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DNA vaccination against bovine viral diarrhoea virus induces humoral and cellular responses in cattle with evidence for protection against viral challenge

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Abstract

The immune response induced by a DNA construct expressing the E2 envelope glycoprotein of bovine viral diarrhoea virus (BVDV) was studied in cattle. Four groups of five calves, were immunised by intradermal injection with a total of 1 mg of plasmid DNA on each of two occasions, with a 3-week dose interval. Group 1 received non-coding plasmid DNA only (control), group 2 received the E2 coding plasmid (0.5 mg) plus non-coding plasmid DNA (0.5 mg) and groups 3 and 4 received the E2 coding plasmid plus plasmid encoding either bovine interleukin 2 (IL-2) or granulocyte macrophage colony stimulating factor (GM-CSF) respectively. Two weeks after the final immunisation, all calves were challenged by intranasal inoculation with 5×10^6 $TCID_{50}$ of homologous virus. On the day of challenge, neutralising antibodies were detectable in 13 of 15 vaccinated calves (one animal in each of groups 3 and 4 remained seronegative at this point). Thereafter, a strong anamnestic serological response was evident in all vaccinated animals. Furthermore, T-cell proliferation following in vitro re-stimulation with BVDV antigen was significantly elevated in the cytokine adjuvanted groups. This enhancement of BVDV specific immune responses in vaccinated animals was reflected in the clinical responses observed post-challenge. In particular, reduced febrile responses provided evidence of a disease sparing effect of vaccination. Significantly, whilst a transient viraemia was detected in all control animals following challenge, no virus was isolated from the leucocytes from 8 out of the 15 vaccinated animals. In groups 2 and 4, three animals remained virus free, although virus was isolated from two animals in each group at a single time point, while in group 3, three out of five animals had detectable viraemia.

In summary, the administration of a DNA vaccine encoding only the E2 glycoprotein of BVDV induced a disease sparing effect in vaccinated calves following challenge and protected more than half of the vaccinated animals from detectable viraemia.

Keywords: DNA; Bovine virus diarrhoea virus; BVDV; Granulocyte macrophage colony stimulating factor; Interleukin 2

1. Introduction

Bovine virus diarrhoea virus (BVDV) is a member of the pestivirus genus, within the *Flavivirus* family. This positive sense RNA virus is an important pathogen of cattle, causing significant economic losses world-wide. These losses can be largely attributed to a reduction in conception rates and an increase in abortions in breeding cattle [1].

Worldwide, there are ongoing efforts to control BVDV infection through eradication and by vaccination. However, a major shortcoming of existing vaccines is a lack of marker status. Such marker vaccines allow distinction between vaccine immunity and infection immunity on the basis of a readily measured marker, for example antibody against a specific viral antigen. To address this, several novel approaches to BVDV vaccination have been described in the literature. Without exception, these involve the delivery of a specific viral antigen, the 53 kDa major envelope glycoprotein, designated E2. High titre polyclonal antisera raised against E2 neutralise numerous viral isolates within a given genogroup [2], and it has been experimentally proven that passively acquired antibody of adequate titre is effective in preventing BVDV infection [3]. For vaccination, E2 has been delivered by numerous methods including as a recombinant protein [4,5], as a constituent of recombinant adenoviruses [6] and as a DNA vaccine construct [7], with varying degrees of success. In particular, in target species studies with recombinant protein vaccines, Bolin and Ridpath [4] were able to show a high degree of protection against clinical disease

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and viraemia following homologous challenge in calves, and Bruschke et al. [5] were able to demonstrate in utero protection following homologous challenge in pregnant sheep.

DNA vaccination potentially provides a particularly attractive control strategy for viral pathogens in the veterinary field [8]. There are good scientific grounds for expecting enhanced efficacy from a DNA vaccine. Following DNA vaccination, antigens are synthesised endogenously within the target cells. Subsequent immune responses to such antigens are thus likely to mimic the pattern resulting from natural intracellular infection. As exposure of susceptible cattle to natural infection is believed to result in life long immunity, at least against antigenically similar strains, a vaccine that can mimic infection immunity is likely to be highly efficacious. Also, DNA vaccines avoid the need to propagate live viruses in complex culture systems.

There are numerous examples of the experimental use of DNA vaccine technology in livestock species including cattle (e.g. [9,10]), pigs (e.g. [11]) and sheep [12]. However, in the only published study to date involving DNA vaccination against BVDV in cattle [7], the authors were able to demonstrate only limited protection against clinical disease. Protection was not evident in all individuals within the group, and correlates of protection, notably the production of neutralising antibodies and in vitro lymphoproliferative responses, were weak or non-existent. These results suggest that further action is required to increase the immunogenicity of the basic E2 plasmid vaccine.

To this end, one well-established means of enhancing humoral and cell-mediated immune responses to DNA vaccination is the co-administration of relevant cytokines or cytokine genes. Numerous studies in mice and primates have indicated the potential of such an approach (e.g. [13–15]). In livestock species, several studies using recombinant cytokines have been performed with the intention of increasing the efficacy of conventional inactivated and modified live vaccines. Such studies include investigation of interleukin 2 (IL-2) in cattle for both modified live [16] and recombinant sub-unit [17] bovine herpes virus-1 vaccines. Our own preliminary studies with the BVDV E2 gene in mice have indicated that both IL-2 and granulocyte macrophage colony stimulating factor (GM-CSF) increased production of neutralising antibodies and enhanced lymphoproliferation [18]. Equally importantly, co-administration of these cytokines led to a more uniform response at the group level. This could reduce the risk of vaccine failure in an outbred cattle population.

In the present study, we examined the utility of co-administration of plasmids encoding the bovine cytokines interleukin 2 and granulocyte macrophage colony stimulating factor to enhance cell-mediated and humoral immune responses against E2 derived from a genogroup 1 BVDV strain. Following vaccination with these constructs, cattle were challenged with the homologous BVDV strain and a range of clinical, virological and immunological correlates of protection were subsequently determined.

Table 1
PCR primers used for amplification of cytokine cDNA

IL-2	Forward primer	5′ATCTGCAGTCAACTCCTGCCACAATGT 3′
	Reverse primer	5′ CGCTGCAGGGTACCCACTTAGTGATCA-AGTCA 3′
GM-CSF	Forward primer	5′CGGATCCATGTGGCTGCAGAATTTAC 3′
	Reverse primer	5′GCCTCTAGATCATTTTTGGCTTGGTTT 3′

2. Materials and methods

2.1. cDNA cloning

Cloning of BVDV E2 from Ky1203 nc (a type Ia BVDV strain) has been described previously [18]. Briefly, the Ky1203 nc E2 sequence obtained following RT-PCR on BVDV infected foetal bovine lung cells (FBL) started at the authentic start of E2 but did not include the carboxy-terminal transmembrane region. The cDNA was cloned into the mammalian expression vector pSecTag (Invitrogen). For amplification of bovine cytokine cDNA, RNA was extracted from concanavalin A stimulated bovine peripheral blood mononuclear cells (PBMNC). Amplification after reverse transcription with random primers was performed with the cytokine specific primers described in Table 1 using *Pfu* DNA polymerase. The primer design was based on the cytokine sequences found on the EMBL database (M12791: bovine IL-2 and BT22385: bovine GM-CSF), including authentic signal sequence, start and stop codons. Both cDNAs were cloned into the expression vector pcDNA3.1 (Invitrogen).

2.2. Experimental design

2.2.1. Vaccination phase

Mixed breed calves ranging in age from 3 to 7 months were obtained from a BVDV free supplier. All calves were housed in a purpose built barn with biosecurity measures in place to avoid possible exposure to adventitious pathogens. Five calves were allocated to each of four treatment groups according to a random block design based on age. Treatment groups were as follows:

- group 1: 500 μg pSecTag + 500 μg pcDNA;
- group 2: 500 μg pSecTag encoding E2 + 500 μg pcDNA;
- group 3: 500 μg pSecTag encoding E2 + 500 μg pcDNA encoding IL-2;
- group 4: 500 μg pSecTag encoding E2 + 500 μg pcDNA encoding GM-CSF.

Subsequently, calves were allocated a second time into smaller husbandry groups based on age and size to ensure fair competition for food. Following an acclimation period of 1 week, all calves were vaccinated on two occasions, 3 weeks apart, on study days −42 and −21. On each occasion,

route of administration was intradermal, with a quarter of the total dose volume (1 ml in PBS) delivered into four separate sites on the neck. Blood samples for serology were obtained on study days −42 and −21.

2.2.2. Challenge phase

Pre-challenge (baseline) measurements of rectal temperatures, clinical scores and haematological profiles commenced on study day −4, and continued on days −1 and 0. On day 0, all the animals were challenged intranasally with 5×10^6 $TCID_{50}$ of Ky1203 nc virus. Thereafter, measurements of all parameters were made daily until study day 10, with a final time point on day 14. Additional blood samples were taken on each occasion for virus isolation, and for serology on days 0, 3, 7, 14 and 21. All calves were euthanised shortly after study day 21.

2.3. Clinical scores

Each husbandry group was observed for 10 min on each of the designated days by a trained veterinarian. Clinical categories assessed were demeanour, degree of dyspnoea, nasal discharge and frequency of coughing. A simple three-point numerical system was used, with a score of 1 for normal, 2 for moderate or 3 for severe symptoms.

2.4. Haematological profiles

EDTA blood was provided from each individual on each designated day for haematological analysis. An automated haematological analyser (Cell Dyn, Abbot) running an established bovine haematology profile was used to determine both total and differential leukocyte counts. Lymphocytes, neutrophils and monocytes were all quantified in the differential count, and where abnormal values were indicated, manual differential counts were made. Results were expressed as counts ($\times 10^9\,l^{-1}$).

2.5. Virus isolation

Blood was collected from each animal on each sampling occasion (5 ml, EDTA treated). To extract BVDV, erythrocytes in each sample were lysed with ammonium chloride lysis buffer. Following centrifugation, the leukocyte pellet was washed twice in PBS, and finally resuspended in 1 ml of PBS. The sample was divided into two and stored at −70 °C. To detect BVDV, each sample was thawed and the cell lysate diluted 1:4 in maintenance medium (minimum essential medium containing 2% foetal calf serum and antibiotic/antimycotic solution). Each diluted sample was seeded onto freshly prepared monolayers of early passage FBL cells prepared in 24-well tissue culture plates and incubated for 5 days at 37 °C. Following freeze–thawing, each sample was passaged onto fresh FBL cells and incubated for a further 5 days. Finally, BVDV was detected by immunofluorescence. Briefly, monolayers were fixed in ice cold acetone and then incubated with a high titre convalescent BVDV antiserum for 30 min. Following washing, bound immunoglobulin was detected by addition of a species specific fluorescent antibody conjugate (rabbit anti-bovine Cy3 labelled; Stratech) for 30 min prior to scoring wells under a fluorescent microscope positive or negative).

2.6. Serology

2.6.1. ELISA

Total IgG responses against the E2 protein were determined as described previously [18]. Test sera from each treatment group were pooled, and diluted in a two-fold series, in duplicate, from an initial dilution of 1:100. Results were expressed as the reciprocal of the final serum dilution which was above the negative threshold (defined as twice the mean of the control serum at a 1:100 dilution). Specific isotypes (IgG_1 and IgG_2) were quantified using isotype specific conjugates at dilutions previously proven to give similar optical density values when incubated with equivalent concentrations of purified reference standard IgG_1 and IgG_2 (Bethyl Laboratories).

2.6.2. Serum neutralisation assays

Serum neutralising (SN) titres against BVDV were determined in an assay based on that of Howard et al. [19]. Briefly, test sera were diluted out in a two-fold series from an initial dilution of 1:2 to a maximum dilution of 1:4096. Next, 50 μl of inoculum containing 100 $TCID_{50}$ of BVDV virus, either Ky1203 nc (homologous) or NADL (heterologous) was added to each well, and the plates were incubated at 37 °C for 2 h. Finally, 50 μl of a suspension of FBL cells at 3×10^5 cells/ml was added to each well, and the plates were incubated for a further 5 days at 37 °C. Scoring was performed by microscopic examination of the monolayer of cells for cytopathic effect (NADL), or the presence of intracellular virus following fixation and immunoperoxidase detection (Ky1203 nc). Results were expressed as the reciprocal of the serum dilution at which 50% neutralisation of virus occurred.

2.7. Antigen specific proliferation

EDTA treated blood was taken from all individuals on study day 9 (control and E2 only groups) or day 10 (both cytokine adjuvanted groups). PBMNC were extracted on a histopaque gradient, washed and resuspended to a final concentration of 1×10^6 cells/ml in RPMI 1640 medium supplemented with 10% foetal calf serum. Cells were plated out in 96-well microtiter plates at 100 μl per well, and equal volumes of purified recombinant E2 protein (10 μg/ml and 1 μg/ml), live Ky1203 nc virus (at 5×10^6 $TCID_{50}$/ml), or PBS only were added in triplicate to cells from each individual. Concanavalin A (5 μg/ml) was used as a positive control. Plates were incubated at 37 °C for 4 days prior to the addition of 1 μCi of tritiated thymidine to all wells.

Following a further 18 h incubation, cells were harvested and thymidine incorporation was determined using a liquid scintillation counter (Packard). Stimulation indices (SI) were calculated for each sample as the ratio of mean counts determined in antigen pulsed wells compared with mean counts from PBS pulsed cells.

2.8. Statistical analysis

Unless otherwise stated, inter-group differences were investigated using the Kruskal Wallis test followed where appropriate by Dunn's multiple range test. In all instances, significance is claimed at $P < 0.05$, and in the context of the Kruskal Wallis test, P values are reported adjusted for ties. All log transformations were $\log_e$, and where a value of zero was recorded in a data set, a value of 1 was added prior to transformation.

Inter-group differences in clinical scores were investigated by comparing the time to the first abnormal clinical observation within each category, the duration of abnormal clinical observations within each category, and the overall clinical score.

Inter-group differences in viraemia were investigated by comparing the duration of the viraemic period. The length of time (in days) between the first and last virus positive sample was calculated for each individual.

Inter-group differences in mean rectal temperatures and haematological counts were compared using repeated measures analysis of variance procedures. Haematological counts were log transformed prior to analysis.

Inter-group differences in mean neutralising antibody titres were compared on study day 7 (homologous virus) or 7 and 10 (heterologous virus), using one way analysis of variance followed by Tukey's multiple range test to compare geometric mean titres. No statistical analysis was performed on the ELISA antibody data as samples from each group were pooled.

Finally, inter-group differences in antigen specific proliferative responses were also compared. To prepare the data for analysis, the specific background count was subtracted for each individual sample, and all values were log transformed prior to one way analysis of variance. Data in the text is presented as group mean ± standard error unless otherwise stated.

3. Results

3.1. Clinical observations

3.1.1. Clinical scores and rectal temperatures

Post-challenge clinical signs of viral infection were first detectable around day 3, but the majority (86% of the total number of observations) of disease signs were recorded between days 6 and 9. The most common observations were a serous nasal discharge (34% of the total number of observations) and/or a dull demeanour (53% of the total number of observations). Occasional coughing was noted in all groups, and there was almost no evidence of dyspnoea. Statistical analysis of clinical scores (data not shown) revealed no significant differences between any experimental groups.

In contrast, rectal temperature profiles post-challenge indicated a protective effect of E2 vaccination (Fig. 1). There was some evidence for a biphasic febrile response,

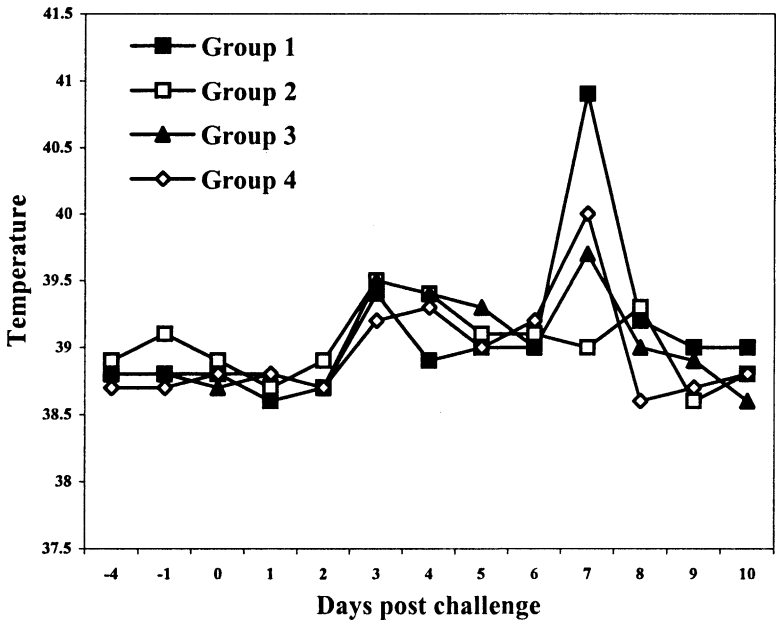

Fig. 1. Temperature fluctuations in relation to viral challenge. Temperatures were taken daily from 4 days before challenge to 10 days after challenge in group 1 (pSecTag + pcDNA), group 2 (pSecTag/E2 + pcDNA), group 3 (pSecTag/E2 + pcDNA/bvIL-2) and group 4 (pSecTag/E2 + pcDNA/bvGM-CSF).

particularly in the unvaccinated control group, where the group mean temperature was moderately elevated on day 3, followed by a peak of $40.9 \pm 0.1\,^{\circ}C$ on day 7. A similar pattern was observed in the IL-2 adjuvanted group, though it should be noted that mean peak temperatures recorded on day 7 ($39.7 \pm 0.2\,^{\circ}C$) were over $1\,^{\circ}C$ lower than recorded in the unvaccinated control group. Statistical analysis revealed a significant treatment × time interaction, indicating differences in the overall temperature profiles of the experimental groups. Temperatures were significantly elevated in the control group ($P < 0.01$) on day 7 compared with all other groups. No differences were observed between the vaccinated groups.

3.1.2. Haematological parameters

In the period prior to challenge, total leukocyte counts were similar in all four treatment groups, and there was little evidence of any marked day to day fluctuation (Fig. 2a). Post-challenge, mean counts fell sharply in all groups between days 1 and 3. This decrease was most marked in the control group, amounting to a fall of around 47% compared with pre-challenge mean values. Cell numbers declined by 37% in both the E2 only and the IL-2 adjuvanted group over the same period, but by only 18% in the GM-CSF adjuvanted group. Counts remained suppressed in all groups until after day 6, recovering thereafter to pre-challenge levels by day 8. No statistical differences were apparent between the treatment groups over time.

The lymphocyte count profiles were similar to those described for the total leukocyte count (Fig. 2b). By day 3 post-challenge, mean counts had fallen by 40% in the unvaccinated control group, and by similar amounts in the E2 only and IL-2 adjuvanted groups. Animals in the GM-CSF adjuvanted group again showed the smallest decrease in lymphocyte numbers, where mean counts fell by only 18%. Lymphocyte numbers remained suppressed in all groups until after day 6, recovering thereafter to pre-challenge levels by day 8. Statistical analysis revealed a marginally non-significant ($P < 0.06$) treatment × time interaction, reflecting the strong trend in the data for a reduced lymphopenia in the GM-CSF adjuvanted group.

Neutrophil and monocyte count profiles followed similar patterns to those described for total leukocyte counts (Fig. 2c and d respectively). In both instances, the most marked differences were apparent between the unvaccinated control group and the GM-CSF adjuvanted group. By day 3, mean neutrophil and monocyte counts had declined by over 50% in the unvaccinated control group compared with less than 25% in the GM-CSF adjuvanted group. No statistical differences were apparent between the treatment groups over time.

3.2. Virus isolation

The frequency of virus isolation from buffy coat samples is summarised in Table 2. Virus was first isolated in the control group on day 4 post-challenge, and the last virus isolation was made on day 7. During this period, virus was detected in all five control calves on at least one occasion. In contrast, virus was only detected in two out of five animals vaccinated with E2 only, three out of five animals in the IL-2 adjuvant group and two out of five animals in the GM-CSF adjuvant group. Furthermore, in six out of seven of these animals, virus was isolated on 1 day only. Statistical analysis revealed an overall significant difference between the groups ($P = 0.05$). However, multiple comparison tests failed to identify exactly which groups differed from each other (a consequence of the P value being so close to 0.05, coupled with the number of ties in the data). Overall, there was strong evidence of a reduction in viraemia as a result of vaccination, but no clear evidence of a beneficial effect of the cytokine adjuvants.

Table 2
Virus isolation following challenge

Group number	Animal number	Day post-challenge									
		1	2	3	4	5	6	7	8	9	10
Group 1 (control)	2091	−	−	−	−	+	+	−	−	−	−
	2094	−	−	−	+	−	+	−	−	−	−
	2106	−	−	−	−	+	−	−	−	−	−
	2110	−	−	−	−	+	−	+	−	−	−
	2118	−	−	−	−	−	+	−	−	−	−
Group 2 (E2 only)	2093	−	−	−	−	−	−	−	−	−	−
	2098	−	−	−	−	−	−	−	−	−	−
	2100	−	−	−	−	−	−	+	−	−	−
	2109	−	−	−	−	−	−	−	−	−	−
	2116	−	−	−	−	+	−	−	−	−	−
Group 3 (E2 + IL-2)	2089	−	−	−	−	−	−	−	−	−	−
	2095	−	−	−	−	−	+	−	−	−	−
	2102	−	−	−	−	+	−	−	−	−	−
	2115	−	−	−	−	+	+	−	−	−	−
	2117	−	−	−	−	−	−	−	−	−	−
Group 4 (E2 + GM-CSF)	2092	−	−	−	−	+	−	−	−	−	−
	2096	−	−	−	−	−	−	−	−	−	−
	2105	−	−	−	−	−	+	−	−	−	−
	2111	−	−	−	−	−	−	−	−	−	−
	2120	−	−	−	−	−	−	−	−	−	−

Following vaccination and challenge, samples were taken daily for 10 days. Virus isolation from buffy coats was performed by immunofluorescent assay after two passages on FBL cells.

3.3. Serological parameters

3.3.1. Total anti-BVD antibody responses

Total anti-E2 antibody responses as determined by ELISA are presented in Fig. 3. Early signs of an anamnestic immune response were observed in the vaccinated groups as early as day 7, and by day 10, mean titres had risen to very high levels in all vaccinated groups, whereas the unvaccinated controls remained seronegative. Titres remained elevated for at least 21 days post-challenge in all vaccine groups. By day 21, all animals in the control group had seroconverted,

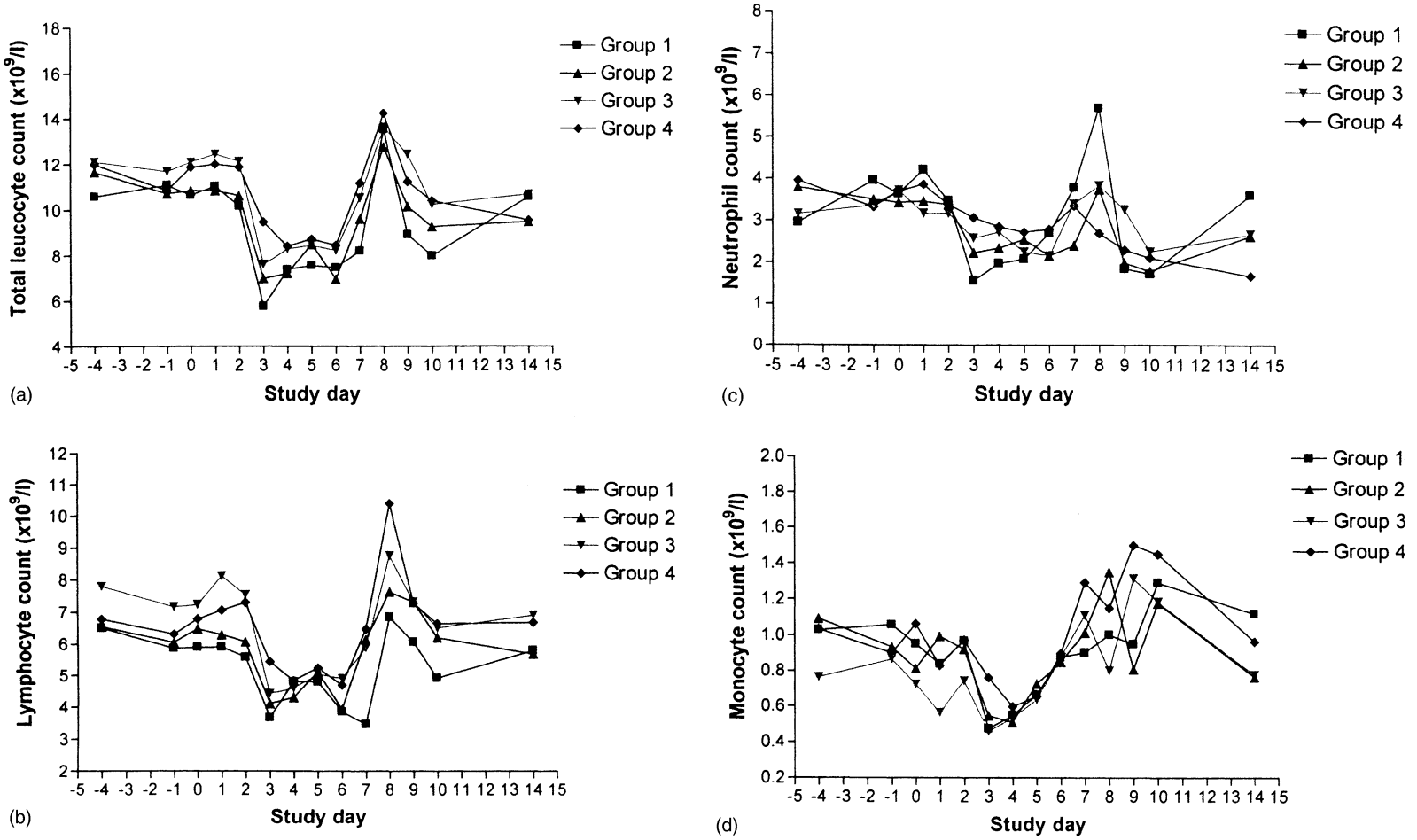

Fig. 2. Leucocyte fluctuations following viral challenge. Blood was taken daily for 10 days following challenge and leucocyte differential counts were performed on a Cell-Dyn 3000 device (Abbott); cell counts in group 1 (pSecTag + pcDNA), group 2 (pSecTag/E2 + pcDNA), group 3 (pSecTag/E2 + pcDNA/bvIL-2) and group 4 (pSecTag/E2 + pcDNA/bvGM-CSF) are shown between day −4 and day 3 for (a) leucocytes, (b) lymphocytes, (c) neutrophils and (d) monocytes.

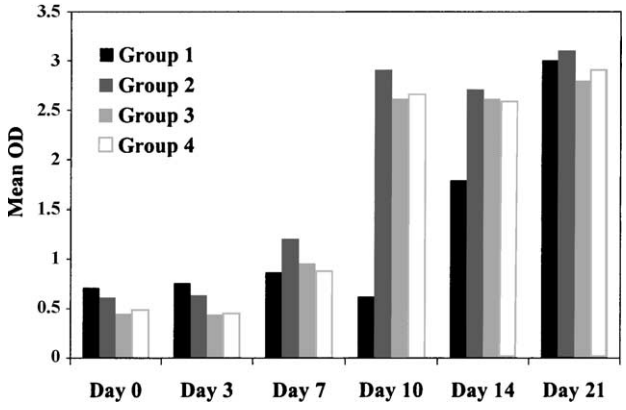

Fig. 3. Kinetics of E2 specific antibodies following viral challenge. Results are given as mean OD of pooled sera diluted 1:400 tested on the E2 specific ELISA. Group 1 (pSecTag + pcDNA), group 2 (pSecTag/E2+pcDNA), group 3 (pSecTag/E2+pcDNA/bvIL-2) and group 4 (pSecTag/E2 + pcDNA/bvGM-CSF).

and titres had risen to similar levels to that observed in the vaccine groups. IgG isotyping revealed a predominant IgG1 response in all groups (data not presented).

3.3.2. Neutralising antibody responses

Neutralising antibody titres are presented in Table 3. Immediately prior to challenge, no neutralising antibody was detectable in the control group, indicating biosecurity measures had been effective in preventing adventitious BVD infection. In contrast, low levels of neutralising antibody were detected against the homologous BVDV vaccine strain in the majority of vaccinated animals (13 out of 15 animals had measurable neutralising antibody titres). By day 7 post-challenge, no seroconversion was apparent in the control group, but there was clear evidence of a strong anamnestic response in all vaccine groups, particularly against the homologous strain. By day 10, the majority of calves in the control group had seroconverted to the homologous strain, but there was still no evidence of recognition of the heterologous strain in any individual in this group. In contrast, the majority of vaccinated animals had titres in excess of the weakest dilution tested (>1:4096) against the homologous strain, and strong titres were also apparent against the heterologous strain. For the homologous assay, statistical analysis was only performed to compare the titres at day 7, as day 0 titres were uniformly low, and day 10 sera were not titred out to an endpoint. Data from the heterologous assay was compared on days 7 and 10. In no instance was any significant difference apparent between the vaccine groups.

3.4. Antigen specific proliferation

Proliferative responses of PBMNC from all calves were measured, following in vitro re-stimulation with recombinant E2 protein or live virus. The data is presented in Fig. 4. No significant proliferation was apparent in the control group in response to any antigen. In contrast, the E2 only group showed modest increases following stimulation with recombinant protein, but not with live virus. However, proliferative

Table 3
Neutralising antibody titres following vaccination and challenge (at day 0) with Ky1203 nc virus as determined by homologous (Ky1203 nc) and heterologous (NADL) assays

	Animal number	Seroneutralisation titres					
		Ky1203 nc			NADL		
		Day 0	Day 7	Day 10	Day 0	Day 7	Day 10
Group 1 (control)	2091	0	0	45	0	0	0
	2094	0	0	22.6	0	0	0
	2106	0	0	45	0	0	0
	2110	0	0	0	0	0	0
	2118	0	0	22.6	0	0	0
Group 2 (E2 only)	2093	11	1024	>4096	0	32	362
	2098	32	1024	>4096	0	90	512
	2100	4	90.5	1448	0	0	45
	2109	64	362	>4096	0	32	724
	2116	16	512	2048	0	16	181
Group 3 (E2 + IL-2)	2089	16	512	>4096	0	64	1024
	2095	8	256	2896	0	11	256
	2102	0	724	>4096	0	22	128
	2115	5.6	64	1448	0	0	32
	2117	5.6	512	>4096	0	22	22
Group 4 (E2 + GM-CSF)	2092	11.3	724	>4096	0	45	1024
	2096	128	181	>4096	0	5	256
	2105	4	362	>4096	0	16	128
	2111	32	128	2896	0	0	32
	2120	0	128	724	0	0	22

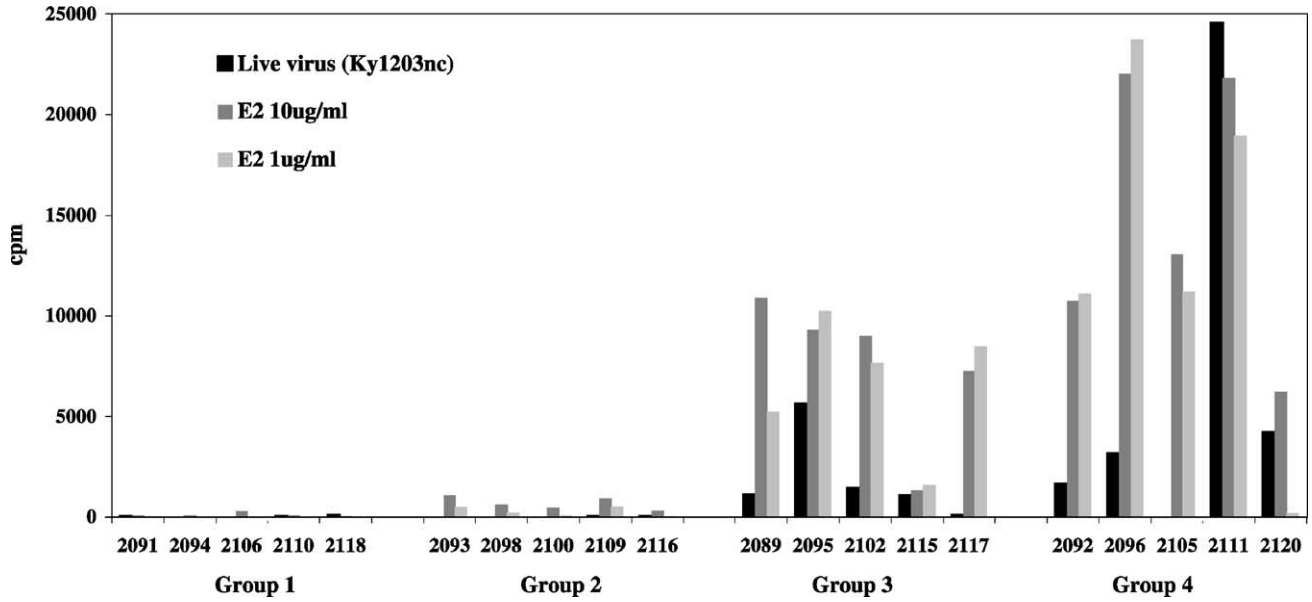

Fig. 4. Proliferation of bovine PBMNC induced by antigen stimulation. PBMNC were stimulated separately with recombinant purified E2 and live Ky1203 nc virus 9 days after challenge for group 1 (pSecTag + pcDNA), group 2 (pSecTag/E2 + pcDNA), 10 days after challenge for group 3 (pSecTag/E2 + pcDNA/bvIL-2) and group 4 (pSecTag/E2 + pcDNA/bvGM-CSF). The assay was performed on each blood sample individually and results are given as cpm for each animal.

responses were elevated in both cytokine adjuvanted groups against live virus, but enhanced proliferation was particularly apparent against recombinant protein. Statistical analysis revealed highly significant differences between the vaccinated groups and the control group with respect to their response to recombinant protein. However, no significant differences were apparent when the antigen was live virus. All vaccinated groups showed significantly higher proliferative responses compared with the control group ($P < 0.001$). Furthermore, although no statistically significant differences were apparent between the two cytokine groups, proliferative responses in both cytokine adjuvanted groups were significantly greater ($P < 0.001$) than that seen in the E2 only group, strongly indicative of enhanced priming of the cell-mediated immune response by the cytokine adjuvants.

4. Discussion

DNA vaccination potentially offers an elegant and cost effective means of providing marker vaccines for the livestock industry [8,20]. Although many challenges to practical implementation remain, the availability of such vaccines would provide valuable tools for incorporation into current and future eradication programs. In the present study, we provide the strongest evidence published to date to support the use of E2-based DNA vaccines to control BVDV infection. The value of IL-2 and GM-CSF as adjuvants for DNA vaccination in cattle is also studied.

The challenge of susceptible healthy cattle with genogroup 1 BVDV strains typically results in a transient leucopenia and a biphasic febrile response, with few overt signs of infection. The virus initially replicates in the nasal epithelium before spreading to the tonsils [21]. Thereafter, virus dissemination occurs through the blood and lymphatic system, with virus first being isolated from the blood around 3 days post-challenge. A viraemic period of 7–10 days is normally observed.

In the present study, a leucopenia was evident in all groups within 3 days of challenge. However, there was a marked and consistent trend for the maintenance of higher numbers of all cell types (lymphocytes, monocytes and neutrophils) in the GM-CSF adjuvanted group. This positive effect of vaccination was also apparent when the febrile response was examined. All vaccination regimes reduced the observed temperature increase in comparison with unvaccinated controls but the E2 only group in particular was protected from the marked pyrexia apparent in the control group. This may correlate with the slightly higher antibody levels in this group at this time point.

The reduction in cell-associated viraemia observed in all the vaccinated groups is very encouraging. Prevention of viraemia is the key efficacy parameter for a BVDV vaccine, as viraemia in a pregnant animal is likely to result in vertical transmission of infectious virus to the foetus [1,22]. Although there was evidence of limited clinical infection in some vaccinated individuals, this compares with some well-established killed BVDV vaccines which are clinically proven to protect the bovine foetus in utero. Such a vaccine succeeds in its clinical objective by preventing vertical transmission of the virus but does not provide barrier immunity against infection [22].

We assessed a range of in vitro correlates of protection to identify the mechanisms by which the DNA vaccines conferred protection, starting with humoral responses.

The importance of humoral immunity in controlling BVDV infection has been the subject of considerable research. Whilst neutralising IgG responses develop too slowly to prevent infection in naive animals, high titres of passively derived antibody confer protective immunity [3,23]. Free virus in blood and lymph is readily neutralised by antibody, and neutralising titres as low as 1:2 can be effective in this regard [4]. Following natural infection, the majority of neutralising antibody against BVDV is contained in the IgG1 fraction [24]. Interestingly, in vitro studies have indicated that IgG1 production is associated with IL-4, suggesting that this is a Th2-associated isotype in cattle [25]. Clearly, pre-existing neutralising antibody, and in particular the effective priming of humoral memory, are important factors in preventing or limiting the consequences of BVDV infection.

In the present study, serological responses post-vaccination were generally weak, though low levels of neutralising antibody were detected against the homologous virus in most individuals. There was no evidence of increased titres in the cytokine adjuvanted groups compared with the E2 only group. This was in marked contrast to the serological responses seen in mice with similar plasmid constructs [26], where co-administration of both IL-2 and GM-CSF significantly augmented humoral responses. Post-challenge, we observed a rapid increase in neutralising titres against the homologous and heterologous virus in vaccinated animals. However, there was no evidence for a beneficial effect of the cytokine adjuvants, either in terms of elevated neutralising titres, or in the number of responding individuals compared with the E2 only group. The antibody produced following challenge was predominantly IgG1. This again contrasts markedly with results observed with the corresponding plasmids in mice, where a switch to a predominant IgG2a response was observed post-vaccination [18].

Several factors may account for these differences. Firstly, the route of administration of the DNA appears to have controlled the major type of immune response induced in all DNA injected groups, outdoing the effect of the cytokines. In the present study, the predominant IgG1 response observed in vaccinated animals is broadly indicative of a Th2 bias. Such responses may be observed following intradermal injection of DNA, as opposed to a predominant Th1 response routinely observed following intramuscular injection of DNA [20,27]. Secondly, as mice are not susceptible to BVDV challenge, post-vaccination serological responses in mice were compared to post-challenge responses in cattle. Thirdly, Th1 and Th2 type responses in cattle are seldom so polarised as often described in the murine model. Individual bovine T helper clones, for example, are often capable of expressing both IFN-gamma and IL-4 [28].

In order to investigate antigen specific T-cell responses, proliferation assays were performed following in vitro re-stimulation with recombinant E2 protein. Lymphoproliferative responses were significantly higher in both cytokine adjuvanted groups compared with the E2 only group. There was a similar trend when live virus was used as the antigen, but this was not statistically significant. The magnitude of the difference between the adjuvanted and non-adjuvanted groups is significant. The possibility that the day of sampling might have influenced this result (groups 1 and 2 were sampled on day 9, groups 3 and 4 on day 10 post-challenge) whilst small, is real. However, the stimulation indices in response to concanavalin A obtained in groups 1 and 2 were slightly higher than in groups 3 and 4 (data not shown) confirming that the cells were capable of proliferation at this time and that the antigen specific proliferation results for groups 3 and 4 did reflect enhanced antigen specific priming. Unfortunately, the differences in proliferation observed are not reflected in greater clinical protection when compared to the E2 only group. The predominant BVDV specific antibody isotype in all groups was shown to be IgG1, and whilst differential cytokine responses were not measured in the present study, one can tentatively speculate that the underlying CD4+ response contained a strong Th2 like element. Studies in BVDV convalescent rather than vaccinated animals also indicate that a highly polarised Th2 type CD4+ response is dominant, with IL-4 being the dominant cytokine secreted by CD4+ T-cells following in vitro stimulation with BVDV infected monocytes [29]. However, a CD8+, IL-2/interferon gamma secreting T-cell population was also identified in that study, though cytotoxicity was not investigated. This is interesting, as in our own study, T-cells from animal 2120, which had no neutralising antibody on the day of challenge, proliferated strongly in response to in vitro re-stimulation with live virus (SI of 22), and no virus was recovered from this animal following challenge. Harpin et al. [7] also showed partial protection in a calf which failed to seroconvert after vaccination with DNA encoding E2, but which was shown to have T-cell memory. These results may indicate a significant role for cell-mediated immunity in preventing infection and dissemination of virus. This theory is further supported by studies with closely related pestiviruses. In one such study, pigs which failed to seroconvert following vaccination with classical swine fever virus (CSFV) sub-unit antigen were protected following challenge [30].

What is clear from the present study is that the availability of increased levels of both IL-2 and GM-CSF at the time of antigen expression enhanced the development of antigen specific helper T-cell responses. The mechanisms behind these effects are not clearly identified in cattle, but it is well known that in mice, GM-CSF plays a central role in dendritic cell maturation, which is likely to correlate with enhanced presentation of antigen in the local draining lymph nodes [31,32]. IL-2 on the other hand has a well-established role as an essential requirement for the development of effector T-cells, and exerts additional direct effects on the maturation of dendritic cells [33]. While the outcome of the viral chal-

lenge shows that this enhancement of T-cell responses did not result in increased control of virus replication, it must also be noted that the E2 glycoprotein is not a major T-cell target and effective control of viraemia may require priming of responses to additional antigens.

Overall, the present study has confirmed that E2-based DNA vaccination has potential for the control of BVDV infection in cattle. Furthermore, co-delivery of plasmids encoding cytokine adjuvants, particularly GM-CSF, significantly enhanced the priming of T-cell responses against the E2 antigen. While the level of protection achieved is not as significant as that obtained with an inactivated vaccine, these results, based on the delivery of a single viral antigen, are the most complete presented to date and do offer scope for further enhancement. In future, we aim to examine the phenotype of the responding T-cell population in more detail and to explore ways of further improving the efficacy of our DNA constructs, particularly with the aim of inducing stronger primary serological responses, possibly through the use of optimised CpG motifs [34], or the creation of chimeric genes encoding BVDV antigens linked to ligands such as CTLA-4 [35] and C3d [36]. Further challenge experiments involving heterologous BVDV strains and extended duration of immunity would also be of value. Although the method, route and dose for delivery still remain to be optimised, these results suggest that DNA vaccines, particularly when complemented by cytokine adjuvants, could provide a potential platform for marker vaccines in the livestock industry.

References

[1] Brownlie J, Thompson I, Curwen A. Bovine virus diarrhoea virus—strategic decisions for diagnosis and control. In Prac 2001;22:176–87.

[2] Bolin S, Moennig V, Kelso Gourley NE, Ridpath J. Monoclonal antibodies with neutralising activity segregate isolates of bovine viral diarrhea virus into groups. Arch Virol 1988;99:117–23.

[3] Howard CJ, Clarke MC, Brownlie J. Protection against respiratory infection with bovine virus diarrhoea virus by passively acquired antibody. Vet Microbiol 1989;19:195–203.

[4] Bolin SR, Ridpath JF. Glycoprotein E2 of bovine viral diarrhea virus expressed in insect cells provides calves limited protection from systemic infection and disease. Arch Virol 1996;141:1463–77.

[5] Bruschke CJ, Moormann RJ, van Oirschot JT, van Rijn PA. A subunit vaccine based on glycoprotein E2 of bovine viral diarrhea virus induces fetal protection in sheep against homologous challenge. Vaccine 1997;15:1940–5.

[6] Elahi SM, Shen SH, Talbot BG, Massie B, Harpin S, Elazhary Y. Recombinant adenoviruses expressing the E2 protein of bovine viral diarrhea virus induces humoral and cellular immune responses. FEMS Microbiol Lett 1999;177:159–66.

[7] Harpin S, Hurley DJ, Mbikay M, Talbot B, Elazhary Y. Vaccination of cattle with DNA plasmid encoding the bovine viral diarrhoea virus major glycoprotein E2. J Gen Virol 1999;80:3137–40.

[8] Babiuk LA, van Drunen Littel-van den Hurk S, Babiuk SL. Immunisation of animals: from DNA to the dinner plate. Vet Immunol Immunopathol 1999;72:189–202.

[9] Schrijver RS, Langedijk JP, Keil GM, Middel WG, Maris-Veldhuis M, van Oirschot JT, et al. Comparison of DNA application methods to reduce BRSV shedding in cattle. Vaccine 1998;16:130–4.

[10] Cantlon JD, Gordy PW, Bowen RA. Immune responses in mice, cattle and horses to a DNA vaccine for vesicular stomatitis. Vaccine 2000;18:2368–74.

[11] van Rooij EM, Haagmans BL, de Visser YE, de Bruin MG, Boersma W, Bianchi AT. Effect of vaccination route and composition of DNA vaccine on the induction of protective immunity against pseudorabies infection in pigs. Vet Immunol Immunopathol 1998;66:113–26.

[12] Scheerlinck J-PY, Casey G, McWaters P, Kelly J, Woollard D, Lightowlers MW, et al. The immune response to a DNA vaccine can be modulated by co-delivery of cytokine genes using a DNA prime-protein boost strategy. Vaccine 2001;19:4053–60.

[13] Chow YH, Huang WL, Chi WK, Chu YD, Tao MH. Improvement of hepatitis B DNA vaccines by plasmids co-expressing hepatitis B surface antigen and interleukin-2. J Virol 1997;71:169–78.

[14] Tsuji T, Hamajima H, Xin KQ, Ishii N. Enhancement of cell mediated immunity against HIV-1 induced by co-inoculation of plasmid-encoded HIV-1 antigen with plasmid expressing IL-12. J Immunol 1997;158:4008–13.

[15] Lee AH, Suh YS, Sung YC. DNA inoculations with HIV-1 recombinant genomes that express cytokine genes enhance HIV-1 specific immune responses. Vaccine 1999;17:473–9.

[16] Reddy PG, Blecha F, Minocha HC, Anderson GA, Morrill JL, Fedorka-Cray PJ, Baker PE. Bovine recombinant interleukin-2 augments immunity and resistance to bovine herpesvirus infection. Vet Immunol Immunopathol 1989;23:61–74.

[17] Hughes HP, Campos M, Godson DL, van Drunen Littel-van den Hurk S, McDougall L, Rapin N, et al. Immunopotentiation of bovine herpes virus subunit vaccination by interleukin-2. Immunology 1991;74:461–6.

[18] Nobiron I, Thompson I, Brownlie J, Collins ME. Co-administration of IL-2 enhances antigen-specific immune responses following vaccination with DNA encoding the glycoprotein E2 of bovine viral diarrhoea virus. Vet Microbiol 2000;76:129–42.

[19] Howard CJ, Brownlie J, Clarke MC. Comparison by the neutralisation assay of pairs of non-cytopathogenic and cytopathogenic strains of bovine virus diarrhoea virus isolated from cases of mucosal disease. Vet Microbiol 1987;13:361–9.

[20] Lweis PJ, Cox GJM, van Drunen Littel-van den Hurk S, Babiuk L. Polynucleotide vaccines in animals: enhancing and modulating the immune responses. Vaccine 1997;15:861–4.

[21] Bruschke CJ, Weerdmeester K, van Oirschot JT, van Rijn PA. Distribution of bovine virus diarrhoea virus in tissues and white blood cells of cattle during acute infection. Vet Microbiol 1998;64:23–32.

[22] Brownlie J, Clarke MC, Hooper LB, Bell GD. Protection of the bovine foetus from bovine viral diarrhoea virus by means of a new inactivated vaccine. Vet Rec 1995;137:58–62.

[23] Bolin SR, Ridpath JF. Assessment of protection from systemic infection or disease afforded by low to intermediate titers of passively acquired neutralising antibody against bovine viral diarrhea virus in calves. Am J Vet Res 1995;56:755–9.

[24] Howard CJ, Clarke MC, Brownlie J. An enzyme-linked immunosorbent assay (ELISA) for the detection of antobodies to bovine viral diarrhoea virus (BVDV) in cattle sera. Vet Microbiol 1985;10:359–69.

[25] Estes DM. Differentiation of B-cells in the bovine. Role of cytokines in immunoglobulin isotype expression. Vet Immunol Immunopathol 1996;54:61–7.

[26] Nobiron I, Thompson I, Brownlie J, Collins ME. Cytokine adjuvancy of BVDV DNA vaccine enhances both humoral and cellular immune responses in mice. Vaccine 2001;19:4226–35.

[27] Cohen AD, Boyer JD, Weiner DB. Modulating the immune response to genetic immunisation. FASEB J 1998;12:1611–26.

[28] Brown WC, Rice-Ficht AC, Estes DM. Bovine type 1 and type 2 responses. Vaccine 1998;17:473–9.

[29] Rhodes SG, Cocksedge JM, Collins RA, Morrison WI. Differential cytokine responses of CD4+ and CD8+ T cells in response to bovine viral diarrhoea virus in cattle. J Gen Virol 1999;80:1673–9.

[30] Rumenapf T, Stark R, Meyers G, Thiel HJ. Structural proteins of hog cholera virus expressed by vaccinia virus: further characterization and induction of protective immunity. J Virol 1991;65:589–97.
[31] Morrissey PJ, Bressler L, Park LS, Alpert A, Gillis S. Granulocyte-macrophage colony-stimulating factor augments the primary antibody response by enhancing the function of antigen-presenting cells. J Immunol 1987;139:1113–9.
[32] Fischer HG, Frosch S, Reske K, Reske-Kunz AB. Granulocyte-macrophage colony-stimulating factor activates macrophages derived from bone marrow cultures to synthesis of MHC class II molecules and to augmented antigen presentation function. J Immunol 1988;141: 3882–8.
[33] Granucci F, Vizzardelli C, Pavelka N, Feau S, Persico M, Virzi E, et al. Inducible IL-2 production by dendritic cells revealed by global gene expression analysis. Nat Immunol 2001;2:882–8.
[34] Chu RS, Targoni OS, Krieg AM, Lehmann PV, Harding CV. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. J Exp Med 1997;186:1623–31.
[35] Boyle JS, Brady JL, Lew AM. Enhanced responses to a DNA vaccine encoding a fusion antigen that is directed to sites of immune induction. Nature 1998;392:408–11.
[36] Dempsey PW, Allison ME, Akkaraju S, Goodnow CC, Fearon DT. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. Science 1996;271:348–50.