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International Journal for Parasitology 29 (1999) 1613–1625

The Australian Society for Parasitology Inc. INTERNATIONAL Journal for PARASITOLOGY

Immunogenicity of a killed whole *Neospora caninum* tachyzoite preparation formulated with different adjuvants

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Received 29 March 1999; received in revised form 5 July 1999; accepted 10 July 1999

Abstract

A killed whole *Neospora caninum* tachyzoite preparation was formulated with various adjuvants and tested for its immunogenicity in cattle. The adjuvants used were: Havlogen, a polymer of acrylic acid cross-linked with polyallylsucrose; Polygen, a non-particulate copolymer; a mixture of Havlogen and Bay R-1005, which is a preparation of free base synthetic glycolipids; and Montanide ISA 773, a water-in-oil emulsion made with a mixture of metabolisable and mineral oils. Immune responses in immunised cattle were compared with those of cattle experimentally infected with culture-derived *N. caninum* tachyzoites. The overall mean serum IFAT titres were significantly higher ($P < 0.05$) in experimentally infected cattle compared with all immunised cattle. Nonetheless, the maximum antibody titres of the immunised cattle, which were obtained following the third immunisation, were within the range of titres previously described for naturally infected cattle. The overall mean serum IFAT titres were significantly higher ($P < 0.05$) in cattle immunised with the killed tachyzoite preparation formulated with Polygen and with the mixture of Havlogen and Bay R-1005, compared with cattle immunised with the Havlogen- and Montanide-based preparations. Two of the four adjuvant preparations were able to induce cell-mediated immune responses similar to those of the experimentally infected cattle. The Havlogen-adjuvanted tachyzoite preparation elicited *N. caninum*-specific proliferation of peripheral blood mononuclear cells statistically similar ($P = 0.095$) to that of the infected animals. Peripheral blood mononuclear cells from animals immunised with the Polygen-adjuvanted tachyzoite preparation produced interferon-γ concentrations of similar magnitude ($P = 0.17$) to those from the infected animals. Polygen was one of two adjuvants that elicited the highest antibody responses, and was the only adjuvant that induced interferon-γ levels similar to those of the infected heifers.

Keywords: Adjuvants; Bovine neosporosis; Immune responses; Killed whole tachyzoite preparation; *Neospora caninum*

1. Introduction

Neosporosis, caused by the apicomplexan protozoan parasite *Neospora caninum*, is usually asymptomatic in non-pregnant cattle; however, in pregnant cattle, transplacental transmission of

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0020-7519/99/$20.00
PII: S0020-7519(99)00116-2

the parasite can occur, resulting in abortion or congenital infection [1, 2]. Bovine neosporosis is now recognised as a major cause of abortion in dairy cattle in several countries worldwide [3]. To date, there is no known effective treatment for bovine neosporosis [3, 4]. Consequently, improved means of control are needed to reduce the economic impact of neosporosis on dairy productivity.

Despite the importance of this disease, studies of the immune responses following experimental *N. caninum* infection have only recently been reported. Khan et al. [5] presented evidence that cell-mediated immunity (CMI) is involved in the resistance of inbred A/J mice to *N. caninum* infection, and that interferon-γ (IFN-γ) and interleukin-12 (IL-12) partially mediate this immunity. Splenocytes from *N. caninum*-infected A/J mice proliferate and produce IFN-γ and IL-12 when grown in the presence of *N. caninum* lysate antigen [5]. Moreover, mice treated with antibodies against IFN-γ or IL-12 become susceptible to *N. caninum* infection [5]. More recently, Lundén et al. [6] showed that peripheral blood mononuclear cells (PBMC) from experimentally infected cattle proliferate and produce IFN-γ, when stimulated with a *N. caninum* lysate antigen. Whether these cellular immune responses correlate with immunity to *N. caninum* infection in cattle remains to be determined. Whether similar immune responses are induced in cattle following immunisation with inactivated *N. caninum* tachyzoite antigens is also unknown. Therefore, the objective of the present study was to investigate if cattle would respond immunologically to a killed whole *N. caninum* tachyzoite preparation formulated with four different adjuvants and to compare limited immune parameters of immunised cattle with those of experimentally infected cattle.

2. Materials and methods

2.1. Animals and study design

Thirty-six heifers, 12–18 months old, seronegative for *N. caninum* by an IFAT [7], were selected. They were given decoquinate (Deccox, Rhône Poulenc Animal Nutrition) and vaccinated against common abortifacient pathogens (Horizon 10, Bayer) prior to the experiment. All treatments and vaccinations were terminated at least 45 days prior to the beginning of the experiment. Heifers were then randomly assigned to six treatment groups of six animals each. One group was infected with culture-derived *N. caninum* tachyzoites as previously described [8]. One uninfected control group was inoculated with uninfected Vero cell cultures (African Green Monkey Kidney cells). The next four groups were inoculated three times at 4-week intervals with a killed whole *N. caninum* tachyzoite preparation formulated with four different adjuvants: adjuvant 1 was Havlogen, adjuvant 2 was Polygen, adjuvant 3 was a mixture of Havlogen and Bay R-1005, and adjuvant 4 was Montanide ISA 773. Throughout the experiment, the infected heifers were kept in separate pens from the control and immunised heifers.

Blood samples were collected by jugular venipuncture at 2-week intervals for 18 weeks beginning 4 weeks prior to infection or immunisation. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Administrative Advisory Committee of the University of California at Davis.

2.2. Neospora caninum *tachyzoites preparation for animal infection, and infection protocol*

Tachyzoites of the *N. caninum* isolate BPA1, originally obtained from the brain of an aborted bovine foetus [9], were harvested from Vero cell monolayers when 80% of the cells were infected. Cells were washed and disrupted through passage into a 22-gauge blunt needle. The i.v. inoculum was further filtered through a sterile 5-μm pore-size filter. Each heifer of the infected group was inoculated i.v. (3×10^7) and i.m. (5×10^7) with tachyzoites resuspended in 5 ml of minimum essential medium. Control heifers were inoculated i.v. and i.m. with an amount of uninfected Vero cells equivalent to that received by the infected group. Each inoculum was given to the cow within 90 min of cell harvesting.

2.3. Preparation of N. caninum tachyzoite lysate

Neospora caninum tachyzoite lysate, prepared from the BPA1 isolate, was used as antigen in cultures of PBMC for proliferation and IFN-γ assays. Tachyzoite-infected Vero cells were harvested, washed in sterile PBS (pH 7.2), and passed through a 22-gauge needle to rupture the cells.

Neospora caninum tachyzoites were purified from contaminating Vero cells by passage through a Sephadex G-25M containing PD-10 column (Pharmacia), washed and stored at −80°C until preparation of the antigen. Briefly, pelleted tachyzoites were pooled, resuspended in cold sterile water containing a mixture of protease inhibitors (complete™ protease inhibitor cocktail, Boehringer-Mannheim) and disrupted by four freeze–thaw cycles. Tachyzoite lysates were further sonicated on ice with 5×20 s bursts at maximum setting using a sonic dismembrator model 300 (Fisher Scientific). Lysates were clarified by centrifugation at 2000 *g* at 4°C for 15 min, supernatant was collected and filtered through a 0.22-μm pore-size filter. A lysate of uninfected Vero cells was prepared the same way as the tachyzoite lysate and used as a control antigen. The protein concentration of the lysates was determined using the bicinchoninic acid (BCA) protein assay method (Pierce). Lysates were stored in aliquots at −80°C.

2.4. Killed N. caninum tachyzoite preparation and immunisation protocol

Antigen for the immunisations was prepared using proprietary methods. Partially purified tachyzoites of the BPA1 isolate were inactivated with 0.01 M (final concentration) of binary ethylenimine [10] for a period of 96 h at 4°C, followed by neutralisation with sodium thiosulfate. One millilitre of the inactivated *N. caninum* tachyzoite preparation, estimated to contain 400 μg ml^{-1} of protein by the BCA assay, was incorporated into each 5 ml dose of antigen and adjuvant formulation as follows.

Each dose of Havlogen-adjuvanted preparation consisted of 1 ml of *N. caninum* antigen mixed with 0.5 ml of Havlogen and 3.5 ml of PBS. Havlogen is a preparation of Carbopol 934P cross-linked with polyallylsucrose (BF Goodrich Specialty Chemicals) and emulsifier, as described in US Patent 3 919 411 (Bayer). Carbopol is a polymer of acrylic acid. Each dose of Polygen-adjuvanted preparation was formulated by mixing 1 ml of *N. caninum* antigen with 0.75 ml Polygen and 3.25 ml of PBS. Polygen (MVP Laboratories) is described as a preparation of low molecular weight, non-particulate copolymer which can form cross-linkages in solution to become a high molecular weight gel. Each dose of Havlogen and Bay R-1005-adjuvanted preparation consisted of 1 ml of *N. caninum* antigen mixed with 0.5 ml Havlogen and 3.5 ml of Bay R-1005. Bay R-1005 (Bayer AG) is a preparation of free base synthetic glycolipids [*N*-(2-deoxy-2-L-leucyloamino-β-D-glucopyranosyl)-*N*-octadecyl-dodecanoylamine hydroacetate] forming micelles in aqueous solution. Each dose of Montanide ISA 773-adjuvanted preparation consisted of 1 ml of *N. caninum* antigen mixed with 3.5 ml of Montanide ISA 773 and 0.5 ml of PBS. Montanide ISA 773 is a preparation of pharmaceutical injectable oils (metabolisable and mineral oil) and highly refined emulsifier obtained from mannitol and purified oleic acid (SEPPIC).

Each 5 ml dose was injected s.c., three times at 4-week intervals, on alternate sides of the neck using a 1-inch 16-gauge needle. Reactions at injection sites were evaluated visually and by palpation following each immunisation.

2.5. Indirect fluorescent antibody test

Serum samples were assayed for *N. caninum*-specific antibodies as previously described [7], using a fluorescein isothiocyanate (FITC)-labelled affinity-purified rabbit anti-bovine IgG (Jackson ImmunoResearch Laboratories). The end-point titre was the last serum dilution showing distinct, whole parasite fluorescence.

2.6. Lymphocyte proliferation assay

Blood, collected in acid citrate dextrose-vacutainer tubes, was diluted 1:1 in Alsever's solution

(Sigma Chemical) and layered on Ficoll-Paque Plus (Pharmacia). After centrifugation at 900 *g* for 30 min at 22°C, PBMC were collected from the interface layer, washed three times in Alsever's solution and once in RPMI 1640 (Gibco BRL) supplemented with 10% FBS (Hyclone), 2 mM L-glutamine (Gibco BRL), 10 mM *N*-2-hydroxyethylpiperazine- *N'*-2-ethanesulfonic acid (HEPES; Gibco BRL), 5×10^{-5} M 2-mercaptoethanol, 100 IU ml^{-1} penicillin and 100 $\mu g\,ml^{-1}$ streptomycin (Sigma) (RPMI-FBS). Peripheral blood mononuclear cells were seeded in triplicates at 1×10^5 cells per well in 96-well flat-bottomed plates (Costar) in RPMI-FBS alone (unstimulated cells), or with 1 $\mu g\,ml^{-1}$ of the T-cell mitogen Con A (Con A-stimulated cells; Sigma C5275), or with 20 $\mu g\,ml^{-1}$ of *N. caninum* tachyzoite lysate (*N. caninum*-stimulated cells), or with 20 $\mu g\,ml^{-1}$ of Vero cell lysate (Vero-stimulated cells). Cells were incubated at 37°C, in a humidified atmosphere of 5% CO_2 for 3 days (for mitogen) and 5 days (for antigen). The concentrations of mitogen and lysate antigen, as well as the incubation times, were predetermined to be optimal for maximum proliferation. DNA synthesis was measured by the incorporation of bromodeoxyuridine (BrdU) using the cell proliferation ELISA, BrdU colourimetric kit (Boehringer-Mannheim) with the following conditions. Cells were labelled for 2 h with 20 μl of the BrdU labelling solution. The monoclonal anti-BrdU conjugated with peroxidase was added for 90 min. The enzyme substrate tetramethylbenzidine was incubated for 10 min. The enzymatic reaction was stopped and O.D. read at a dual wavelength of 450/650 nm using a UVmax kinetic microplate reader (Molecular Devices Corporation). The results were expressed as stimulation indices (SI), calculated as the ratios between the mean O.D. of cells cultured with mitogen or antigen and the mean O.D. of cells cultured with medium alone. In some cases, PBMC were depleted of adherent cells before being tested in the proliferation assay. For that purpose, 2×10^6 cells ml^{-1} of PBMC in RPMI-FBS were incubated for 2 h at 37°C, non-adherent cells (NAC) were aspirated, counted and seeded as described above for PBMC.

2.7. Interferon-γ assay

Duplicate plates of *N. caninum*-, Con A-, and Vero-stimulated PBMC, prepared as described above, were incubated for 5 days. Plates were centrifuged at 400 *g* for 10 min, supernatants collected and stored at −80°C. Supernatants were assayed in duplicate using a commercial sandwich ELISA for bovine IFN-γ (IDEXX). Concentrations of IFN-γ were calculated using a standard curve obtained with serial dilutions of a recombinant bovine IFN-γ (Novartis). Interferon-γ concentrations in supernatants from cells grown with medium alone were subtracted from concentrations in supernatants from Con A- or *N. caninum*-stimulated cells. Data were expressed in ng ml^{-1}.

2.8. Statistical analysis

Serum antibody responses (IFAT titres) and CMI responses (stimulation indices SI, IFN-γ production) were analysed using one-way repeated measures analysis of variance (ANOVA) with treatment as the grouping factor and time as the repeated measures factor [11]. Data were $\log_{10}$-transformed before statistical analyses were performed using Minitab 9.2 for Windows (Minitab). Post hoc Tukey's pairwise comparisons were performed when significant differences between treatment groups were detected. All statistical analyses were considered significant at the $P < 0.05$ level.

3. Results

No clinical signs were apparent in the infected, immunised or control heifers. No reactions were observed or palpated at the site of inoculation of any of the four killed tachyzoite formulations used in this trial experiment.

3.1. Serum antibody responses

All 36 heifers were seronegative with a titre $\leq$1:80 prior to infection and immunisation. *Neospora caninum*-specific antibody responses

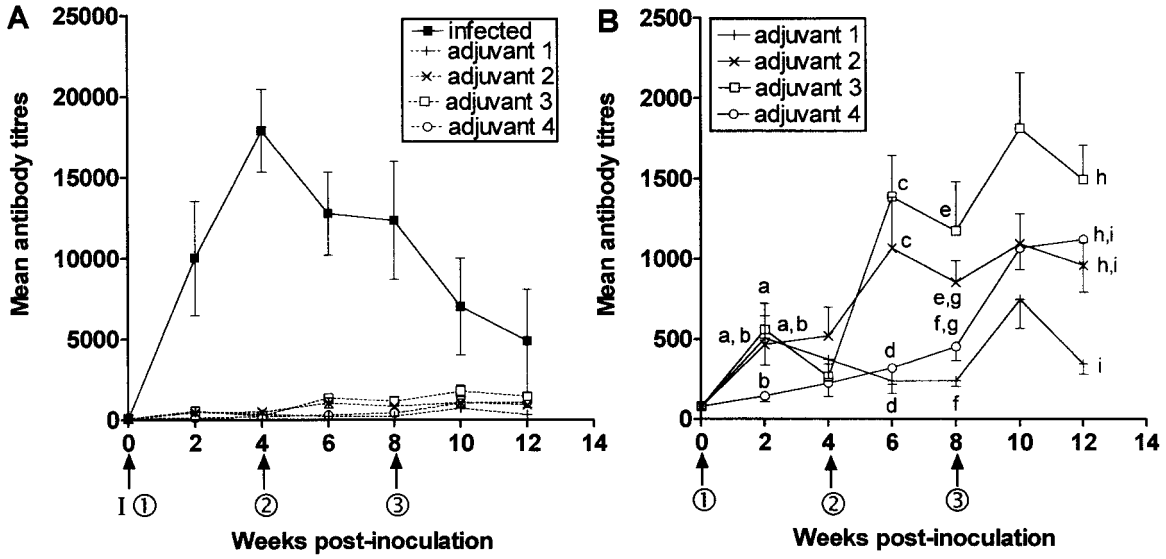

Fig. 1. Time course of the serum antibody response of heifers experimentally infected with culture-derived *Neospora caninum* tachyzoites and heifers immunised with a killed whole *N. caninum* tachyzoite preparation formulated with different adjuvants. Six heifers were infected, as described in Materials and methods, at week 0 (indicated by the letter I). For immunised groups, primary immunisation was given at week 0 (circled 1) and booster immunisations at week 4 (circled 2) and week 8 (circled 3). (A) The same scale for the mean reciprocal antibody titres from infected and immunised heifers, showing significantly higher titres ($P < 0.05$) in infected animals compared with immunised ones at all time-points except week 12. (B) The mean reciprocal antibody titres of heifers from the four adjuvant vaccine groups ($n = 6$ per adjuvant group) on a different scale. Results were expressed as mean titres $\pm$ S.E.M. Statistical analyses were performed on $\log_{10}$-transformed values using repeated measures ANOVA. At each time-

increased in tachyzoite-infected cattle by week 2 p.i., reached a maximum at week 4 p.i. (mean titres $\pm$ S.D.: 17 920 $\pm$ 5724), then steadily decreased to a minimum of 4907 $\pm$ 7133 (mean $\pm$ S.D.) by the end of the experiment at week 12 (Fig. 1A). All Vero-inoculated control heifers remained seronegative (titre $\leq$1:80) throughout the experiment. Fig. 1B shows the serum antibody responses of the immunised animals on a lower scale than Fig. 1A. The earliest antibody responses occurred in the immunised animals by week 2 after the first immunisation. Maximum antibody responses in all immunised groups were obtained 2 weeks after the third immunisation, with mean antibody titres ranging from 747 to 1813 (Fig. 1B). The serological response of the immunised animals was significantly lower ($P < 0.05$) than that of the tachyzoite-infected cattle at weeks 2, 4, 6, 8 and 10 p.i. (Fig. 1A). The overall mean antibody titres were significantly greater ($P < 0.05$) in animals that received the adjuvants preparations 2 and 3 compared with those animals in adjuvants 1 and 4. Tukey's pairwise comparisons revealed that antibody titres of adjuvant 3 group were significantly higher ($P < 0.05$) than those of adjuvant 4 group at weeks 2, 6 and 8. Antibody titres of adjuvant 2 group were significantly higher ($P < 0.05$) than those of adjuvant 4 group at week 6. Antibody titres of adjuvants 2 and 3 groups were significantly higher ($P < 0.05$) than those of adjuvant 1 group at week 8. Mean antibody titres were not statistically different between the adjuvants groups 2 and 3 ($P = 0.147$) or between the adjuvants groups 1 and 4 ($P = 0.605$). Significant interaction between treatment group and time ($P < 0.001$) and significant individual variation within each adjuvant group

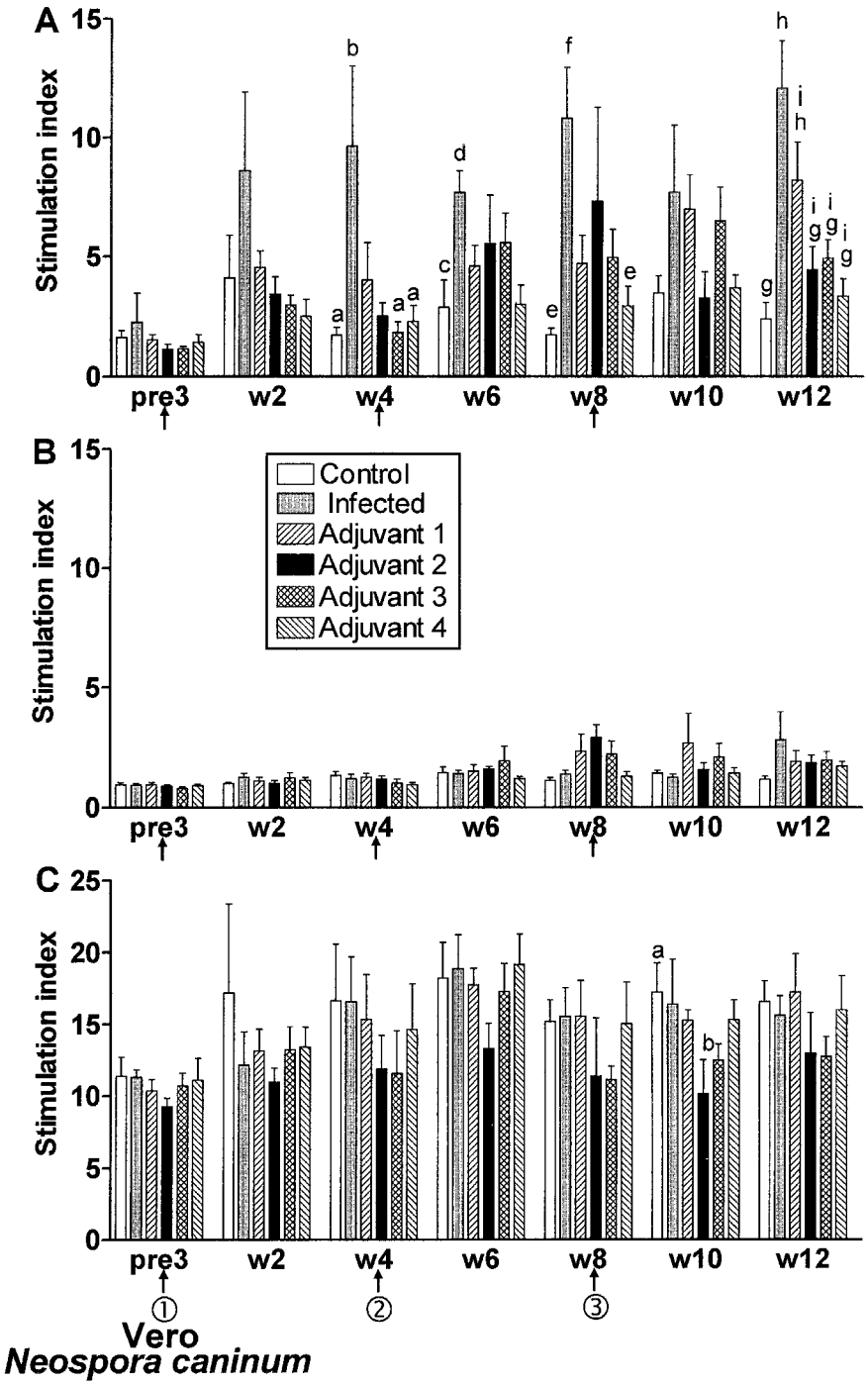

Fig. 2—*Caption opposite*

($P < 0.05$) were observed following immunisation.

3.2. Peripheral blood mononuclear cells proliferative responses to N. caninum lysate

There were no significant differences among treatment groups ($P = 0.798$) prior to infection and immunisation. Specific *N. caninum*-induced PBMC proliferation was detected by week 2 p.i. in four tachyzoite-infected animals, while it did not occur until week 4 p.i. in the other two infected animals. Mean PBMC proliferation remained elevated in the infected animals until the end of the experiment at week 12 p.i. (Fig. 2A). The mean PBMC proliferative responses of the infected animals were significantly higher ($P < 0.05$) than those of the Vero-inoculated controls at weeks 4, 6, 8 and 12 p.i.

Only immunised animals which received adjuvant 1 had significantly higher SI ($P < 0.05$) than the Vero-inoculated controls at week 12. Moreover, the animals immunised with the adjuvant 1 vaccine had mean SI that were statistically similar ($P = 0.095$) to those of the infected animals at all time-points. All six animals in the adjuvant 1 group had SI above the cut-off value following the third immunisation. Peripheral blood mononuclear cells from the adjuvant 4 group had significantly lower SI ($P < 0.05$) than those from infected animals at weeks 4, 6, 8 and 12. Animals from the adjuvants groups 2 and 3 had significantly lower ($P < 0.05$) proliferative responses than the infected animals at week 12. The magnitude of the *N. caninum*-specific proliferative responses was highly variable among the individual animals in the various adjuvant groups ($P < 0.05$). As a result, PBMC proliferative responses were not significantly different ($P > 0.05$) at all time-points among the animals that received adjuvants 2, 3 or 4. However, at week 12, cattle immunised with the adjuvant 1 preparation had significantly higher ($P < 0.05$) proliferative responses than cattle immunised with the adjuvant 4 preparation (Fig. 2A).

Prior to infection or immunisation, SI above the cut-off value 3.7 (calculated as the mean of all 108 pre-infection and pre-immunisation SI values + 2 S.D.) were observed in *N. caninum*-stimulated PBMC from three out of the 36 heifers. *Neospora caninum* tachyzoite lysate also occasionally stimulated PBMC from some of the Vero-inoculated control animals with SI above the cut-off value. The reason for this non-specific proliferative response was not known, but the possibility that *N. caninum* antigen could be mitogenic for lymphocytes was further investigated. Table 1 shows that removal of adherent cells from PBMC prior to culture resulted in a significant decrease in proliferative responses to *N. caninum* antigen, while it did not affect, or in some cases even marginally increased, the proliferation to Con A.

Fig. 2. Time course of the proliferative response of peripheral blood mononuclear cells stimulated with 20 $\mu g\,ml^{-1}$ of *Neospora caninum* tachyzoite lysate (A), 20 $\mu g\,ml^{-1}$ of Vero cell lysate (B) and 1 $\mu g\,ml^{-1}$ of Con A (C) in heifers experimentally infected with culture-derived *N. caninum* tachyzoites, heifers immunised with a killed whole *N. caninum* tachyzoite preparation formulated with four different adjuvants and control uninfected heifers. Six heifers were inoculated with uninfected Vero cell culture material (control) and six were infected with tachyzoites (infected), as described in Materials and methods, at week 0 (indicated by Vero and *Neospora caninum*, respectively). For immunised groups ($n = 24$, 6 per adjuvant group), primary immunisation was given at week 0 (circled 1) and booster immunisations at week 4 (circled 2) and week 8 (circled 3). Mononuclear cells were cultured with antigen for 5 days and concanavalin A for 3 days, and DNA synthesis measured by the incorporation of bromodeoxyuridine as described in Materials and methods. Data were expressed as stimulation indices ±S.E.M.. Statistical analyses were performed on $\log_{10}$-transformed values using repeated measures ANOVA. At each time-point, different letters above bars indicate significant difference ($P < 0.05$) based on post hoc Tukey's pairwise comparison. Mean background O.D.s (in the absence of antigen or mitogen) range from 0.07 to 0.12 for the controls, from 0.06 to 0.31 for the infected, from 0.06 to 0.11 for the adjuvant 1, from 0.1 to 0.64 for the adjuvant 2, from 0.07 to 0.21 for the adjuvant 3, and from 0.07 to 0.12 for the adjuvant 4 groups. At weeks 10 and 12, stimulation indices range from 0.82 to 5.82 for the controls, from 1.2 to 20.98 for the infected, from 2.6 to 15 for the adjuvant 1, from 1.61 to 8.56 for the adjuvant 2, from 1.71 to 10.45 for the adjuvant 3, and from 1.6 to 5.37 for the adjuvant 4 groups. Pre = pre-infection or pre-immunisation; w = week following infection or first immunisation.

Table 1
The proliferation of peripheral blood mononuclear cells from *Neospora*-infected and immunised cattle to *Neospora* lysate antigen was dependent on the presence of adherent cells

Treatment group		Stimulation indices (SI) in cultures with:	
		Neospora lysate antigen	Concanavalin A
Control	PBMC	2.96	13.92
	NAC	1.18	15.33
Infected	PBMC	16.46	16.71
	NAC	1.45	10.73
Adjuvant 1	PBMC	11.53	24.97
	NAC	1.91	27.17
Adjuvant 2	PBMC	4.91	15.1
	NAC	1.4	18.52
Adjuvant 3	PBMC	8.04	11.74
	NAC	1.15	15.58
Adjuvant 4	PBMC	5.42	13.38
	NAC	1.15	16.13

PBMC = peripheral blood mononuclear cells; NAC = non-adherent cells, removed after incubation of PBMC on plastic flasks for 2 h at 37°C. Peripheral blood mononuclear cells or NAC were seeded at 1×10^5 cells per well in medium containing 1 μg ml^{-1} of Con A or 20 μg ml^{-1} of *Neospora* tachyzoite lysate, and incorporation of bromodeoxyuridine into DNA synthesis was measured after 5 days, as described in Materials and methods. Stimulation indices were defined as the ratios between the mean O.D. of cells cultured with *Neospora* tachyzoite lysate antigen or Con A and the mean O.D. of cells cultured with medium alone. Data are given from one representative animal of each treatment group.

3.3. Peripheral blood mononuclear cell proliferative responses to Vero cell lysate and Con A

Uninfected Vero cell lysate was used as a control antigen in the assay. Peripheral blood mononuclear cell proliferative responses to the Vero cell lysate with SI > 3 were randomly observed in animals of the various treatment groups. However, in all cases the SI were markedly lower than those observed in PBMC stimulated with *N. caninum* tachyzoite lysate (Fig. 2B).

There were no group differences in PBMC responses to Con A stimulation prior to and following infection and immunisation, except at week 10 when the PBMC mitogenic responses were significantly lower ($P < 0.05$) in the adjuvant 2 group when compared with the Vero control group (Fig. 2C). These results suggest that, except for the case of adjuvant 2 group at week 10, the functional integrity of the lymphocytes was not affected by experimental infection or immunisation.

Spontaneous PBMC proliferation in the absence of mitogen or antigen was observed occasionally (at one or two time-points post-infection and immunisation) in two infected animals and two animals from the adjuvant 3 group, and more consistently (five to six time-points post-immunisation) in three animals from the adjuvant 2 groups. Because of this high background, negligible SI were observed in those animals following stimulation with either mitogen or antigen. However, removal of adherent cells from PBMC resulted in decrease of the background and appearance of a good SI to both mitogen and antigen. Decrease in background and increase in SI were also observed when lower cell concentrations (5×10^4 cells per well) were used (data not shown).

3.4. Production of IFN-γ in N. caninum*-stimulated PBMC*

No group differences ($P > 0.05$) in IFN-γ levels produced by *N. caninum*-stimulated PBMC were observed prior to infection and immunisation.

Interferon-γ production was increased as early as week 2 p.i. in infected animals, reached a maximum at week 8 p.i. and slightly decreased thereafter (Fig. 3A). Interferon-γ levels were significantly higher ($P < 0.001$) in infected animals at weeks 4, 6, 8, 10 and 12 p.i. compared with the Vero controls. Production of IFN-γ in *N. caninum*-stimulated PBMC was also increased following immunisation (Fig. 3A). Interferon-γ levels from the animals that received the adjuvant 2 preparation were significantly higher ($P < 0.05$) than those from the Vero controls at weeks 6 and 12. Interferon-γ levels were significantly higher ($P < 0.05$) at week 6 in animals of the adjuvant 3 group compared with the control group. Animals that received the adjuvant 4 preparation had IFN-γ levels significantly lower ($P < 0.05$) than those of the infected animals at weeks 4, 6, 8, 10 and 12. Animals that received the adjuvant 1 preparation had significantly lower IFN-γ levels ($P < 0.05$) than those of the infected animals at week 6. Animals that received the adjuvant 3 preparation had levels of IFN-γ significantly lower than those of the infected animals at week 4. Only animals that received the adjuvant 2 preparation had levels of IFN-γ statistically similar ($P = 0.17$) to those of the infected animals at all time-points. However, IFN-γ was never produced in one of six animals in this group.

Due to considerable individual variation, no significant differences ($P > 0.1$) in IFN-γ production were observed among animals in the adjuvant 1, 2 and 3 preparation groups. Cattle in the adjuvant 2 group had significantly higher levels ($P < 0.05$) of IFN-γ than the animals which received adjuvant 4 preparation at weeks 10 and 12.

3.5. Production of IFN-γ in Vero lysate- and Con A-stimulated PBMC

At all time-points throughout the experiment, the production of IFN-γ in PBMC stimulated with Vero cell lysate was markedly lower than that observed in PBMC stimulated with *N. caninum* lysate (Fig. 3B).

There were no significant group differences ($P > 0.05$) in IFN-γ levels produced by Con A-stimulated PBMC prior to and following infection and immunisation (Fig. 3C).

4. Discussion

Although CMI is the major component of the immune response to intracellular protozoan infections, including infection with the closely related protozoan *Toxoplasma gondii* [12], a protective role of humoral responses has also been reported. Passive transfer of antiserum from *T. gondii*-immune animals has been shown to confer protection in naive recipient animals [13, 14]. A recent paper reported that B-cell deficient C57BL/6 mice become susceptible to *N. caninum* infection [15], suggesting a role for B cells and/or antibodies in resistance of mice to *N. caninum* infection. Whether antibodies play a protective role in *N. caninum* infection in cattle remains to be investigated. In the present study, the serum IFAT titres of immunised cattle were significantly lower than those of the experimentally infected cattle, though they were within the range of titres previously observed in naturally infected cattle [7]. This difference in antibody titres may be due partly to the route of delivery of the antigen, via i.v. and i.m. inoculation for the experimental infection and s.c. administration for the immunisation. Another explanation for the difference in antibody titres between experimentally infected and immunised cattle is the nature of the inoculum, i.e. live tachyzoites in experimentally infected cattle vs killed tachyzoites in immunised cattle.

In this study, limited cell-mediated immune responses were assessed by measuring proliferative responses and production of IFN-γ by PBMC in response to *N. caninum* lysate antigen. The population of lymphocytes in peripheral blood is not representative of all lymphoid and non-lymphoid organs of the peripheral immune system [16]; however, the requirements of this study did not allow for the acquisition of sequential biopsies of spleen or lymph nodes. Flow cytometry was performed to analyse cell surface phenotypes in per-

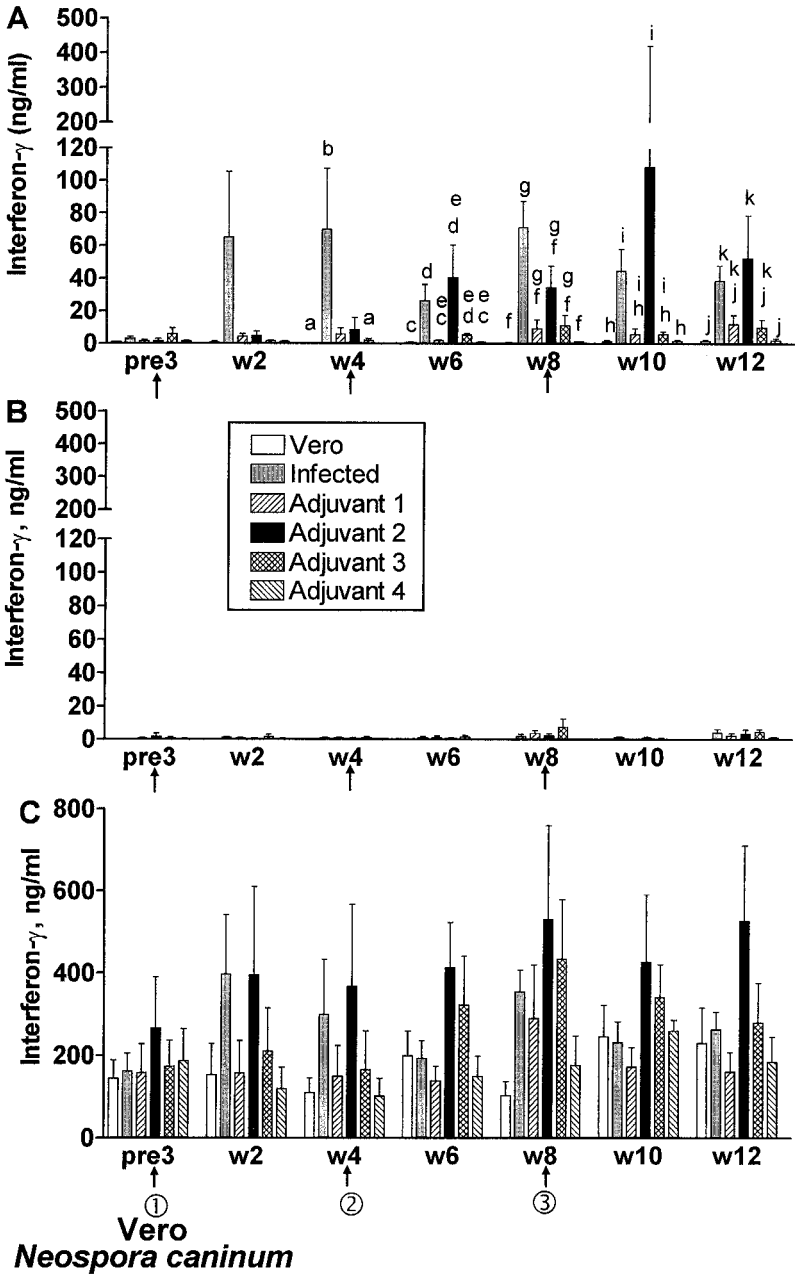

Fig. 3—*Caption opposite*

ipheral blood. However, there were no changes in any of the T-cell subsets following *N. caninum* infection and no clear trends in lymphocyte parameters following immunisation (data not shown).

Significant *N. caninum*-specific PBMC proliferative responses were induced in infected (weeks 4, 6, 8 and 12 p.i.) and adjuvant 1 group (week 12) cattle as compared with the Vero controls. Animals which received adjuvant 1 had proliferative responses similar to those of the infected animals at all time-points. The phenotype of these *N. caninum*-responsive cells, as well as the nature of the *N. caninum* antigens which triggered their proliferation, still remain to be determined. Previous reports indicate that $CD4^+$ T cells from experimentally infected cattle proliferate when stimulated with low molecular weight *N. caninum* antigens [17].

The reason why proliferative responses to *N. caninum* antigen were also induced in some Vero controls and in some heifers prior to infection or immunisation is not known, though similar observation of non-specific proliferation to a *N. caninum* lysate was previously reported [6]. The possibility that *N. caninum* could simply act as a T lymphocyte mitogen is unlikely, as the PBMC proliferative responses to *N. caninum* antigen were dependent on the presence of adherent cells (Table 1), mainly composed of monocytes, in peripheral blood and probably acting as antigen-presenting cells. This dependence on antigen-presenting cells may explain the longer optimal time (5 days vs 3 days for the mitogen Con A) for PBMC to proliferate when stimulated with *N. caninum* lysate. It is also possible that the whole *N. caninum* tachyzoite lysate used as antigen in the proliferation assay contained cross-reactive T-cell epitopes from closely related parasites, notably the agents of bovine sarcocystosis or coccidiosis, which are two very common parasitoses in cattle [18, 19]. It is noteworthy that despite these proliferative responses in our control animals, the proliferative responses of the infected animals were significantly greater than those of the control uninfected animals. Interestingly, *T. gondii* antigens have also been reported to induce lymphocyte proliferation in naive subjects [20, 21].

Significant levels of IFN-γ were produced in cultures of *N. caninum*-stimulated PBMC from infected animals (weeks 4, 6, 8, 10 and 12 p.i.) and some immunised animals (adjuvant 2 group at weeks 6 and 12; adjuvant 3 group at week 6). Animals which received adjuvant 2 even had levels of IFN-γ comparable with those of the infected animals at all time-points. The importance of IFN-γ in host resistance against *N. caninum* infection was indicated by the recent work of Khan et al. [5], in which mice treated with an antibody against IFN-γ became susceptible to the disease. Interferon-γ has also been shown to inhibit intracellular multiplication of *N. caninum* in vitro [22]. Previous experiments using in vivo treatment of mice with antibody against IFN-γ confirmed the critical role of this cytokine in the control of both acute [23] and chronic [24] *T. gondii* infection. The specific cell(s) secreting IFN-γ and the role of this cytokine in vivo in cattle with natural *N. caninum* infection remains to be determined. However, $CD4^+$ T cells from experimentally infected cattle were shown to pro-

Fig. 3. Time course of interferon-γ production in peripheral blood mononuclear cells stimulated with 20 $\mu g\,ml^{-1}$ of *Neospora caninum* tachyzoite lysate (A) 20 $\mu g\,ml^{-1}$ of Vero cell lysate (B) and 1 $\mu g\,ml^{-1}$ of Con A (C). Six heifers were inoculated with uninfected Vero cell culture material (control) and six were infected with tachyzoites (infected), as described in Materials and methods, at week 0 (indicated by Vero and *Neospora caninum*, respectively). For immunised groups ($n = 24$, 6 per adjuvant group), primary immunisation was given at week 0 (circled 1), and booster immunisations at week 4 (circled 2) and week 8 (circled 3). Values represent mean interferon-γ in $ng\,ml^{-1} \pm$ S.E.M. Statistical analyses were performed on $\log_{10}$-transformed values using repeated measures ANOVA. At each time-point, different letters above bars indicate significant difference ($P < 0.05$) based on post hoc Tukey's pairwise comparison. At weeks 10 and 12, interferon-γ levels range from 0 to 4.2 $ng\,ml^{-1}$ for the controls, from 2 to 83 $ng\,ml^{-1}$ for the infected, from 0.4 to 36 for the adjuvant 1, from 0.8 to 475 for the adjuvant 2, from 0 to 11 for the adjuvant 3, and from 0 to 6.4 for the adjuvant 4 groups. Pre = pre-infection or pre-immunisation; w = week following infection or first immunisation.

duce IFN-γ when stimulated in vitro by *N. caninum* antigens [17].

This first adjuvant trial shows that a killed whole *N. caninum* tachyzoite preparation induced different immune responses depending on the adjuvant formulation. Although the mechanism of action of adjuvants remains mainly speculative, a recent review [25] describes five ways in which an adjuvant can exert its effects: immunomodulation, antigen presentation, induction of $CD8^+$ cytotoxic T-lymphocyte (CTL) responses, antigen targeting and depot generation.

The Montanide adjuvants are commercially available oil adjuvants (Seppic) for water-in-oil emulsions, and are approved for use in veterinary vaccines. Water-in-oil emulsions form a short-term depot at the site of delivery of the antigen, attracting macrophages or other antigen presenting cells to the site and thus resulting in proper targeting of the antigen [25]. The Montanide adjuvants have been used in vaccine trials against several micro-organisms, including *Plasmodium falciparum* [26], and have been found in all cases to induce a better antibody response than the other adjuvants tested. Cellular immune responses with induction of cytotoxic T-lymphocytes have also been induced by Montanide ISA 720-adjuvanted vaccine [27]. However, in our study, heifers that received the Montanide ISA 773 (adjuvant 4) vaccine had amongst the lowest levels of antibody responses. Furthermore, Montanide ISA 773 failed to induce proliferative responses higher than those of the Vero controls at any time-point, and it was one of two adjuvants that did not elicit IFN-γ levels higher than those of the Vero controls.

Havlogen is believed to act as a long-term depot adjuvant, slowly releasing the antigen as the adjuvant dissociates, resulting in a sustained immune response. Due to the added carbohydrate polyallylsucrose and the emulsion system, Havlogen may also target the antigen to antigen-presenting cells [25, 28]. In the present study, Havlogen was able to induce proliferative responses comparable with those of the infected animals. However, the elicited antibody responses were amongst the lowest and the production of IFN-γ was never higher than that of the Vero controls.

Adjuvant 3 is a mixture of Havlogen and Bay R-1005. Bay R-1005 is not a B-cell mitogen, but rather acts as a second signal following the antigenic stimulus [29]. The potentiating effect of Bay R-1005 on humoral immune response may explain the high antibody titres observed in the present study in heifers receiving the combined Havlogen-Bay R-1005 adjuvant, as Havlogen alone was shown to induce one of the lowest serum antibody responses. The proliferative responses were, however, never higher than those of the Vero controls, and the IFN-γ levels were higher than those of the Vero controls only at week 6.

The lack of information about the composition of Polygen (adjuvant 2), other than it is a low molecular weight, non-particulate copolymer adjuvant (MVP Laboratories), precludes us from speculating about its mechanism of action. However, in the present study, Polygen was one of two adjuvants that elicited the highest antibody responses and was the only adjuvant that induced IFN-γ levels statistically similar to those of the infected heifers. In view of the essential role of IFN-γ in protective immunity against *N. caninum* infection in mice [5] and against the closely related *T. gondii* pathogen [24], the potential of Polygen to stimulate strong IFN-γ production is of primary importance and is certainly worthy of further investigation. The high background proliferation and the resulting negligible SI observed in three of six animals in the adjuvant 2 group may account for the overall low proliferation index in this group.

Acknowledgements

The authors thank Dr Rolf Steiger (Novartis, Basel, Switzerland) for the gift of recombinant bovine interferon-γ, Dr Ian Gardner for valuable advice in statistical analysis, and Dr Joan Rowe and Dr Marcello Schonmann for veterinary assistance. The authors also acknowledge Karen Sverlow, Linda Smith and Joyce Duxbury for skillful technical help.

This work was supported by Bayer Corporation, Agricultural Division, Research Agreement 95K210.

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