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Co-administration of IL-2 enhances antigen-specific immune responses following vaccination with DNA encoding the glycoprotein E2 of bovine viral diarrhoea virus

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Abstract

Induction of effective immunity requires the delivery of a protective antigen with appropriate co-stimulatory signals. For bovine viral diarrhoea virus (BVDV) this antigen is the major viral glycoprotein E2. Neutralising antibodies are directed towards the E2 protein and passive transfer of antibodies in serum or colostrum can completely protect against viral infection. DNA vaccination of mice with a construct encoding the E2 glycoprotein induced neutralising antibody levels that were potentially sufficient to prevent virus replication in a challenge system. The co-delivery of interleukin-2 (IL-2) further enhanced the levels of antibody raised. The strong IgG2a component of the antigen-specific antibody suggests a Th1 bias to the immune response induced following vaccination.

Keywords: BVDV; DNA vaccination; Cytokine

1. Introduction

Bovine viral diarrhoea virus (BVDV) infection is endemic throughout much of the world with up to 70% of individuals in susceptible cattle populations showing evidence of virus infection. The impact of even the most clinically mild infection upon reproductive performance and animal productivity is severe and opens the potential for the generation

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of persistently infected animals (McClurkin et al., 1984). Indeed, establishment of infection in the foetus during the first trimester may result in abortion but any live calves born will be persistently infected with BVDV and shed high levels of virus throughout their life. Therefore, the key to the control of BVDV infection through vaccination is the prevention of foetal infection. Blocking of virus transmission can be achieved in the presence of neutralising antibodies directed against the E2 glycoprotein (Howard et al., 1989; Brownlie et al., 1995; Bolin and Ridpath, 1996; Brownlie et al., 1998). This fact makes the control of BVDV infection through vaccination a real possibility. Several eradication schemes have been implemented in northern Europe. The use of marker vaccines in such campaigns would be of great advantage as they would allow distinction between serological responses induced by vaccination or natural infection.

Molecular techniques have established that the genome of this pestivirus is a single-stranded RNA molecule of approximately 12.5 kb length which encodes a single virus polyprotein (Collett et al., 1988; Deng and Brock, 1992). The organisation and protein processing pathways of BVDV and the closely related classical swine fever virus (CSFV) are very similar to those of the human hepatitis C virus. The structural glycoproteins of BVDV, namely C, E^{rns}, E1 and E2, are encoded toward the 5′-end of the viral RNA, with E2 being the immunodominant, major viral glycoprotein. The non-structural proteins, Npro, NS2-3, NS4A, NS4B, NS5A and NS5B, are less well studied. In some cases their enzymatic functions have been elucidated but their contribution to protective immunity has yet to be defined.

Several reports confirm that immunisation with the E2 glycoprotein cloned in live recombinant virus vectors will induce neutralising antibodies against homologous virus and in some cases, protection against subsequent viral challenge (Rumenapf et al., 1991; Van Zijl et al., 1991; Peeters et al., 1997; Elahi et al., 1999; Kweon et al., 1999; Toth et al., 1999). In contrast, while baculovirus expressed E2 protein can induce significant levels of virus neutralising antibody, some reports suggest only limited induction of protective immunity, particularly against heterologous virus (Hulst et al., 1993; Bouma et al., 1999; Bruschke et al., 1997, 1999). As the studies with live recombinant viruses confirm that the E2 glycoprotein of CSFV and BVDV encodes epitopes sufficient for complete protection against viral challenge, it seems likely that the recombinant protein injections failed to deliver the co-stimulatory signals essential for effective immunity. This may indicate the importance of cell-mediated immune mechanisms in cross-strain protection even when the only BVDV antigen provided is the E2 protein.

Recent developments in vaccine technology have tried to clarify the nature of the antigen-non-specific signals that are delivered by a live virus vector but which are so difficult to mimic with adjuvanted protein preparations. The ideal and the aim of this study was the specific modulation of the immune response to a defined antigen while eliminating the potential hazards associated with the use of live virus vaccines or inflammatory adjuvants.

In many cases DNA vaccine technology (reviewed in Cohen et al., 1998) has been particularly effective in inducing anti-viral immunity. The exact mechanisms by which DNA stimulates the high Th1-type responses, effective against intracellular pathogens, are still being elucidated and may reflect bias in the route of delivery, the target cells

expressing antigen or the effect of co-stimulatory CpG sequences in the plasmid backbone. In other cases, a range of different cytokines has been delivered with the antigen to stimulate or even to re-direct the Th1/Th2 bias of an immune response. Several cytokines have been shown to be effective including interleukin-1 (IL-1), IL-2, IL-4, IL-12, and granulocyte-macrophage colony-stimulating factor (GMCSF) (for example Geissler et al., 1997; Chow et al., 1998; Kim et al., 1998). The data to date have failed to identify the ideal cytokine and the effects measured can vary in different systems or in combination with different antigens. However, several studies have described the efficacy of IL-2 in stimulating both cellular and humoral immune responses to viral antigens through its stimulation of Th0 and Th1 cells (Hughes et al., 1991, 1992; Hazama et al., 1993; Reddy et al., 1993; Chow et al., 1997). Particularly in the bovine, IL-2 has been shown to enhance the secretion of all classes of immunoglobulin (Collins and Oldham, 1995; Estes, 1996). Given the essential role of antibody in the clearance of BVDV infections and the central role of cell-mediated responses in immunity to virus infection, IL-2 was chosen as a Th1 cytokine for inclusion in the current study. As DNA vaccination has been effective in a wide range of different systems, it was our intention to examine the responses to DNA-encoded E2 in vivo and to use IL-2 as a co-stimulant to enhance both Th1 immune responses and antibody production.

2. Materials and methods

2.1. RNA extraction

BVDV RNA was extracted from foetal bovine lung cells infected with BVDV strain Ky1203nc using RNA stat-60 (AMS biotechnology). One millilitre of reagent was used to homogenise 10^7 cells. Following chloroform extraction and isopropanol precipitation, RNA was resuspended in 15 μl of DEPC treated water. Murine RNA was extracted from concanavalin A stimulated murine splenocytes using the same technique.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

RNA (5 μl) was mixed with 1.5 μl of random hexamers (10 pmol/μl) and cDNA synthesised by 200 U of Superscript II RT (Gibco) (final volume 25 μl) at 42°C for 50 min. To 5 μl of cDNA, 2.5 μl of forward and reverse primer (10 pmol/μl) were added before amplification with Pfu polymerase (Stratagene) (final volume 50 μl). PCR was subjected to denaturation at 94°C for 1 min, annealing for 1 min and elongation for 2 min at 72°C, for 30 cycles. PCR primers are described in Table 1.

2.3. DNA cloning

PCR fragments cloned into pGEM-3Zf(-) (Promega) were sequenced before subcloning into pSecTag (Invitrogen) for mammalian cell expression of E2 (pSecTag/E2) or into pcDNA3 for murine interleukin 2 (pcDNA/IL2), respectively. The E2 cDNA

Table 1
PCR primers used to isolate the BVDV E2 and murine IL-2 coding sequences[a]

Primer	Position in BVDV strain NADL	Sequence and comments
24	2462–2478	5′ CCGGATCCGCACCTAGACTGCAAACC 3′, forward primer for E2 authentic start
25	3484–3470	5′ CGCTGCAGTCAGGCGAAGTAATCCCG 3′, reverse primer for E2 authentic 3′ end with in-frame stop codon
27	3484–3469	5′ GTCGACTCAATGATGATGATGATGATGGGCGAAGTAATCCCGG 3′, reverse primer for the cloning of E2 in pMelBac providing a 6×His tag
36m		5′ TTGGATCCTCAAGTCCTGCAGGCATGTACA 3′, forward PCR primer for murine IL-2 including native start codon
37m		5′ GGCTCTAGATTATTGAGGGCTTGTTGAGATG 3′, reverse PCR primer for murine IL-2 including native stop codon

[a] Primer 25 was used to amplify E2 while removing the C-terminal hydrophobic tail of the protein for expression in pSecTag. Primer 27 was used to amplify the E2 gene, adding a C-terminal 6×His tag, prior to cloning in baculovirus and protein expression in insect cells.

amplified by primer 24 and 27 (with a 6-histidine tag) was cloned into pMelBac (Invitrogen) giving pMelBac/E2, and recombined with the Bac-N-Blue virus (Invitrogen) for insect cell expression of E2 protein used as ELISA antigen.

2.4. Sequencing

DNA sequencing was performed with the Thermosequenase sequencing kit (Amersham Pharmacia Biotech) on an ALF Express according to the manufacturer's recommendations.

2.5. Expression of E2 and IL-2 in mammalian cells

Plasmid DNA transfection of freshly confluent foetal bovine lung (FBL) and COS7 cells was performed with Lipofectamine (Gibco) as specified by the manufacturer. Expression of E2 in FBL and in COS7 and expression of IL-2 in COS7 cells was demonstrated by an immunoperoxidase assay. The biological activity of the IL-2 was demonstrated using culture supernatant from pcDNA/IL2 transfected cells to stimulate bovine concanavalin A-induced lymphoblasts. Briefly, bovine PBMNC were purified on a Histopaque gradient from whole calf blood diluted 1:2 with phosphate buffered saline (PBS). Cells were resuspended in 10 ml of RPMI 1640 (supplemented with 20% FBS, 50 μM 2-mercaptoethanol, 2 mM glutamine, 27 mM sodium carbonate, 1 mM sodium pyruvate, 200 U/ml penicillin and 100 μg/ml streptomycin) at a starting density of 2×10^6 ml^{-1}. They were incubated with concanavalin A (5 μg/ml) for 4 days. After centrifugation at 650 g for 8 min, they were resuspended at 4×10^5 ml^{-1}, plated on a 96 well-plate (100 μl/well) and incubated for 36 h with serial dilutions of pcDNA/IL2 transfection supernatants. Proliferation was quantified by the XTT cell proliferation kit (Boehringer Mannheim).

2.6. Immunoperoxidase staining

Cells transfected with plasmid DNA were washed and fixed with ice-cold 80% acetone. After washing, cells were incubated with bovine anti-BVDV hyperimmune serum 2359 (0.5 ml diluted 1/138 in 5% rabbit serum in PBS-T), washed with PBS-T before 0.5 ml of anti-bovine horseradish peroxidase (HRP) conjugate (diluted 1/2000 in 5% rabbit serum in PBS-T) was added. AEC (3-amino-9-ethyl carbazole in *N*,*N*-dimethyl formamide) substrate (0.5 ml) was added and cells were examined for brown cytoplasmic staining.

2.7. Injection of mice

Three groups of five female Balb/c mice (age 6–8 weeks) were injected intramuscularly, twice at 4-week intervals, with DNA diluted in sterile PBS. Mice were bled on weeks 0, 4, 6 and 8. The aim was to investigate the immune response to the DNA vaccine encoding the BVDV E2 and to assess the effects of co-delivery of IL-2.

Group 1: 50 μg of plasmid pSecTag/E2 and 50 μg of pcDNA.
Group 2: plasmids pSecTag/E2 (50 μg) and pcDNA/IL2 (50 μg).
Group 3: (negative control) vector DNAs, pcDNA and pSecTag (50 μg each). To provide a positive control for the isotyping investigation, two groups of four female Balb/c mice were injected intramuscularly twice with different forms of the same control antigen.
Group 4: recombinant β-galactosidase protein (10 μg). (First injection adjuvanted with CFA, second injection with IFA.)
Group 5: plasmid pcDNA/LacZ encoding β-galactosidase (100 μg).

2.8. E2 antigen preparation and ELISA

ELISA antigen for the detection of E2 antibodies was recombinant E2 protein expressed from a baculovirus recombined with pMelBac/E2 in Sf21 insect cells according to Invitrogen's protocol. Expression of the protein was demonstrated by SDS-PAGE under denaturing conditions followed by Western blotting as described (Ausubel et al., 1987). A C-terminal 6 histidine tag added via the reverse PCR primer allowed protein purification (Ni-NTA spin kit, Qiagen). The elution product in 8 M urea was diluted (1:50 or 1:100) in carbonate buffer to coat plates overnight. Plates were washed with PBS-T and blocked for 1 h. After further washes, the mouse sera (diluted 1/25 in PBS-T with 5% pig serum) were added, incubated for 2 h at 37°C and washed three times. Anti-murine HRP conjugate was incubated under the same conditions. After three washes, OPD substrate (*o*-phenylenediamine dihydrochloride, Sigma) was added (200 μl) and incubated in the dark for 30 min at room temperature. The reaction was stopped with 2 M sulphuric acid (50 μl/well) and the OD was read at 490 nm. Sera were considered positive when the OD after subtraction of the background was five times higher than the OD of the negative control animals.

The positive control mouse sera were assayed in a similar manner, but using plates coated with β-galactosidase protein (500 ng/well, Sigma G-5635).

2.9. Antibody isotyping

The determination of murine antibody isotype in total serum samples or of E2 antigen-specific antibody was determined using the mouse IgG1 and IgG2a ELISA quantitation kit (Bethyl) as described above but with IgG1 and IgG2a goat anti-mouse-HRP conjugates. The dilution of each conjugate was optimised using the manufacturer's reference serum in order to give similar OD when detecting the same amount of respective target immunoglobulin.

2.10. Serum neutralisation assay

Twofold dilutions of murine sera in 96 well plates were mixed with 50 μl of virus Ky1203nc containing 100 $TCID_{50}$. A 50 μl suspension of FBL cells (3×10^5 cells/ml) was added and incubated for 4 days at 37°C. Neutralisation of the noncytopathogenic virus was demonstrated by an immunoperoxidase assay as described previously. The neutralising antibody titre is calculated as the dilution of serum giving a 50% inhibition of virus production. Titres >1:10 are considered positive in this assay.

3. Results

3.1. Demonstration of expression of E2 and IL-2

The E2 cDNA from BVDV strain Ky1203nc was cloned in pMelBac, providing a secretion signal for expression in Sf21 insect cells. Following co-transfection of pMelBac/E2 with Blue-N-Bac virus DNA, the apparent molecular weight of the recombinant protein expressed, demonstrated by western blot (Fig. 1), was 52 kDa, slightly smaller than native E2 as a consequence of the removal of the C-terminal hydrophobic membrane anchor during the RT-PCR. The same E2 cDNA was cloned into pSecTag and following transfection of COS7 and FBL cells, expression of the protein was demonstrated by an indirect immunoperoxidase assay (Fig. 2). In COS7 cells, around 50% of the cells expressed the E2, while the transfection efficiency was lower in FBL.

The cDNA encoding murine IL-2 was cloned in pcDNA 3.1 following RT-PCR. Expression of the cytokine was demonstrated by an indirect immunoperoxidase assay on transfected COS7 cells. To confirm biological activity, serial dilutions of the supernatant from transfected cells were used on concanavalin A stimulated bovine lymphoblasts. The murine interleukin-2 had a strong mitogenic effect on bovine lymphoblasts (Fig. 3).

3.2. Immune response to E2

ELISA antibodies to the BVDV E2 protein were first detected in murine serum, 2 weeks after the second DNA injection (Fig. 4). In group 1 (E2 only), two out of five mice exhibited marked anti-E2 responses. In group 2 (E2+IL-2), all five mice seroconverted strongly to BVDV E2 protein. No valid statistical analysis could be performed due to the small size of the groups. However, the mean ELISA titre (1340) induced by the

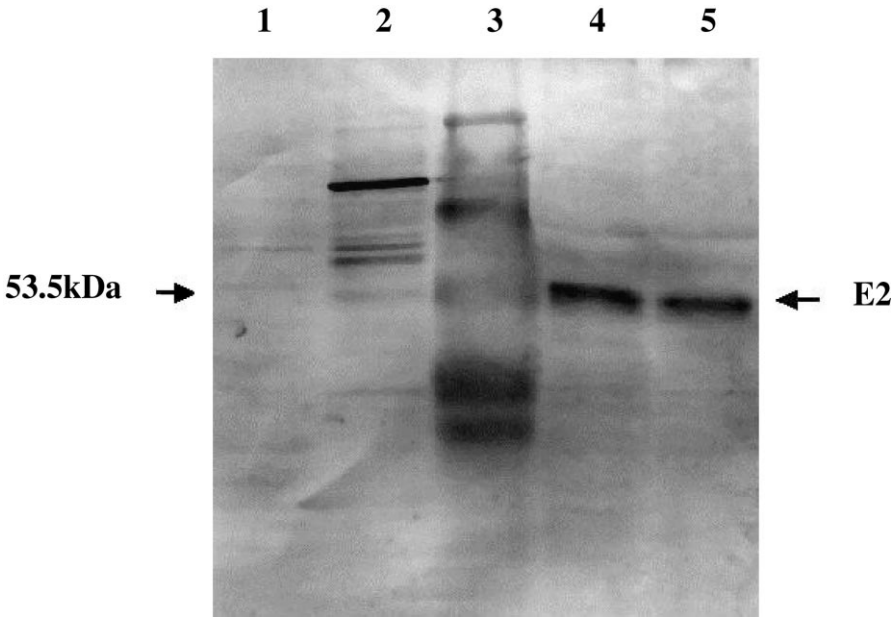

Fig. 1. Western blot demonstrating expression of E2 in insect cells. All samples were incubated with anti-BVDV serum 2359 diluted 1/100 and anti-bovine HRP conjugate. Lane 1: uninfected SF21 cells; lane 2: positive control ELISA antigen produced from BVDV infected FBL cells; lane 3: marker; lane 4 and 5: Sf21 cell lysates from two different baculovirus clones expressing E2.

co-administration of E2+IL-2 was increased compared to the mean titre (50) in group 1 (E2 only).

3.3. Demonstration of the induction of neutralising antibodies

When tested for the presence of serum neutralising antibodies against BVDV strain Ky1203nc, 7 of 10 mice injected with E2 encoding DNA were positive (Table 2). There was a 4-fold increase in the mean titre of group 2 compared to group 1. The highest titre

Table 2
Neutralisation titres 6 weeks after vaccination[a]

Group 1 (pSecTag/E2+pcDNA)		Group 2 (pSecTag/E2+pcDNA/IL2)	
Mouse 1	40	Mouse 6	320
Mouse 2	80	Mouse 7	113
Mouse 3	<10	Mouse 8	1812
Mouse 4	320	Mouse 9	<10
Mouse 5	57	Mouse 10	<10

[a] The neutralising antibody titre is calculated as the dilution of serum giving a 50% inhibition of virus production. Values >10 are considered positive in this assay.

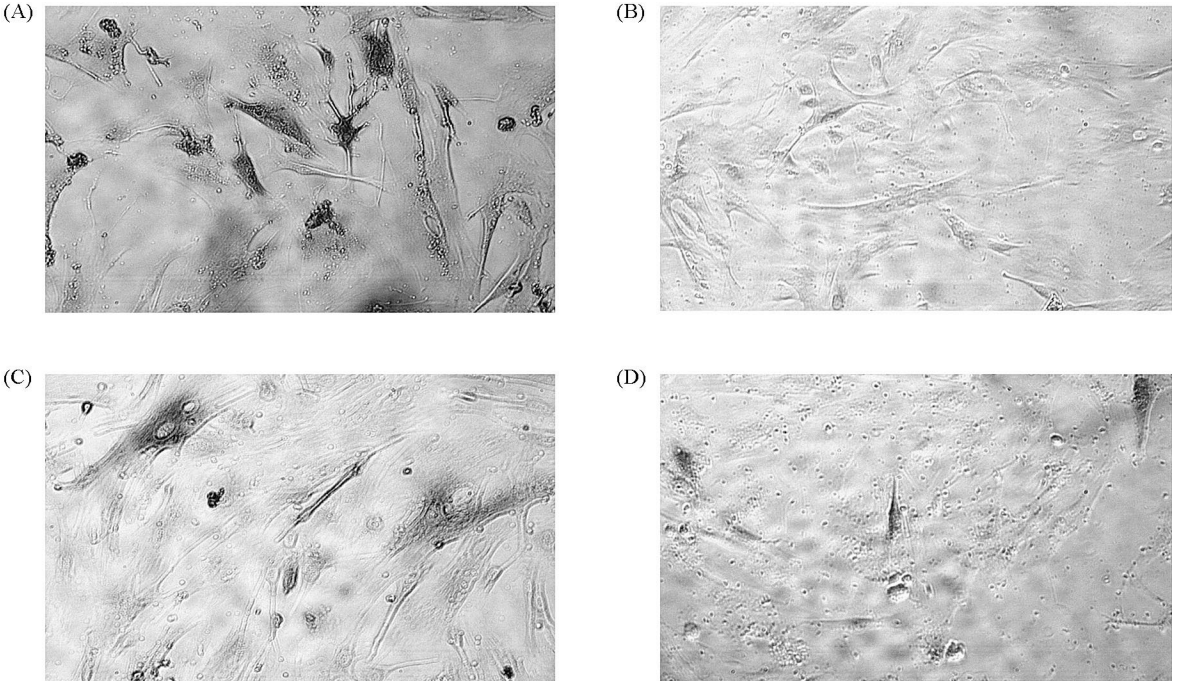

Fig. 2. Indirect immunoperoxidase assay on FBL cells (incubated with anti-BVDV serum 2359). (A) The cells were transfected with 1 μg of DNA (pSecTag/E2) and 8 μg of lipofectamine per well. (B) Cells treated as above excluding the addition of DNA. (C) Cells were infected with BVDV two days prior to the immunoperoxidase assay. (D) One microgram of pcDNA/LacZ was added per well together with 8 μg of lipofectamine. Expression of β-galactosidase was demonstrated by the addition of X-gal after fixation with formaldehyde/glutaraldehyde.

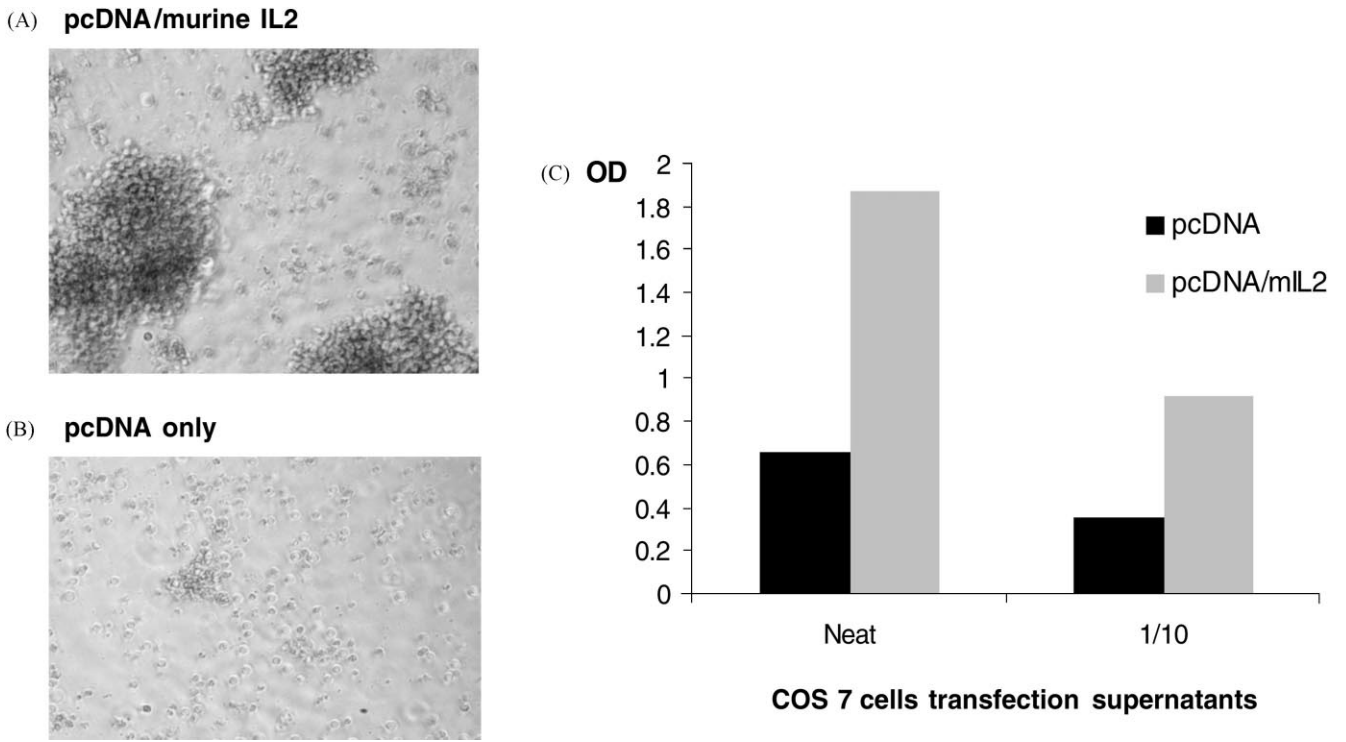

Fig. 3. Increase of proliferation induced by murine IL-2. Bovine lymphoblasts were incubated with concanavalin A for 24 h prior to incubation with culture supernatant (neat or 1/10 dilution) of cells transfected with the plasmid encoding murine IL-2. The stimulation induced by murine IL-2 is illustrated: (A) bovine blasts induced by murine IL-2; (B) negative control supernatant; (C) quantification of proliferation using the XTT kit (Boehringer Mannheim).

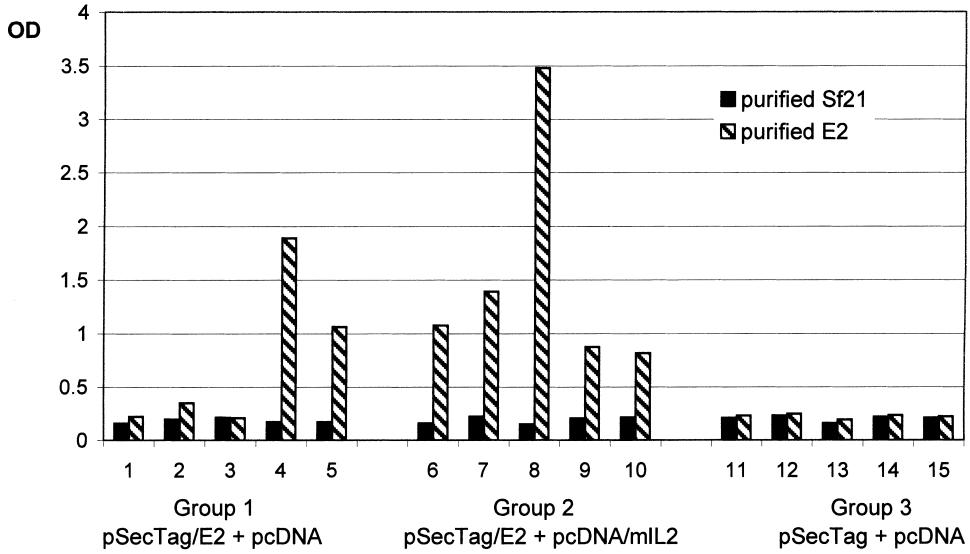

Fig. 4. Analysis of week 6 mouse sera in an E2-specific ELISA. The sera (diluted 1/25) were tested against the Ni-NTA purified E2 recombinant protein and uninfected Sf21 cell lysates purified in the same way (used at the same dilution).

(1812) was observed in the IL-2-adjuvanted group in the animal which also showed the highest ELISA antibody titre.

3.4. Antibody isotype

The IgG isotype produced by vaccination can indicate the type of immune response induced, with IgG1 being associated with Th2 and IgG2a with Th1 responses in mice. Measurement of the E2-specific antibody in groups 1 and 2 revealed predominantly subclass IgG2a antibodies with IgG1 being hardly detectable (Fig. 5). This is in contrast to the assessment of isotype in total serum antibody, where IgG1 levels were almost 50% of IgG2a. To confirm that the extreme Ig2a bias in E2-specific antibody was not an artefact, the isotype of antibodies induced following injection of mice with another antigen were compared — mice were injected with β-galactosidase either in the form of recombinant protein or as the pcDNA/LacZ plasmid construct. As expected, antigen-specific IgG1 was readily quantifiable in the mice injected with β-galactosidase protein (group 4), while the pcDNA/LacZ vaccinated mice (group 5) had very low IgG1 levels (Fig. 6) similar to those of the group 1 (E2 DNA vaccine) mice.

4. Discussion

The initial experiments confirmed the biological activity of the E2 and IL-2 constructs when transfected into cells in vitro. The protein products could be readily detected by

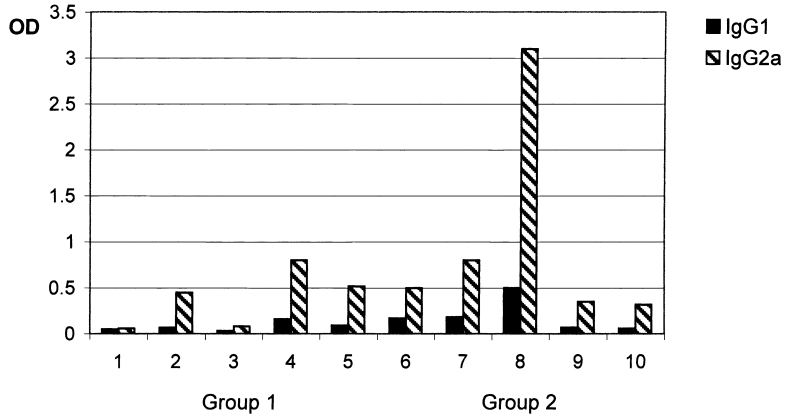

Fig. 5. E2-specific IgG1 and IgG2a antibodies in week 6 mouse sera (group 1 and 2) as determined by ELISA.

immunoassay and the murine IL-2 induced the proliferation of bovine lymphoblasts in vitro. This observation is all the more surprising as in the reciprocal experiment, bovine IL-2 fails to stimulate murine lymphoblasts (Collins et al., 1994). When the plasmid pSecTag/E2 alone or with pcDNA/IL2 was injected into mice, both ELISA and virus neutralising E2-specific antibodies were detected two weeks following the second vaccination. The co-administration of IL-2 enhanced the ELISA antibody response (comparing data for group 1 with group 2) with both the number of animals seroconverting and the levels of antibody produced being consistently higher in the E2+IL-2 group. This confirms the utility of IL-2 as a co-stimulant, augmenting the levels of antibody associated with both Th1- and Th2-type responses (Estes, 1996). Virus neutralising activity is also of great biological significance and may not always be accurately reflected by the ELISA antibody levels (West and Ellis, 1997). However, when

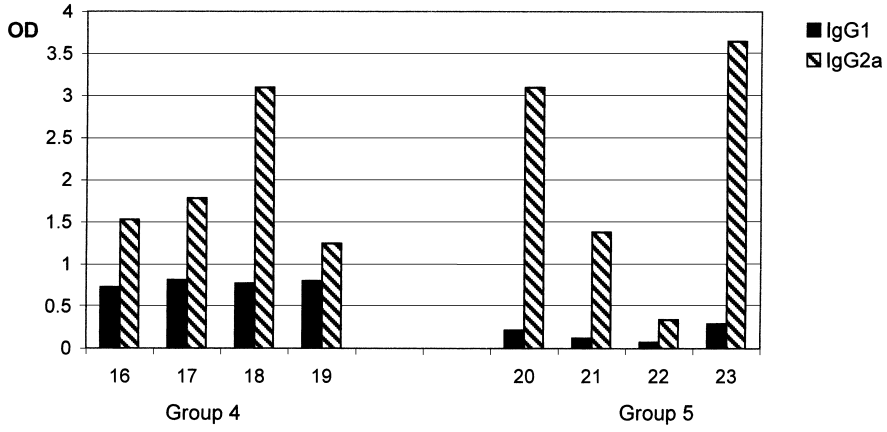

Fig. 6. β-galactosidase-specific IgG1 and IgG2a antibodies in groups 4 and 5 as determined by ELISA.

neutralising antibody titres were assessed, there was a reasonable correlation with the ELISA data. It is clear that the highest neutralising titres were again observed in the E2+IL-2 group. However, potentially significant levels of neutralising antibody were measured in four out of five mice in the E2 only group. The biological significance of these antibodies cannot be proven in the current model system (mice are not susceptible to infection by BVDV) but similar levels of antibody have been reported to prevent serum viraemia following experimental infection of cattle with BVDV (Bolin and Ridpath, 1996). In agreement with the data of Harpin et al. (1997) this confirms that E2 is an effective antigen when delivered in the form of a DNA vaccine and that potentially significant neutralising titres can be induced when the antigen is targeted to the endoplasmic reticulum for glycosylation.

The use of DNA vaccines has demonstrated the potential of this technology to modulate the Th1/Th2 bias of an immune response (Raz et al., 1996; Donnelly et al., 1997). In our experiment, this bias was measured by assessing the levels of IgG1 and IgG2a isotypes of antigen-specific antibody. When comparing the IgG2a to IgG1 ratio for β-galactosidase antigen injected either as a conventional, adjuvanted recombinant protein or as a DNA vaccine, our results confirm previous observations. Injection of the protein antigen induces high levels of antigen-specific antibodies of both the IgG2a and IgG1 isotypes. In contrast, high levels of IgG2a antibody with an apparent suppression of antigen-specific IgG1, a pattern frequently associated with Th1 responses, is induced following intramuscular injection of DNA. This experiment does not contain a group of mice injected with recombinant E2 protein but in both groups 1 and 2 (injected with DNA constructs encoding the E2 protein) the E2-specific antibody response was almost entirely IgG2a with IgG1 levels being too low for calculation of a meaningful IgG2a/IgG1 ratio. This is again consistent with the DNA vaccination inducing an anti-E2, Th1-biased response. Additionally, the administration of IL-2 further enhanced the levels of IgG2a ELISA antibody measured, although the neutralising antibody component was not particularly augmented by this treatment. This is again consistent with a Th1 bias of the response which would tend to increase IgG2a, the isotype more frequently associated with cell mediated immune mechanisms which act through complement fixation and Fc receptor binding rather than direct neutralising activity.

5. Conclusion

The data presented here confirm that delivery of the BVDV E2 sequences as a DNA vaccine can induce high levels of virus neutralising antibodies consistent with a strong Th1 immune response and that co-administration of IL-2 enhanced the production of E2-specific antibody. The efficacy of these constructs in a viral challenge system are now awaited.

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