Vaccination of cattle with a DNA plasmid encoding the bovine viral diarrhoea virus major glycoprotein E2

Serge Harpin,1 David J. Hurley,1, 4 Majambu Mbikay,3 Brian Talbot2 and Youssef Elazhary1

1 Faculté de Médecine Vétérinaire, Université de Montréal, Département de Pathologie et Microbiologie, Section Virologie, C.P. 5000, St-Hyacinthe, Québec, Canada, J2S 7C6
2 Département de Biologie, Faculté des Sciences, Université de Sherbrooke, Québec, Canada, J1K 2R1
3 Institut de Recherches Cliniques de Montréal, Université de Montréal, Montréal, Canada
4 Department of Veterinary Science and Biology/Microbiology, College of Agriculture and Biological Sciences, South Dakota State University, USA

Bovine viral diarrhoea virus (BVDV) is an economically important pathogen of cattle that is ubiquitously distributed worldwide. In this study, cattle were immunized by intramuscular injections with plasmid DNA expressing the BVDV type 1 major glycoprotein E2. Animals either received injections of naked DNA (N-DNA) or DNA in cationic liposomes (L-DNA). Both DNA preparations induced virus-specific neutralizing antibodies in vaccinates, although the response was much lower in N-DNA-immunized animals. N-DNA-vaccinated animals also showed virus-specific lymphocyte proliferation responses to type 1, live BVDV in vitro, whereas L-DNA vaccination induced no such responses. After 16 weeks, DNA-vaccinated and mock-vaccinated animals were challenged with a USDA-certified BVDV type 1 strain. Four significant observations were made: (1) N-DNA-vaccinated calves showed limited protection from virus challenge, (2) L-DNA-vaccinated animals did not show any signs of protection, (3) the challenge induced strong memory responses in the production of serum neutralizing antibodies to both genotypes (type 1 and 2 of BVDV), and (4) the challenge induced a mucosal memory response in nasal secretions of both L- and N-DNA-vaccinated animals.

Introduction

Bovine viral diarrhoea virus (BVDV) is an enveloped single-stranded RNA virus classified as a member of the genus pestivirus within the Flaviviridae family (Horzinek, 1991). The BVDV genome is approximately 12.5 kb in length and contains one large open reading frame which encodes a hypothetical polyprotein of approximately 450 kDa, which is proposed to be processed co- and post-translationally by either host or viral proteases (Collett et al., 1988). Processing of the standard BVDV polyprotein results in at least 12 individual proteins, three of which are structural glycoproteins. Antibodies to the BVDV major glycoprotein E2 (formerly known as gp53) have been shown to possess virus-neutralizing activity (Donis et al., 1988). E2 subunit vaccines have been reported to induce protection in immunized animals (Bolin & Ridpath, 1996; Bruschke et al., 1997). BVDV is an important pathogen of cattle, causing severe and even fatal infections, which result in significant losses to cattle production worldwide. Two distinct genotypes of BVDV exist. One is the classical BVDV strain termed type 1 and the second, known as type 2, was recently found to be involved in atypical outbreaks of severe acute BVDV in Canada and the USA (Pellerin et al., 1994; Ridpath et al., 1994; Harpin et al., 1995).

Commercially available inactivated and modified-live BVDV (MLV) vaccines have been extensively used for more than 30 years, but since their introduction the problem of BVDV-related infections appears to have become worse instead of better (Bolin, 1995). Both MLV and inactivated BVDV vaccines have significant shortcomings. MLV vaccines contain a limited antigen mass and require the opportunity to replicate in the host to establish significant immune activity. They are also susceptible to interference from maternal antibody in the system (Schulz, 1993). Furthermore, MLV viruses are a potential source of in utero infections and/or
immunosuppression (Liess et al., 1984; Roth & Kaeberle, 1983). MLV vaccines are produced in bovine cell cultures and thus have the potential to introduce other biological agents into herds as contaminants. Even if they provide a large preformed antigen mass, inactivated viruses are quite expensive to produce and susceptible to loss of important immunogenic activities during the process of inactivation.

The use of cloned viral genes holds great promise for the development of new vaccines to control BVDV. DNA vaccination can serve as an alternative to conventional immunization with MLV or inactivated vaccines to induce protection (Robinson et al., 1993; Sedegah et al., 1994; Xiang et al., 1994). Direct injection of plasmid DNA into animals offers several advantages over classical vaccine preparations and virus vectors for vaccination. Simple, rapid and inexpensive production of plasmid DNA, thermal stability of the plasmid product, and the potential for a long shelf-life of stabilized plasmid DNA are characteristics that make genetic vaccination very attractive for the next generation of vaccines against BVDV. This technology is insensitive to preformed antibody (Siegrist et al., 1997) and DNA vaccines are capable of inducing both humoral and cellular immunity by providing access to newly synthesized antigen in both the MHC class I- and class II-restricted pathways (Donnelly et al., 1997; Hasset & Whitton, 1996). Most DNA vaccination studies have been carried out and designed in mice to determine the efficiency of DNA immunization. Our initial studies of DNA immunization with the E2 gene of BVDV in mice demonstrated the potential of the method (Harpin et al., 1997).

In this report, we examined DNA vaccination for BVDV in cattle, the natural host. Vaccination was carried out by injecting calves with DNA encoding the BVDV major glycoprotein E2 either as naked plasmid dissolved in saline (N-DNA) or as plasmid entrapped in cationic liposome (L-DNA). The virus-specific neutralizing antibody and the virus-specific lymphocyte proliferation responses were studied. We also evaluated the response to challenge with the NY-1 strain of type 1 BVDV (produced by the NVSL and USDA-certified as a vaccine challenge strain).

Methods

- **Cells and virus.** Madin–Darby bovine kidney (MDBK) cells free of BVDV were grown in Earle’s minimum essential medium (MEM, Gibco BRL), supplemented with gentamicin (50 µg/ml, Sigma) and 10% donor horse serum (Gibco BRL). For this study, BVDV type 1 strain Singer, and type 2 strain 125 were obtained from the National Veterinary Service Laboratory of the United States Department of Agriculture (NVSL–USDA, Ames, Iowa, USA). The type 1 challenge virus (NVSL–USDA BVDV/NY-1 lot #97-12) was prepared by the USDA Animal and Plant Health Inspection Service, Center for Veterinary Biologics, in Ames, IA, USA. Virus was stored at −80 °C until use.

- **Plasmid DNA expression vector.** Cloning and expression of the BVDV E2 glycoprotein from a cytopathic type 1 strain (BVDV/NADL) yielding pcDNA/gp53 has been previously described (Harpin et al., 1997). The new gp53 expression vector used in this study and designated as pCMVigp53 (where i stands for intron) was constructed by replacing the region containing the cytomegalovirus (CMV) promoter in the pcDNA/gp53 vector with a BgIII–HindIII fragment containing the CMV promoter and an intron from the pCl-neo plasmid (Promega). Plasmid pCMVintron was used as plasmid control. All plasmids used in this study were prepared according to the manufacturer’s directions using the Qiagen DNA purification kit. For use in vaccination, the DNA was dissolved in PBS at a final concentration of 1 mg/ml. The DNA aliquots were stored at −20 °C until the time of injection.

- **Entrapment of plasmid DNA into liposomes.** Liposomes were prepared as unilamellar vesicles containing the cationic lipid DOTAP as DOTAP/DOPE (1:1 molar ratio). DOTAP is N-[1-(2,3-dioleoyloxy)-propyl]-N,N,N-trimethylammonium chloride (Avanti Polar Lipids), and DOPE is the neutral lipid dioleoyl phosphatidylethanolamine (Avanti Polar Lipids). Briefly, a film made of an equimolar mixture of DOTAP and DOPE was first prepared by the standard hydration and re-hydration method (Bangham et al., 1965). The film was re-hydrated in PBS and sonicated for 30 s in a bath sonicator to form a clear liposomal suspension. Liposomes were stored at 4 °C until use. One day before the time of vaccination, a DNA:liposome ratio of 1:10 (w/w) was prepared as previously reported (Felgner et al., 1987). For use in vaccination, the DNA–liposome formulation was kept at 4 °C at a final concentration of 0.5 mg of plasmid DNA/ml. A total of 2 ml of the mixture (1 mg DNA) was injected per calf.

- **DNA immunization and challenge.** BVDV- and BVDV-antibody-negative cattle, 7 to 9 months of age, were used in this study. The animals were housed in an isolated barn and handled under strict precautions to prevent the introduction of BVDV. Calves were intramuscularly immunized in the thigh muscle. Three calves received 1 mg of pCMVigp53 in a liposome preparation (L-DNA), three calves received 1 mg of pCMVigp53 as naked DNA (N-DNA) and two calves received 1 mg of control plasmid pCMVintron. For the challenge study, two calves were added to serve as unvaccinated and unchallenged controls. Those two calves were housed separately from the challenged animals in order to prevent horizontal transmission of the virus. Booster injections were given at weeks 3 and 6. Blood samples were drawn from each calf every 2 weeks. On week 16 after vaccination, the animals were challenged intranasally with 1:2 ml of 10^7 TCID50/ml of BVDV/NY-1. A volume of 0.6 ml per nare was delivered dropwise to each DNA-vaccinated and mock-vaccinated animal. Clinical signs were observed daily from 3 days prior to challenge to day 14 post-challenge. During the challenge period, blood samples for antibody and lymphocyte proliferation assays were collected on days 3, 0, 2, 4, 6, 8, 10, 14 and 21. Nasal secretions were collected at weeks 0, 3, 5 and 8 during the vaccination phase and on day 21 post-challenge.

- **Virus neutralization assay.** The virus neutralization assays were conducted using heat-inactivated (30 min at 56 °C) samples (serum, nasal secretion). Two hundred TCID50 of BVDV (Singer or 125 strains) was pre-incubated with twofold dilutions of the heat-inactivated sample for 1 h at 37 °C. This mixture (50 µl) was then added to duplicate wells of microtitre plates containing 80–90% confluent MDBK cells for 1 h at 37 °C. MEM (150 µl) was added to each well. The plates were incubated at 37 °C for 4 days. The reciprocal of the highest dilution that completely inhibited virus cytopathic effect in the two test wells was reported as the virus neutralization titre.

- **Proliferative response of bovine mononuclear cells.** Bovine leukocytes were enriched by centrifugation of peripheral blood to collect the buffy coat. The mononuclear (MN) cells were purified from the buffy coat by flotation on Histopaque 1.083 (Sigma), and suspended at a
Serum neutralizing antibodies in immunized calves

To evaluate the usefulness of a DNA vaccine in the natural host of BVDV, two groups of three calves each were immunized with 1 mg of pCMVigp53 in a liposome preparation or as naked DNA. This was carried out on three occasions, 3 weeks apart. As negative controls, two calves were immunized with control plasmid pCMVintron in the same way. Table 1 summarizes the development of serum neutralizing antibodies during this study. We observed that neutralizing antibodies first appeared at week 8 for some animals immunized with the plasmid DNA. All calves injected with L-DNA seroconverted at week 8. However, only one calf in the N-DNA group had a significant titre during the whole vaccination period. No neutralizing antibody (SN titre < 4) was detected in the animals receiving mock plasmid.

Table 1 also indicates clear evidence of a memory response in the production of serum neutralizing antibodies to BVDV after challenge. This occurred in animals vaccinated with plasmid pCMVigp53, both N-DNA and L-DNA. There was a significant difference in the neutralizing antibody responses after challenge between each of the DNA-vaccinated groups and the mock-vaccinated animals (P < 0.01). The serum neutralizing titres of DNA-vaccinated animals showed a marked rise 1 week after challenge. At week 3 post-challenge, titres of DNA-vaccinated animals were approximately 128-fold higher than those of mock-vaccinates. However, no significant differences in serum neutralization titres were observed between N-DNA-vaccinated and L-DNA-vaccinated animals (P > 0.05). The two unvaccinated, unchallenged control animals remained seronegative (SN titre < 4) during the challenge trial.

Characterization of the cellular immune response

Bovine MN cells were isolated and stimulated in vitro with live type 1 (Singer strain) or type 2 (strain 125) BVDV. Control wells were incubated with cells alone. The baseline response
was established by using the average SI of the mock-vaccinated animals. Bovine MN cells from two N-DNA-vaccinated calves (1 and 3) exhibited an increase in proliferation following stimulation with the Singer strain compared to MN cells from mock-vaccinated calves at week 4 after vaccination (Fig. 1). This response slowly decreased during the remainder of the vaccination period. However, L-DNA-vaccinated calves did not show any measurable increase in MN cell response to the Singer strain of BVDV (Fig. 2). No proliferative response was observed after stimulation with BVDV type 2 in any of the DNA-vaccinated calves (data not shown).

At week 16, after the introduction of the challenge virus, the level of cell proliferation dropped during the first week of challenge for all challenged animals, and then increased until it returned to approximately the pre-challenge baseline. No significant differences in the pattern or magnitude of the response were seen among the groups of DNA- or mock-vaccinated animals after challenge.

### Nasal secretion neutralizing antibodies to BVDV after challenge

There was no evidence of neutralizing antibodies to BVDV in nasal secretions from any of the animals during the vaccination period. Nevertheless, during the challenge period there was a significant seroneutralizing mucosal response in animals vaccinated with plasmid DNA pCMVigp53 (Table 2) as compared to the mock-vaccinated animals. Only DNA-vaccinated animals showed evidence of neutralizing antibody in nasal secretion after challenge. No mucosal responses (SN titre < 4) were observed in the 3 weeks following challenge in mock-vaccinated animals or in unvaccinated, unchallenged control animals.

### Immune responses to heterologous virus

To evaluate heterotypic immune responses induced by DNA vaccination against type 1 virus, we measured the

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**Table 2. Neutralizing antibodies to BVDV/Singer and BVDV/125 in nasal secretions after challenge**

Calves were challenged intranasally with 1–2 ml of 10^7 TCID_50/2 ml of BVDV/NY-1 and nasal secretions were collected on day 21 post-challenge.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>SN titre†</th>
<th>BVDV/Singer</th>
<th>BVDV/125</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-DNA-1</td>
<td>512</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>L-DNA-2</td>
<td>128</td>
<td>&lt; 4</td>
<td></td>
</tr>
<tr>
<td>L-DNA-3</td>
<td>512</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>N-DNA-1</td>
<td>512</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>N-DNA-2</td>
<td>128</td>
<td>&lt; 4</td>
<td></td>
</tr>
<tr>
<td>N-DNA-3</td>
<td>512</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Mock-1</td>
<td>&lt; 4</td>
<td>&lt; 4</td>
<td></td>
</tr>
<tr>
<td>Mock-2</td>
<td>&lt; 4</td>
<td>&lt; 4</td>
<td></td>
</tr>
<tr>
<td>Mock-U</td>
<td>&lt; 4</td>
<td>&lt; 4</td>
<td></td>
</tr>
</tbody>
</table>

* Three calves received 1 mg of pCMVigp53 in a liposome preparation (L-DNA); three calves received 1 mg of pCMVigp53 as naked DNA (N-DNA); two calves received 1 mg of control plasmid pCMVintron (mock-1 and mock-2) and two calves were unvaccinated and unchallenged control animals (mock-U).

† Virus neutralization (SN) titre was expressed as the reciprocal of the highest dilution that completely inhibited virus cytopathic effect in the two test-well cultures. A factor of 2 between results is considered to be significant.
Partial protection from BVDV DNA vaccination

Table 3. Development of serum neutralizing antibodies to BVDV/125 from DNA-vaccinated calves after challenge

Calves were challenged intranasally with 1-2 ml of $10^{7.9}$ TCID$_{50}$/2 ml of BVDV/NY-1.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Weeks post-challenge</th>
<th>SN titre†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>L-DNA-1</td>
<td>&lt; 4</td>
<td>16</td>
</tr>
<tr>
<td>L-DNA-2</td>
<td>&lt; 4</td>
<td>&lt; 4</td>
</tr>
<tr>
<td>L-DNA-3</td>
<td>&lt; 4</td>
<td>&lt; 4</td>
</tr>
<tr>
<td>N-DNA-1</td>
<td>&lt; 4</td>
<td>&lt; 4</td>
</tr>
<tr>
<td>N-DNA-2</td>
<td>&lt; 4</td>
<td>&lt; 4</td>
</tr>
<tr>
<td>N-DNA-3</td>
<td>&lt; 4</td>
<td>32</td>
</tr>
<tr>
<td>Mock-1</td>
<td>&lt; 4</td>
<td>&lt; 4</td>
</tr>
<tr>
<td>Mock-2</td>
<td>&lt; 4</td>
<td>&lt; 4</td>
</tr>
<tr>
<td>Mock-U</td>
<td>&lt; 4</td>
<td>&lt; 4</td>
</tr>
</tbody>
</table>

* Three calves received 1 mg of pCMVg53p in a liposome preparation (L-DNA); three calves received 1 mg of pCMVg53 as naked DNA (N-DNA); two calves received 1 mg of control plasmid pCMVintron (mock) and two calves were unvaccinated and unchallenged control animals (mock-U).
† Virus neutralization (SN) titre was expressed as the reciprocal of the highest dilution that completely inhibited virus cytopathic effect in the two test-well cultures. A factor of 2 between results is considered to be significant.

 responses to a cytopathic type 2 strain (BVDV/125). There was no evidence of serum neutralizing antibodies (SN titre $< 4$) to type 2 strain 125 in any of the animals during the vaccination period. However, after challenge, memory responses were observed by the production of neutralizing antibodies to the type 2 virus. Beginning with the measurements at 14 days after challenge, all DNA-vaccinated animals showed a marked increase in serum neutralizing antibody titre (Table 3). The titres of both N-DNA and L-DNA vaccinates increased from undetectable at the time of challenge to peak at 3 weeks post-challenge. However, the titres were at least 32-fold lower than the titres obtained with the homologous virus. Mock-vaccinated and unvaccinated, unchallenged control animals remained negative (SN titre $< 4$) for BVDV type 2 during the challenge period.

There was also evidence of nasal secretion neutralizing antibodies to type 2 strain 125 in DNA-vaccinated animals (Table 2). Only DNA-vaccinated animals showed evidence of neutralizing antibody in nasal secretion after challenge. Once again, the titres were considerably lower for the heterologous virus. No mucosal responses (SN titre $< 4$) were observed to the type 2 strain in mock-vaccinated animals or in unvaccinated, unchallenged control animals after the challenge.

Table 4. Clinical signs assessment

The clinical score represents the sum of all values associated with the assessment of clinical signs (i.e. respiration, nasal discharge, eyes, attitude, gastrointestinal, body temperature).

<table>
<thead>
<tr>
<th>Clinical sign</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiration</td>
<td></td>
</tr>
<tr>
<td>Severe dyspnoea</td>
<td>4</td>
</tr>
<tr>
<td>Mild dyspnoea</td>
<td>3</td>
</tr>
<tr>
<td>Short and rapid</td>
<td>2</td>
</tr>
<tr>
<td>Cough</td>
<td>1</td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Nasal discharge</td>
<td></td>
</tr>
<tr>
<td>Thick, crusted</td>
<td>4</td>
</tr>
<tr>
<td>Purulent</td>
<td>3</td>
</tr>
<tr>
<td>Muco-purulent</td>
<td>2</td>
</tr>
<tr>
<td>Serous</td>
<td>1</td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Eyes</td>
<td></td>
</tr>
<tr>
<td>Purulent</td>
<td>3</td>
</tr>
<tr>
<td>Serous</td>
<td>2</td>
</tr>
<tr>
<td>Watery</td>
<td>1</td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Attitude</td>
<td></td>
</tr>
<tr>
<td>Dead, comatose</td>
<td>4</td>
</tr>
<tr>
<td>Severe depression</td>
<td>3</td>
</tr>
<tr>
<td>Moderate depression</td>
<td>2</td>
</tr>
<tr>
<td>Mild depression</td>
<td>1</td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td></td>
</tr>
<tr>
<td>Bloody diarrhoea</td>
<td>4</td>
</tr>
<tr>
<td>Watery diarrhoea</td>
<td>3</td>
</tr>
<tr>
<td>Loose faeces</td>
<td>2</td>
</tr>
<tr>
<td>Pasty faeces</td>
<td>1</td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Body temperature (°C)</td>
<td></td>
</tr>
<tr>
<td>$&gt; 41$</td>
<td>4</td>
</tr>
<tr>
<td>40.5–40.9</td>
<td>3</td>
</tr>
<tr>
<td>40.0–40.4</td>
<td>2</td>
</tr>
<tr>
<td>39.5–39.9</td>
<td>1</td>
</tr>
<tr>
<td>$&lt; 39.5$</td>
<td>0</td>
</tr>
</tbody>
</table>

Evidence of protection in DNA-vaccinated calves

Following challenge with a BVDV type 1 virus (BVDV/NY-1), the animals were assessed for evidence of disease on the basis of a composite clinical score (Table 4). Due to the small number of animals in the experiment, the results were assessed per animal rather than as an average (Fig. 3). In mock-vaccinated animals, the symptoms occurred as two peaks at approximately day 4 and day 7. In N-DNA-vaccinated animals, the results show that calf N-DNA-3 had no signs of disease throughout the challenge period and appeared to be completely protected from disease. The remaining N-DNA-vaccinated animals showed reduced levels and duration of clinical signs compared with the controls, indicating partial...
Mock-1; animals were challenged intranasally with 1 according to Table 4. Clinical signs are plotted individually. BVDV/NY-1. Clinical signs were observed daily from 3 days prior to challenge to 14 days after challenge. Clinical scores were assigned to vaccinates were not protected. The small numbers (three) of animals preclude a quantitative evaluation of the protection observed. In the present work, we chose to use intramuscular injection because this technique is already widely used and well accepted by practitioners in the field. However, in an attempt to investigate the effect of changes in DNA presentation, plasmid DNA was used as naked DNA (N-DNA) or was entrapped in cationic liposomes (L-DNA) which have previously shown some promise in genetic immunization studies of small animals (Donnelly et al., 1997).

Overall, the results show that animals immunized with the plasmid pCMVgpp53, as either L-DNA or N-DNA, developed an immune response against BVDV. However, there were significant differences between the responses to N-DNA and L-DNA. N-DNA-immunized animals showed variable levels of protection from disease after challenge. In contrast, the L-DNA-vaccinates were not protected. The small numbers (three) of animals preclude a quantitative evaluation of the protection observed. These observations are surprising when pre-challenge seroneutralizing results are taken into account. Until recently, it has been assumed that BVDV protection in cattle is almost exclusively due to the production of seroneutralizing antibodies. However, the L-DNA-immunized animals in this study all produced seroneutralizing antibodies in the vaccination period. Yet these animals were not protected. This is in contrast with the work of Bolin & Ridpath (1996) which reported that a vaccine-induced neutralizing antibody titre > 2 protected calves from clinical signs of disease induced by homologous virus challenge exposure. A similar examination of the results for N-DNA-immunized animals shows that two of the three calves (N-DNA-1 and -2) produced only a low level of seroneutralizing response during the vaccination period and the third one (N-DNA-3) had titres equivalent to those of the L-DNA vaccinates. This latter observation correlates well with the level of protection observed since the titre of animals (N-DNA-3) was the one that appeared completely protected. The lack of correlation between L-DNA and N-DNA protection and antibody titres is in some way resolved when the cell proliferation results are included in the scheme. Once again the results vary between animals and between treatment groups yet several distinct conclusions can be drawn. In general, the N-DNA-immunized animals showed higher levels of cell proliferation compared to the unvaccinated animals. However, DNA vaccines against porcine viruses were shown to provide protection from disease in pigs (Pirzadeh & Dea, 1998; Gerds et al., 1997). In bovine species, DNA constructs encoding bovine herpesvirus-1 (BHV-1) glycoprotein D were shown to induce an immune response that partially protected cattle from respiratory disease (Cox et al., 1993). As with our study, this research group also investigated the effects of different routes of DNA immunization and reported that intradermal delivery was more effective than an intramuscular injection in generating a protective response against BHV-1 as monitored by a reduction in clinical signs of disease (van Drunken Littel-van den Hurk et al., 1998). In the present work, we chose to use intramuscular injection because this technique is already widely used and well accepted by practitioners in the field. However, in an attempt to investigate the effect of changes in DNA presentation, plasmid DNA was used as naked DNA (N-DNA) or was entrapped in cationic liposomes (L-DNA) which have previously shown some promise in genetic immunization studies of small animals (Donnelly et al., 1997).

Figure 3. Clinical scores from N-DNA-vaccinated calves after challenge. *Mock-1; ●, mock-2; □, N-DNA-3; ▽, N-DNA-2; △, N-DNA-1. The animals were challenged intranasally with 1:2 ml of 10**7.5 TCID50/ml of BVDV/NY-1. Clinical signs were observed daily from 3 days prior to challenge to 14 days after challenge. Clinical scores were assigned according to Table 4. Clinical signs are plotted individually.
animals whereas the L-DNA vaccines did not show this at all. The N-DNA animal (N-DNA-3) that demonstrated the highest cell proliferation results in addition to the high antibody titre also showed the highest level of protection to development of disease after virus exposure. All previous studies of E2 protection did not include lymphoproliferative studies. However, in a recent report, Cortese et al. (1998) determined the efficacy of an MLV type-I isolate of BVDV vaccine in protecting calves from infection with a virulent type-II isolate, and showed that induction of a cellular immune response correlated with protection. Our own work using an inactivated BVDV vaccine has since confirmed this (Y. Chofry, B.G. Talbot, D. Hurley & Y. Elazhary, unpublished observation).

Thus, it can be hypothesized in view of our results and from those of Cortese et al. (1998) that, for maximum protection, the vaccine must induce not only a seroneutralizing antibody but also a cellular response. It is not known whether the lymphoproliferative response to the whole virus depends on E2, although we have determined that another viral protein, NS3, expressed in an adenovirus vector does not induce such an effect in mice (Elahi et al., 1999).

The BVDV/E2 gene used in our DNA construct was cloned from the BVDV cytopathic type 1 NADL strain. In the field, several different genotypes of BVDV are circulating in the cattle population, thus we also measured the reactivity to a cytopathic type 2 strain (BVDV/125) to evaluate heterotypic immune responses induced by DNA vaccination. As demonstrated in Table 3, the challenge induced a strong memory response in the production of serum neutralizing antibodies (titres were 256-fold higher than mock vaccinates) to BVDV type 2. Our finding that a type 1 E2 gene will induce neutralizing antibody to type 2 virus is an interesting observation considering the high variability between the two E2 sequences. This is more understandable if one takes into account the fact that all E2-specific seroneutralizing antibodies obtained to date are configuration-specific. It is quite likely that despite sequence differences between the E2 of different strains, the configurations of some neutralizing epitopes are similar. An ideal BVDV vaccine should thus be able to induce antibodies capable of neutralizing a wide variety of virus genotypes in a heterotypic response if the antigen expresses an E2 consensus configuration.

BVDV infects cattle at mucosal surfaces, primarily the respiratory tract (Bolin, 1990). Thus, mucosal immunity is believed to play an important role in protection against BVDV infection. After virus challenge, we examined the development of BVDV-specific antibodies in nasal secretions. Three weeks after challenge, mucosal neutralizing antibodies were observed only in DNA-vaccinated animals. Significantly, these neutralizing antibodies were observed at similar titres after vaccination with both N-DNA and L-DNA. However, only N-DNA-vaccinated animals showed some level of protection. It should be noted that nasal virus-neutralizing antibodies do not normally appear in nasal secretions in a primary response until at least 4 weeks after infection. Thus, nasal secretion neutralizing antibodies are probably more important for secondary and memory responses. Their actual role in protection needs further investigation.

In summary, we have demonstrated that vaccination of cattle using a DNA construct encoding only the E2 protein of BVDV was able to induce both neutralizing antibody production and lymphocyte proliferation. It appeared that both humoral and cellular immune responses were implicated in the development of a protective response in cattle. This work shows the great potential of genetic vaccines for the control of BVDV.

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B.T. and Y.E. contributed equally to this work.

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