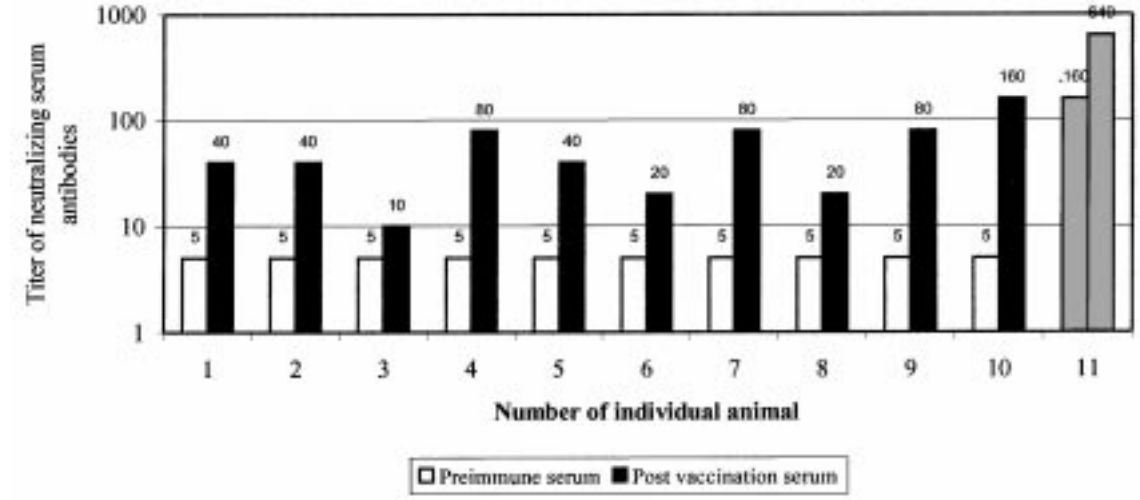
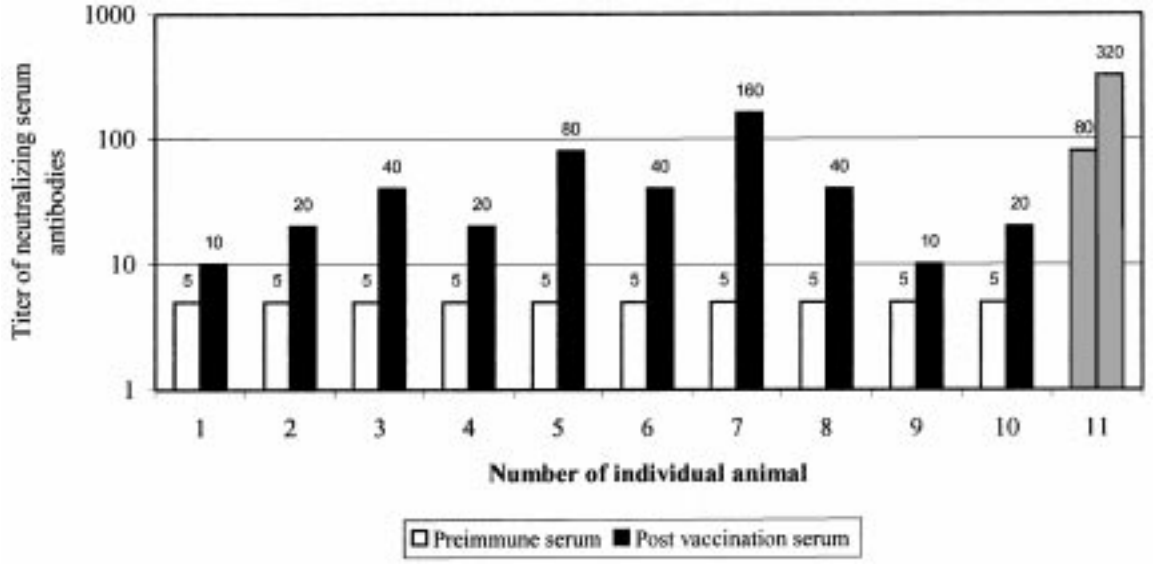


Fig. 3. The results of neutralization test obtained by the analysis of the sera of the individual Balb/c mice that were inoculated with the DNA of recombinant plasmid pCR3.1-EAV-O7-BX-C3 harboring and expressing ORF 7 of equine arteritis virus (EAV). The individual animals are indicated with number 1 to 10. The white and black columns represent the data of pre and post DNA vaccinated animals, respectively. The column 11 (gray color) served as internal positive control and indicates the average of maximum and minimum neutralizing titer obtained from the serum of a New Zealand white rabbit that was immunized with inactivated EAV.



(a)



(b)

Fig. 4. The results of neutralization tests obtained by the analysis of the sera of the individual Balb/c mice that were inoculated in two independent experiments (a and b) with the DNA of recombinant plasmid pCR3.1-EAV-O5-BX-C14 harboring and expressing ORF 5 of equine arteritis virus (EAV). The individual animals are indicated with number 1 to 10. The white and black columns represent the data of pre and post DNA vaccinated animals, respectively. The column 11 (gray color) served as internal positive control and indicates the average of maximum and minimum neutralizing titer obtained from the serum of a New Zealand white rabbit that was immunized with inactivated EAV.

obtained from the Balb/c mice (pre and post DNA vaccination) were analyzed using neutralization test. As shown in Table 2 and Fig. 6 it was found that the majority of the DNA vaccinated animals (90%) developed a significant neutralizing antibody against EAV.

DNA Vaccination of Mice With Vector Construct Expressing Viral ORFs 3 and 4

Although the functional activity of the gene products of two EAV ORFs 3 and 4 is not known, the eventual activity of these viral proteins *in vivo* was investi-

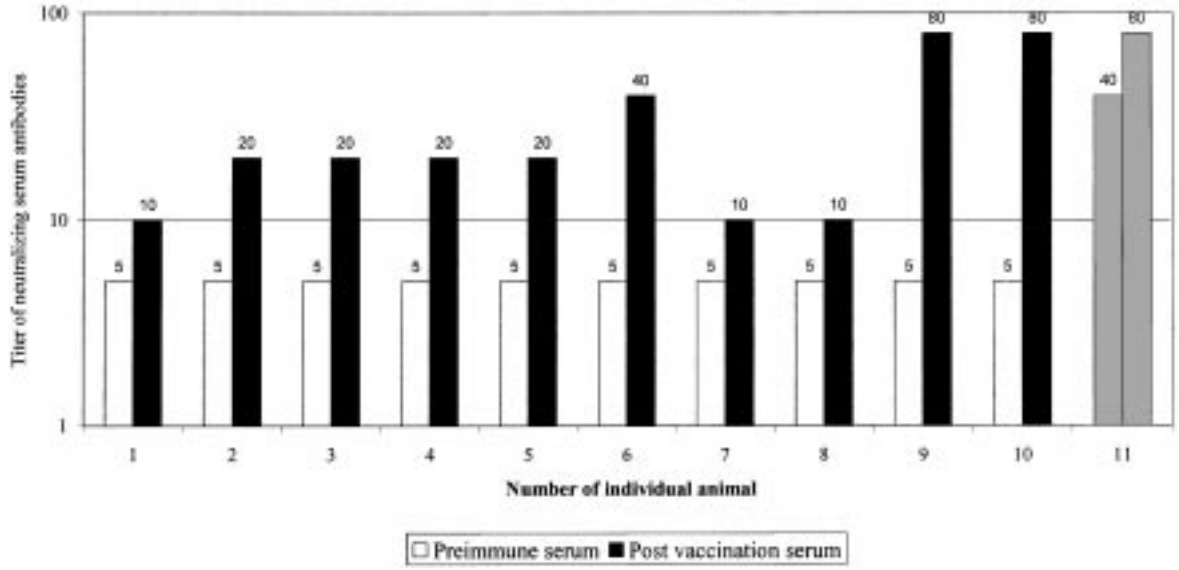


Fig. 5. The results of neutralization test obtained by the analysis of the sera of the individual Balb/c mice that were inoculated with the DNA of recombinant plasmids pCR3.1-EAV-O5-BX-C14 and pCR3.1-EAV-O7-BX-C3 harboring and expressing ORFs 5 and 7 of equine arteritis virus (EAV). The individual animals are indicated with number 1 to 10. The white and black columns represent the data of pre and post DNA vaccinated animals, respectively. The column 11 (gray color) served as internal positive control and indicates the average of maximum and minimum neutralizing titer obtained from the serum of a New Zealand white rabbit that was immunized with inactivated EAV.

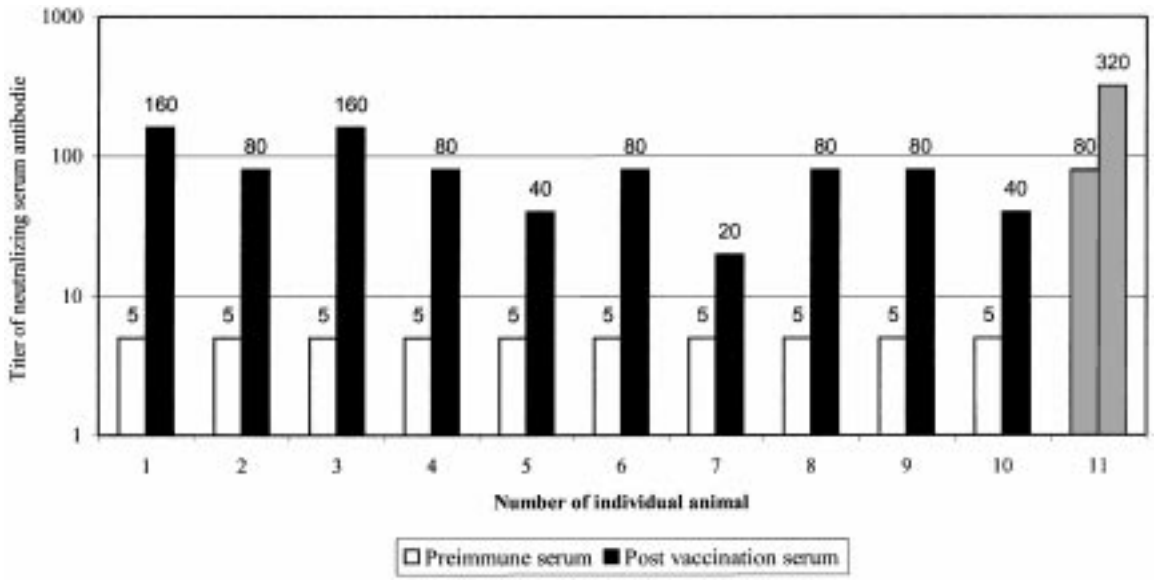


Fig. 6. The results of neutralization tests obtained by the analysis of the sera of the individual Balb/c mice that were inoculated with the DNA of recombinant plasmid pCR31-EAV-O5-del-121 harboring and expressing the N-terminal hydrophilic ectodomain of G_L envelope glycoprotein (amino acid residue 1–121 of ORF 5) of equine arteritis virus (EAV). The individual animals are indicated with number 1 to 10. The white and black columns represent the data of pre and post DNA vaccinated animals, respectively. The column 11 (gray color) served as internal positive control and indicates the average of maximum and minimum neutralizing titer obtained from the serum of a New Zealand white rabbit that was immunized with inactivated EAV.

gated. Two recombinant plasmids pCR3.1-EAV-O3-BX-C1 and pCR3.1-EAV-O4-BX-C3 were constructed (Table 1) in which the cDNA of the EAV ORFs 3 and 4 were inserted into the corresponding site of the expression vector pCR3.1. The sera obtained from the Balb/c mice (pre- and post DNA-vaccination) that were inoculated with these recombinant plasmids were analyzed using neutralization test. The results of these studies are summarized in Table 2. No neutralizing antibodies were detected in the immunized Balb/c mice under the conditions used.

Discussion

EAV causes economically important infectious diseases in horse farms worldwide. The development of an efficient vaccine against equine arteritis virus (EAV) is of particular importance, since it focuses attention on the prevention of the equine arteritis. The infection is maintained in horse population, because chronic carrier animals shed EAV in their semen and transmit it venereally to mares during the mating season. The objective of our study was to proof the possibility of the development of a model system for prevention of horses against an EAV infection by cDNA vaccination.

The ability of these recombinant plasmids expressing the gene products of the individual viral ORFs in induction of an immune response in mouse system was investigated. The pre- and post vaccinated sera of the individual animals were screened by neutralization tests (NT). These analyses revealed that the EAV ORFs 5, and 7 were able to induce neutralizing antibodies against EAV. This is in agreement with the observation of Pirzadeh and Dea (4) reporting that an immune response was demonstrated in mice and pigs that were vaccinated with plasmid DNA encoding ORF 5 of PRRSV harboring a viral glycoprotein associated with neutralizing epitopes. Highest NT-titers were observed when the animals were administered with the cDNA of ORF 5 and/or with the cDNA of the neutralization determinants of EAV that is located on the N-terminal ectodomain of the gene product of ORF 5 between the amino acid positions 1–121. This is in agreement with the fact that the neutralizing determinants of EAV are located on the gene product of ORF 5 (large envelope glycoprotein) within the N-terminal ectodomain (amino acid positions 1–121) as reported by (15).

As far as the protection of the equine arteritis virus in general is concerned, it is known that the persistently infected carrier stallion is the critical natural reservoir of equine arteritis virus. However, the real risk of importing frozen chilled semen contaminated with the equine arteritis virus is a serious factor by distribution and transmission of this veterinary important viral infection (16). Consequently the genetic screening of the equine biological material including frozen semen by RT-PCR for detection of an eventual EAV infection is strongly recommended.

The results of the experiments allow the conclusion that an EAV-specific neutralizing immune response can be induced in Balb/c mice after administration of DNA vector constructs harboring and expressing viral cDNA of EAV ORFs 5 and 7. However, further studies in the autologous animal system horse are required to assess the real protective potential of the DNA vaccination in the natural host.

Recently (17) reported that the ORF la encodes the replicative subunit that is involved in membrane association of the arterivirus replication complex. EAV ORF la encodes hydrophobic domains that seem to be involved in the membrane association of the viral replication complex. This replicase subunit contains domains that seem to be participated as integral membrane protein and to be membrane-associated in infected cells (17). Pedersen et al. (18) reported that the EAV ORF la replicase induces endoplasmic reticulum-derived double-membrane vesicles, which carry the viral replication complex. The formation of a membrane-bound scaffold for the replication complex seems to be a distinct step in the arterivirus life cycle (18).

The ORF lb encodes part of the EAV replicase. Proteolytic processing of the gene product of the ORF lb is mediated by nsp4 serine protease and plays an essential role for virus replication (19). An analysis of EAV mutants showed that the selective blockage of ORF lb processing affected different stages of the viral reproduction (19). Recently Snijder and co-workers (20) reported about transcriptional activity of a short genomic region that is located between the replicase gene and ORF 2 of the equine arteritis virus genome. These investigators reported that this particular region of the viral genome that previously has been assumed to be untranslated contains 5′ part of a novel gene (ORF 2a) that is conserved in all

arteriviruses. The 3′ part of EAV ORF 2a overlap with the 5′ part of the former ORF 2 (20).

Based on this knowledge and the recent analysis concerning the genetic diversity of the equine arteritis virus (21,22), the construction of a new generation of DNA vaccine against EAV infection must be seriously considered e.g. the expression of immunogenic proteins in plant is a suitable and future-aided way for development and application of the vaccine against infectious diseases.

Acknowledgment

The general support and critical comments of D.W. Janott and A. Donetti is gratefully acknowledged.

References

1. den Boon J.A., Snijder E.J., Chirnside E.D., de Vries A.A., Horzinek M.C., and Spaan W.J., J Virol *65*, 2910–2920, 1991.
2. Balasuriya U.B.R., Hedges J.F., Nadler S.A., McCollum W.H., Timoney P.J., and MacLachlan N.J., J Gen Virol *80*, 1949–1958, 1999.
3. Patton J.F., Balasuriya U.B.R., Hedges J.F., Schweidler T.M., Hullinger P.J., and MacLachlan N.J., Arch Virol *144*, 817–827, 1999.
4. Pirzadeh B. and Dea S., J Gen Virol *79*, 989–999, 1998.
5. Giese M., Virus Genes *17*, 219–232, 1998.
6. Glišin V., Crkvenjakov R., and Byus C., Biochemistry *13*, 2633–2637, 1974.
7. Rösen-Wolff A. and Darai G., Virus Res *19*, 115–126, 1991.
8. Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F., and Higgins D.G., Nucleic Acids Res *25*, 4876–4882, 1997.
9. Rösen-Wolff A., Ben-Hur T., Becker Y., and Darai G., Virus Res *10*, 315–324, 1988.
10. Rösen-Wolff A., Scholz J., and Darai G., Virus Res *12*, 43–52, 1989.
11. Bradford M., Anal Biochem *72*, 248–254, 1976.
12. Laemmli U.K., Nature *227*, 680–685, 1970.
13. Welzel T.M., Kehm R., Tidona C.A., Muranyi W., and Darai G., Virus Genes *17*, 185–198, 1998.
14. Balasuriya U.B.R., MacLachlan N.J., DeVries A.A.F., Rossitto P.V., and Rottier P.J.M., Virology *207*, 518–527, 1995.
15. Balasuriya U.B., Patton J.F., Rossitto P.V., Timoney P.J., McCollum W.H., and MacLachlan N.J., Virology *232*, 114–128, 1997.
16. Pycock J.F., The Veterinary Record *26*, 699, 1998.
17. van der Meer Y., van Tol H., Locker J.K., and Snijder E., Journal of Virology *72*, 6689–6698, 1998.
18. Pedersen K.W., van der Meer Y., Roos N., and Snijder E., J Virol *73*, 2016–2026, 1999.
19. van Dinten L.C., Rensen S., Gorbalenya A.E., and Snijder E.J., J Virol *73*, 2027–2037, 1999.
20. Snijder E., van Tol H., Pedersen K.W., Raamsman M.J.B., and De Vries A.A.F., iruses J Virol *73*, 6335–6345, 1999.
21. Stadejek T., Björklund H., Ros Bascunana C., Ciabatti I.M., Scicluna M.T., Amaddeo D., McCollum W.H., Autorino G.L., Timoney P.J., Paton D.J., Klingeborn B., and Belak S., J Gen Virol *80*, 691–699, 1999.
22. Hedges J.F., Balasuriya U.B.R., Timoney P.J., McCollum W.H., and McLachlan N. J., J Virol *73*, 3672–3681, 1999.