# A preliminary study to evaluate the immune responses induced by immunization of dogs with inactivated *Ehrlichia canis* organisms

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**ABSTRACT**

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*Ehrlichia canis* is an intracellular pathogen that causes canine monocytic ehrlichiosis. Although the role of antibody responses cannot be discounted, control of this intracellular pathogen is expected to be by cell mediated immune responses. The immune responses in dogs immunized with inactivated *E. canis* organisms in combination with Quil A were evaluated**.** Immunization provoked strong humoral and cellular immune responses, which were demonstrable by Western blotting and lymphocyte proliferation assays. By Western blotting antibodies to several immunodominant *E. canis* proteins were detected in serum from immunized dogs and antibody titres increased after each immunization. The complement of immunogenic proteins recognized by the antisera were similar to those recognized in serum from infected dogs. Upon challenge with live *E. canis*, rapid anamnestic humoral responses were detected in the serum of immunized dogs and primary antibody responses were detected in the serum from control dogs. Following immunization, a lymphocyte proliferative response (cellular immunity) was detected in peripheral blood mononuclear cells (PBMNs) of immunized dogs upon stimulation with *E. canis* antigens. These responses were absent from non-immunized control dogs until after infection with live *E. canis,* when antigen specific-lymphocyte proliferation responses were also detected in the PBMNs of the control dogs. It can be thus concluded that immunization against canine monocytic ehrlichiosis may be feasible. However, the immunization regimen needs to be optimized and a detailed investigation needs to be done to determine if this regimen can prevent development of acute and chronic disease.

**Keywords**: *Ehrlichia canis*, canine monocytic ehrlichiosis, immune responses, immunization

## INTRODUCTION

Canine monocytic ehrlichiosis caused by *Ehrlichia canis* is an infectious, non-contagious, tick-transmitted disease of dogs. It has a worldwide distribution, except Australia and New Zealand, and closely follows the distribution of the tick vectors, *Rhipicephalus sanguineus* and *Dermacentor variabilis* (Donatien & Lestoquard 1935; Lewis, Hill & Ristic 1978; Keefe, Holland, Salyer & Ristic 1982; Kelly 2000). Clinically, the disease is characterized by three successive phases, the acute, the subclinical and the chronic phase (Kelly 2000). Haematological changes are evident in all phases and are characterized by thrombocytopenia, mild anaemia and variable leukocyte responses (Walker, Rundquist,

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Taylor, Wilson, Andrews, Barck, Hogge, Huxoll, Hildebrandt & Nims 1970; Amyx, Huxoll, Zeiler & Hildebrandt 1971; Seamer & Snape 1972). The acute phase usually resolves spontaneously into a subclinical phase, followed by the chronic phase in dogs which are unable to mount an effective immune response against the parasite. The chronic phase may be mild or severe depending on the strain of the parasite, age and breed of the animal, as well as the presence of concurrent disease. The chronic severe form is characterized by weight loss, pale mucous membranes, abdominal tenderness and bleeding tendencies with epistaxis reported in 30–50 % of the cases (Greene & Harvey 1984; Kelly 2000). It has been reported that German Shepherds are more prone to the chronic severe form of the disease, associated with specific and non-specific immunosupression induced by the *E. canis* infection (Huxoll, Amyx, Hemelt, Hildebrandt, Nims & Gouchenour 1972; Nyindo, Huxoll, Ristic, Kakoma, Brown, Carson & Stephenson 1980; Ristic & Holland 1993).

Immunization against *E. canis* infection is not available at present. Current methods of disease prevention include tick control by routine use of acaricides and prophylactic treatment with tetracycline. Treatment with tetracycline, doxycycline and imidocarb dipropionate is effective against *E. canis* infection, although this is not without limitations, especially in the chronic severe form of the disease. There is one anecdotal report of unsuccessful immunization of dogs against canine ehrlichiosis with inactivated cell culture-derived *E. canis* antigens using an adjuvant that enhances humoral immunity (Ristic & Holland 1993). Although both humoral and cell mediated immune responses are provoked during infection, it is documented that humoral immunity plays little role in protection against *E. canis* infection *in vivo.* It is generally recognized that cell mediated immunity is important in protection against intracellular parasites. This would also apply to *E. canis* because of its intracellular location (Kakoma, Carson, Ristic, Huxoll, Stephenson & Nyindo 1977; Nyindo *et al*. 1980; Ristic & Holland 1993).

Previous reports have highlighted that immunization against phylogenetically related organisms such as *Ehrlichia ruminantium* and *Ehrlichia risticii,* using inactivated organisms can induce protection against live challenge (Rikihisa 1991; Dame, Mahan & Yowell 1992; Van Vliet, Jongejan & Zeijst 1992; Martinez, Maillard, Coisne, Sheikboudou & Bensaid 1994; Mahan, Andrew, Tebele, Burridge & Barbet 1995; Martinez, Perez, Sheikboudou, Debus & Bensaid 1996; Mahan, Kumbula, Burridge & Barbet 1998; Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa & Rurangirwa 2001; Mahan, Smith, Kumbula, Burridge & Barbet 2001). In this study, we demonstrate that immunization of German Shepherd dogs (GSDs) with inactivated *E. canis* organisms in combination with Quil A induces humoral and cellular immune responses which exert a suppressive effect on rickettsaemia following live *E. canis* challenge.

## MATERIALS AND METHODS

### Propagation of *E. canis* in DH82 canine macrophages

*Ehrlichia canis* (Oklahoma strain) was cultured in DH82 cells in Eagles Minimal Essential Medium (EMEM, Earles base), containing 10 % fetal calf serum, 0.292 g/ℓ glutamine (GIBCO), 25 mM sodium bicarbonate and 25 mM Hepes. The cultures were grown in 75 $cm^2$ tissue culture flasks (Costar 3275, MA, USA) at 37 °C. The medium was changed twice a week until more than 80 % of the cells were infected as determined by evaluation of Giemsa-stained cytospin preparations of cells in the supernatant. At this stage, cells were either harvested for live challenge of dogs by gently tapping the flask or fresh medium was added to the existing medium twice weekly until all the cells were infected and the DH82 cell monolayer had disintegrated. The contents of these flasks were frozen at –80 °C for subsequent preparation of *E. canis* antigen for use in Western blotting or lymphocyte proliferation tests or for purification of DNA which was used in polymerase chain reaction (PCR) assays.

### Preparation of *E. canis* antigen

The frozen *E. canis* infected DH82 cell cultures were thawed, sonicated and centrifuged at low speed (1 000 x *g*) to remove cellular debris and to release *E. canis*. The supernatants were placed over an equal volume of 25 % sucrose in phosphate buffered saline (PBS) and centrifuged at 7 500 x *g* for half an hour. The resultant pellet containing the *E. canis* organisms was washed three times in PBS and resuspended in PBS**.** The protein concentration of this stock antigen was estimated by the Lowry method (Lowry, Rosebrough, Farr & Randall 1951), using bovine serum albumin (BSA) in PBS as the standard.

For immunization, freshly harvested live *E. canis* organisms were inactivated with an equal volume of

0.8 % β-propiolactone in sterile PBS on ice for 2 h and frozen overnight at –20 °C as described by Mahan *et al*. (1995) for inactivation of *E. ruminantium*. Inactivation of the organisms was confirmed by staining with 6-carboxy-fluoresceine-diacetate (6-CFDA; Sigma, St. Louis, MO, USA) which only stains live cells. Final confirmation of inactivation of the organisms was achieved by inoculating 10 µℓ of the final immunogen into a centrifugation-shell vial (Sterilin, Feltham, England) containing DH82 cells. These cultures were incubated at 37 °C for 4 weeks, when the cover slip in the shell vial was harvested and examined under the microscope for *E. canis* organisms after staining with modified Wright's stain. A lack of recovery of infected DH82 cells in these vials was proof that the organisms had been inactivated adequately. Positive control cultures were also set up which were inoculated with normal viable organisms from which infected DH82 cells were recovered.

**Immunization of experimental dogs**

Initially, ten healthy 6 to 8-month-old GSDs (two groups of five dogs: control and infected) based at the Zimbabwe Republic Police (ZRP) Dog Training School, Harare (in 1996/7), were committed to this study with authorization from the relevant ZRP officials. All the dogs had been previously vaccinated against canine parvovirus, rabies, distemper, hepatitis and leptospirosis infections as per the recommendation of the individual vaccine manufacturers and were dewormed on a monthly basis. An effective ectoparasite control programme involving weekly dipping with acaricides was in place at the ZRP kennels. Pre-immunization screening (clinical, haematological and biochemical) demonstrated that all dogs were healthy (data not shown). However, after commencement of the experiment, only two control dogs were available, because the other three contracted parvovirus infection and died. Since there were no replacements available, one group containing five immunized and the other two control dogs remained. The dogs were bled by jugular venipuncture for analysis of serum, which was stored at –20 °C, for antibodies to *E. canis*. All the dogs were found to have had no previous exposure to *E. canis*, since they were sero-negative by Western blotting based on the absence of reactivity with the immunodominant 27 kDa protein and other immunodominant *E. canis* proteins (Brouqui, Dumler, Raoult & Walker 1992; Mahan, Tebele, Mukwedeya, Semu, Nyathi, Wassink, Kelly, Peter & Barbet 1993). The five dogs, Alka, Duke, Spider, Zita and Zulu, were immunized three times subcutaneously with the inactivated vaccine, at 2-week intervals. Each dose of the inactivated *E. canis* vaccine was prepared in 1 mℓ of sterile PBS containing 200 µg of *E. canis* antigen mixed with 100 µg of Quil A adjuvant (Superfos, Denmark). The two control dogs, Nita and Nondo, were inoculated at the same time with 100 µg of Quil A in 1 mℓ of PBS.

**Immunological monitoring**

Pre-immunization (day 0), post-immunization (2 weeks after each immunization), pre-challenge (45 days after third immunization) and post-challenge (1, 3, 11 and 37 weeks after challenge) antibody responses to *E. canis* antigens were monitored by Western blotting as described previously (Mahan *et al.* 1993). *Ehrlichia canis* antigen (20–30 µg per lane) was electrophoretically separated on 12 % sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) using the Laemmli system (Laemmli 1970). The resolved antigens were electro-transferred to nitrocellulose membranes and blocked for 3 h at room temperature in washing buffer 1 pH 8.0 (Tris buffered saline [TBS: 0.1 M Tris HCL and 0.9 % NaCl] containing 0.25 % gelatine and 0.25 % Tween 20). The membranes were reacted overnight with pre-immunization sera diluted in the blocking buffer at 1/100; post-immunization sera at 1/100–1/8 000 and post challenge at 1/100–1/32 000. The reactions of the antisera were developed by incubation with horse radish peroxidase labelled Protein G, followed by 4CN peroxidase substrate (purchased from Kirkegaard and Perry, Maryland, USA). The results of the Western blots were photographed using a Polaroid MP4 Land camera and Polaroid 57 speed film. The positive serum sample that was used in these Western blots was from a dog experimentally infected previously with *E. canis* (Oklahoma strain). The negative control serum was from a dog which had never been exposed to *E. canis.*

To verify the presence of cell-mediated immune responses, lymphocyte proliferation tests (LPTs) were performed 4 days before challenge on two immunized dogs (Duke and Zulu) and one control dog (Nondo). The assay was repeated 7 weeks post-challenge on the same two immunized dogs (Duke and Zulu) and both control dogs (Nita and Nondo). Briefly, 20 mℓ of blood were collected from the jugular vein of each dog into Vacutainer tubes containing (EDTA), centrifuged at 4 000 x *g* for 15 min. The buffy coat was harvested and made up to 5 mℓ with sterile PBS. This diluted buffy coat was gently layered on 4 mℓ of Histopaque (Sigma diagnostics, St. Louis, MO, USA) and centrifuged for 30

min at 400 x *g* at room temperature to recover peripheral blood mononuclear cells (PBMNs). The interface containing the PBMNs was harvested, mixed with 10 mℓ of sterile Alsever's solution (0.55 g citric acid, 20.5 g D-glucose, 4.2 g NaCl and 8 g trisodium citrate in 1 ℓ of distilled water) and centrifuged at 450 x *g* for 10 min. The resulting pellet was washed three times in 50 mℓ of Alsever's solution (180 x g, 10 min each) to eliminate platelets. The final pellet containing PBMNs was re-suspended in 0.5 mℓ of PBS and the cell viability assessed with the trypan blue exclusion stain. The concentration of the cells was adjusted to 2 $x10^6$/mℓ with RPMI complete medium (supplemented with 10 % foetal calf serum, 25 mM Hepes, 50 µg gentamicin sulphate, 27 mM sodium carbonate, 5 $x10^{-5}$ M 2-mercaptoethanol). Aliquots of PBMNs (2 $x10^5$ cells in 100 µℓ) from each dog were dispensed into U-bottomed 96 well tissue culture plates (Costar, MA, USA), and stimulated in duplicate with 5, 10 or 20 µg antigen (pre-challenge tests) and 10 µg *E. canis* antigen (post-challenge tests).

The same *E. canis* antigen was used for all LPTs. Negative control cultures contained wells of cells without antigen and positive control cultures contained 5 µg of Concanavalin A which had been previously shown to be the optimal concentration for inducing proliferation in PBMNs (data not shown). The plates were incubated at 37 °C in 5 % $CO_2$ in air atmosphere for 4 days when 0.5 µci of $^3$H-thymidine (Amersham, England) was added to each well and incubated overnight. After harvesting the cells with a PHD cell harvester (Model 200A, Cambridge Technology Inc., Watertown, MA), the incorporated radioactivity was counted in a Beckman LS 6500 scintillation counter and expressed as counts per minute.

### Challenge of dogs with live *E. canis*

Immunized and control group dogs were challenged intravenously 45 days after the last immunization, with $10^7$ *E. canis* (Oklahoma strain)-infected DH82 cells, suspended in 5 mℓ of media. Viability of the infected cells was confirmed by trypan blue exclusion stain. This challenge dose was not pre-tested due to lack of additional uninfected dogs. However, this *E. canis* Oklahoma infected cell culture challenge dose was chosen based on studies reported by Iqbal & Rikihisa (1994) who had shown that it induced mild but significant clinical and haematologic changes consistent with canine monocytic ehrlichia infections and resulted in establishment of chronic infection in which *E. canis* could be detected for a long period after the infection had been initiated.

### Isolation of *E. canis* from peripheral blood of infected dogs

To determine if the immune responses induced by immunization would suppress the rickettsaemia, isolation of *E. canis* was attempted from the immunized and control dogs during the first 2 weeks after challenge with live *E. canis*. Twenty millilitres of blood in EDTA Vacutainer tubes were obtained aseptically from each dog on days 9 and 14 after live *E. canis* challenge. Peripheral blood mononuclear cells were isolated from these samples as described above for LPTs. After the final wash, the cells were resuspended in1 mℓ of EMEM without antibiotics and 0.5 mℓ of the cell suspension was inoculated onto centrifugation-shell vials with confluent layers of DH82 cells. Fresh medium (1 mℓ) was added to each vial and the vials incubated at 37 °C. Media was changed twice a week for 4 weeks when cover slips were harvested, air-dried, fixed in methanol, stained with modified Wright's stain and examined under the microscope for the presence of *E. canis* organisms.

### Detection of *E. canis* in peripheral blood of challenged dogs by PCR

To demonstrate that all the dogs had been successfully challenged with *E. canis*, a 16S *E. canis*-specific PCR was conducted on PBMNs of all dogs. The latter were isolated from each dog on days 4, 9, 14, 22 and 30 after live *E. canis* challenge as described above. For PCR, 0.5 mℓ of the final cell suspension was used. The suspended cells were placed in Eppendorf tubes and centrifuged at 12 000 x *g* for 30 seconds. The supernatant was removed and the cells resuspended in 1 mℓ of saponin lysis buffer (0.22 % NaCl, 0.015 % saponin and 1 mM EDTA) which was added to lyse any remaining erythrocytes which might inhibit the PCR (Panaccio & Lew 1991). After 2 min at room temperature, the tubes were centrifuged at 12 000 x *g* for 30 s and the saponin lysis buffer was removed. After two further washes in 1 mℓ of sterile PBS, the cell pellet was resuspended in 100 µℓ of proteinase K buffer (PK buffer; 0.1M Tris HCl [pH 7.5] and 0.15 M NaCl, 1 % SDS) and stored at –20 °C. DNA was obtained from the isolated buffy coats as follows. The cells were thawed at 37 °C and freeze-thawed twice to disrupt the cells. After further addition of 100 µℓ of PK buffer, the cell debris was digested with 10 µℓ of lysozyme (10 mg/mℓ) for 1 h at 37 °C and then further digested overnight with 10 µℓ of proteinase K enzyme (10 mg/mℓ) at 37 °C, followed by 1 h at 56 °C. DNA extraction was performed by the phe-

nol-chloroform method (Maniatis, Fritsh & Sambrook 1982). The extracted DNA was transferred to sterile tubes and stored at –20 °C until PCR was performed.

*Ehrlichia canis* DNA was extracted from infected DH82 cell cultures as described above and used to validate the *E. canis* PCR and as a positive control for subsequent test PCRs. A PCR assay was conducted using *Ehrlichia* general primers, HE3 (5'-GGTACCGTCATTATCTTCCC-3') and HE2 (5'-GTGGCAGACGGGTGAGTAATGC-3') as an internal control for the assay. These 16S ribosomal DNA (16S rDNA) general primers amplify DNA from organisms in the *Ehrlichia* group (*E. canis*, *E. chaffenssis*, *E. ruminantium* (Savadye, Kelly & Mahan 1998; Peter, Deem, Barbet, Norval, Simbi, Kelly & Mahan 1995; Peter, Barbet, Alleman, Simbi, Burridge & Mahan 2000; Dumler *et al.* 2001) and an unknown ehrlichial agent found in Zimbabwe in non-heartwater areas (Savadye, Kelly & Mahan 1998). The *E. canis* specific primer used in combination with HE2, was HE(C) (5'-CAATTATTTATAGCCTCTGGCTATAGG-3'), and this combination of primers amplifies DNA specifically from *E. canis* (Peter *et al.* 1995). The *Ehrlichia* general primers and the *E. canis* specific primers amplify a 350 base pair DNA fragment by PCR. *Ehrlichia ruminantium* PCR primers, AB128 and AB129 (Mahan, Waghela, McGuire, Rurangirwa, Wassink & Barbet 1992), were used to test for specificity of the PCR conditions and to ensure that there was no contamination with *E. ruminantium* DNA, which is routinely grown at the laboratory where these experiments were conducted. These primers amplify a 279 base pair DNA fragment. The *E. canis* and *E. ruminantium* PCR assays were performed as described previously (Savadye, Kelly & Mahan 1998; Peter *et al.* 1995; 2000). The specificity of the PCR assay was validated by amplifying a 350 bp DNA fragment from *E. canis* DNA but not from *E. ruminantium* DNA, a phylogenetically closely related species, using the 16S rDNA primers specific for detection of *E. canis*. Further evidence of the assay's specificity and its optimal operation was obtained by the use of general 16S rDNA *Ehrlichia* primers and *E. ruminantium* specific primers on the same template DNA samples. This combination of primers amplified 350 bp and 279 bp fragments from the *E. ruminantium* DNA, but only the 350 bp fragment from the *E. canis* DNA sample (data not shown). To improve the detection of the amplified DNA, Southern blotting followed by hybridization to the $^{32}$P dCTP labelled *E. canis* specific 350 bp DNA fragment was performed. The result of this hybridization was visualized by autoradiography (Mahan *et al.* 1992). The 350 bp DNA fragment (used as probe) was amplified from genomic *E. canis* DNA, excised from a 0.8 % low melting temperature agarose gel and purified by a standard phenol:chloroform extraction method (Maniatis *et al.* 1982). The purified DNA fragment was labelled with $^{32}$P dCTP by the random primer extension method (Boehringer Mannheim).

## RESULTS

### Responses to immunization

*Clinical and haematological responses*

The dogs in the immunized and control groups developed an oedematous swelling at the site of injection which fluctuated in size from approximately 5–25 mm, but subsided by 4 weeks post-immunization. Similar swellings were also noted in the control dogs that had been injected with Quil A alone. There were no other clinical or haematological changes that could be attributed to immunization in any of the dogs (data not shown).

### Detection of *E. canis* specific antibody and lymphocyte proliferation responses

The kinetics of antibody titre development and reactions following the various immunizations, was determined by Western blotting. Serum from all *E. canis* immunized dogs reacted with the immunodominant 27 kDa protein and lower and higher molecular mass proteins of *E. canis* (Fig. 1A). The antibody recognition patterns of *E. canis* proteins of the serum from immunized dogs was similar to the pattern of the positive control serum used in the Western blotting assay and of infected dogs after challenge (see Fig. 1B). The antibody titres and the antibody binding signals increased markedly after each immunization (Fig. 1B), but had decreased during the period leading to challenge with live *E. canis* (Fig. 2). Fig. 1A shows that the two non-immunized control dogs, Nita and Nondo, remained sero-negative throughout the immunization period, but strongly sero-converted following challenge with live *E. canis* (Fig. 1B and 2).

Cell mediated immune responses were only tested in two of the immunized dogs, Duke and Zulu, as representatives of this group. Proliferative responses of their respective PBMNs to *E. canis* antigens were detected (Fig. 3), which were much higher than those of control cultures, and of the non-immu-

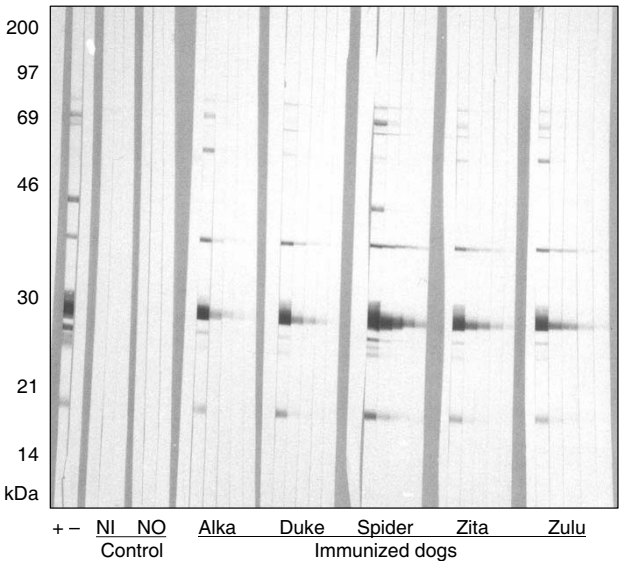


FIG. 1A Western blots to evaluate *E. canis* specific antibody levels in the sera of the two control dogs, Nita (NI) and Nondo (NO), and the five immunized dogs, Alka, Duke, Spider, Zita, Zulu, 2 weeks post third immunization. Lane 1, positive control, Lane 2, negative control; Lane 1 for each dog is a pre-immunization serum reaction (1/100); Lanes 2 and 3 for the control dogs are post-immunization reactions at 1/100 and 1/1 000; Lanes 2–6 for the immunized dogs are post-immunization reactions at 1/100, 1/1 000, 1/2 000, 1/4 000, 1/8 000. Molecular mass markers are shown on the left in kDa

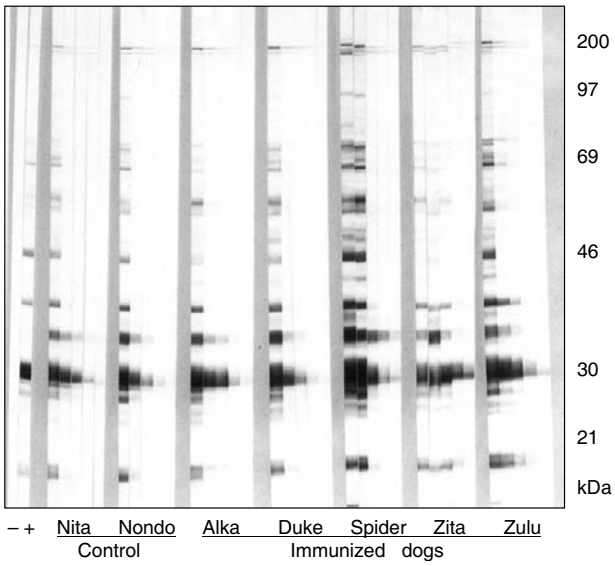


FIG. 1B Western blots to demonstrate the rise in *E. canis* specific antibody titres in the sera of the two control dogs, Nita and Nondo, and the five immunized dogs, Alka, Duke, Spider, Zita, Zulu, 37 weeks post-challenge. Lane 1, negative control; Lane 2, positive control. The five lanes for the two control and immunized dogs show serum reactions at dilutions of 1/100, 1/1 000, 1/2 000, 1/4 000 and 1/8 000. Molecular mas markers are shown on the right in kDa

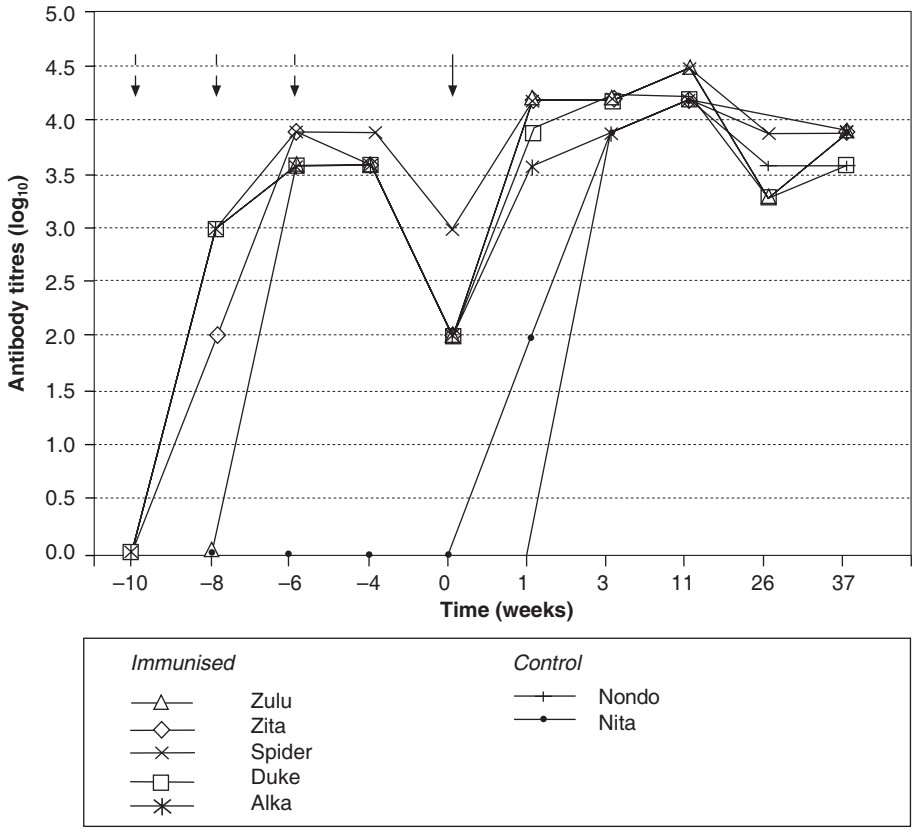


FIG. 2

Kinetics of antibody titres determined by Western blots before and after immunization or challenge with *E. canis*. Following immunization antibody titres increased and after challenge anamnestic and primary antibody responses were detected in the immunized and control dogs, respectively

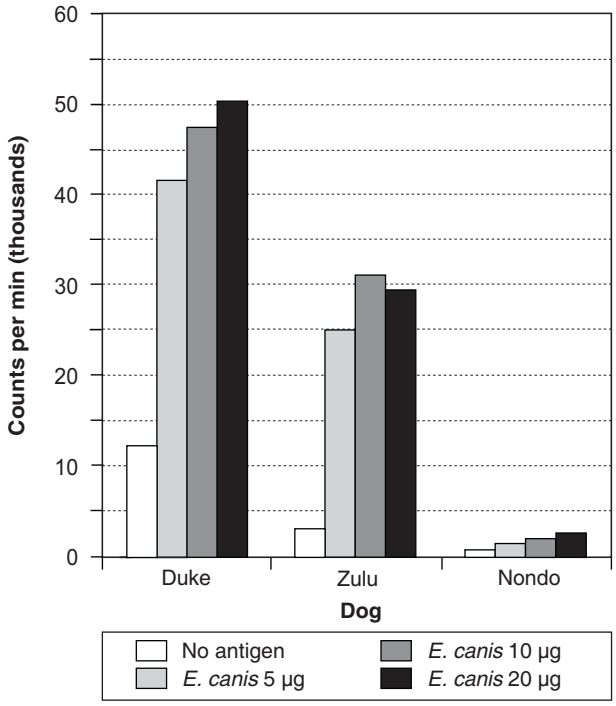


FIG. 3 Pre-challenge lymphocyte proliferation tests performed with PBMNs of the immunized dogs, Duke and Zulu, and the control dog, Nondo. PBMNs of each dog were co-cultured with *E. canis* antigens and proliferation was only detected in PBMNs of immunized dogs in the presence of *E. canis* antigen at 5, 10 or 20 µg of antigen

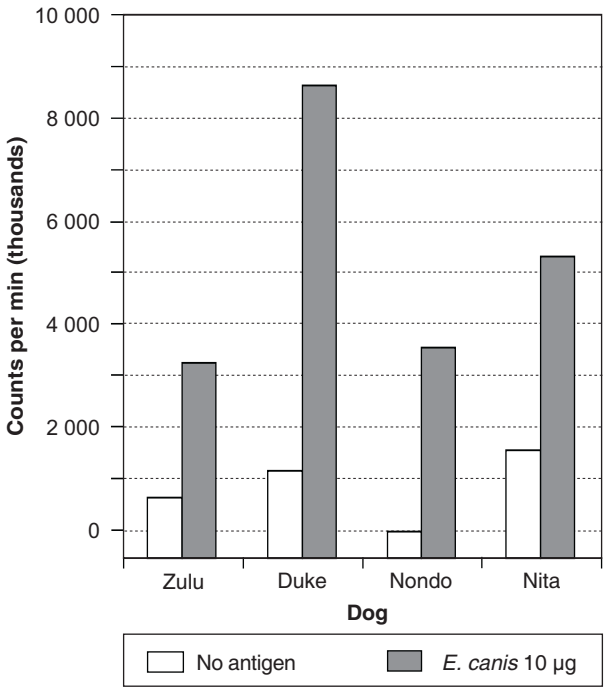


FIG. 4 Post-challenge lymphocyte proliferation tests performed with PBMNs of the immunized dogs, Duke and Zulu, and the control dogs, Nondo and Nita. PBMNs of each dog were co-cultured with *E. canis* antigens and proliferation was detected in PBMNs of all dogs in the presence of *E. canis* antigen at 10 µg of antigen

nized dog, Nondo. The PBMNs of the other three immunized dogs were not tested in LPT. The positive control Concanavalin A induced strong proliferation in these PBMNs (data not shown) confirming the culture conditions were satisfactory for induction of proliferative cellular responses.

### Immunological responses of the dogs after challenge with live *E. canis*

At the time of challenge, the antibody titres in the immunized dogs had decreased (Fig. 2). The live *E. canis* challenge induced rapid anamnestic antibody responses in the five immunized dogs. One week post-challenge, the antibody titres in the immunized dogs increased dramatically to levels between 1/4 000 to1/16 000 while only one control dog (Nita) had developed a detectable antibody titre of 1/100. At 3 weeks post-challenge, antibody titres ranged from 1/8 000 to 1/16 000 in all immunized and control dogs, and by 11 weeks post-challenge, they were higher (Fig. 2). Western blot analyses performed on sera obtained at 37 weeks post-challenge from all dogs demonstrated reactions with all the immunogenic *E. canis* proteins (Fig. 1B).

Cell mediated immune responses demonstrated by LPTs were detected in PBMNs from Zulu and Duke (immunized) and Nondo and Nita (controls) at 7 weeks post-challenge. A proliferative response was detected to *E. canis* proteins in all animals (Fig. 4) and in the control dogs represent their primary exposure to *E. canis* and coincided with high titred antibody responses detected by Western blotting. Having demonstrated that humoral and cellular responses were present after immunization, its effect on rickettsaemia was analyzed. Immunization seemed to suppress the development of rickettsaemia following challenge with live *E. canis*. This statement is supported by the fact that it was not possible to isolate *E. canis* from the PBMNs of the challenged immunized dogs in cell culture on days 9 and 14 post-challenge. In contrast, isolation of *E. canis* organisms was successfully achieved from the PBMNs of the two control dogs on day 9 (Nita and Nondo) and on day 14 (only Nita). Confirmation that all dogs were challenged with live *E. canis* organisms was achieved by detection of *E. canis* in the blood by a 16S *E. canis*-specific PCR assay (data not shown), and by the fact that anamnestic and primary immune responses were detected in

the immunized and control dogs after challenge, respectively.

## DISCUSSION

The major objective of this experiment was to detect immune responses of dogs to immunization with inactivated *E. canis* in conjunction with Quil A. Quil A was included as an adjuvant since it is a potent stimulator of both humoral and cell mediated immune responses and causes minimal side effects (Gupta & Siber 1995). Strong humoral responses were induced in the immunized dogs to various *E. canis* proteins. The complement of proteins recognized by serum of immunized dogs was generally similar to that recognized after infection with live *E. canis* and as reported by others (Nyindo, Kakoma & Hansen 1991; Brouqui *et al.* 1992; Mahan *et al.* 1993). The antibodies detected by Western blotting were of the IgG class since horseradish peroxidase conjugated Protein G only binds with IgG and was used as the second step reagent (Eliasson, Andersson, Olsson, Wigzell & Uhlen 1989). Such antibody responses following immunization were, however, short lived as titres declined from between 1/4 000 and 1/8 000 to 1/100 and 1/1 000 at the time of challenge, 6 weeks after the third immunization (Fig. 2). The decline in antibody titres was probably attributed to a short exposure to *E. canis* proteins during the immunization period, since the organisms used were inactivated and hence persisted for a short period in the dogs. Infection with *E. canis* on the other hand caused persistent antibody production which were detectable for up to 37 weeks, that being the end of the study. However, immunization induced B cell memory responses, since, following challenge with live *E. canis*, rapid anamnestic antibody responses were detected. Antibody titres in the immunized dogs increased from pre-challenge levels of 1/100 –1/1 000 to 1/8 000–1/16 000 1 week post-challenge (Fig. 2). In contrast, following *E. canis* infection, the rise in antibody titres was more gradual in the control dogs and was indicative of a primary exposure. The antibody titres in these control dogs only reached those of the immunized group by 3 weeks post-challenge. The protective role of antibodies to *E. canis in vivo* is uncertain because dogs become fully susceptible to reinfection with homologous *E. canis* after treatment with tetracyclines despite the presence of high antibody titres (Amyx *et al.* 1971; Buhles, Huxoll & Ristic1974). *In vitro*, however, antibodies against *E. canis* have been shown to suppress growth of *E. canis* organisms (Lewis *et al.* 1978).

Cell mediated immune responses were induced in the immunized dogs. Peripheral blood mononuclear cells from the immunized dogs proliferated when stimulated with *E. canis* antigen in LPTs. These assays are frequently used for detection of T cell activation and indicate incorporation of $^{3}$H-thymidine into the newly synthesized DNA of lymphocytes proliferating following antigen stimulation (Mwangi, Mahan, Nyanjui, Taracha & McKeever 1998; Mwangi, McKeever, Nyanjui, Barbet & Mahan 2002). Phenotypic characterization of the responding cell populations or their cytokine responses in the LPTs was not done to define cell populations involved or their respective cytokine responses to antigen stimulation. Cell mediated immune responses are considered critical for the control of infections by intracellular pathogens since the intracellular location of the organisms renders them inaccessible to circulating antibodies. There are two reports on the induction of CMI responses in dogs infected with *E. canis*. Kakoma *et al*. (1977) demonstrated cytotoxicity against monocytes from dogs infected with *E. canis* using autologous lymphocytes. Nyindo *et al.* (1980) used leukocyte migration inhibition tests to demonstrate that dogs infected with *E. canis* produced a migration inhibition factor. Proliferative cellular responses to *E. canis* antigens were also present in the two control dogs following the challenge with live *E. canis,* demonstrating that infection induced activation of cellular immunity, although the nature of this immunity was not fully explored. These proliferation assay results are in contrast with results obtained from experiments where PBMNs from bovines immune to infection with *E. ruminantium* were stimulated with lysate antigens. Peripheral blood mononuclear cells from such animals did not proliferate in the presence of antigen lysate and only proliferated when *E. ruminantium*-infected autologous endothelial cells or monocytes were used for stimulation (Mwangi *et al*. 1998). These observations represent different pathways of induction of immunity to infection to the respective pathogens in their specific hosts.

The immune responses induced by immunization appeared to inhibit rickettsaemia. While isolation of *E. canis* from the mononuclear cells of both unvaccinated control dogs, Nita and Nondo was possible, cultures from all the immunized dogs were negative.

This preliminary study shows that immunization of dogs with inactivated *E. canis* in conjunction with Quil A induces strong humoral and cell mediated immune responses. Immunization appears to inhib-

it rickettsaemia resulting from challenge with live *E. canis* and this may be of benefit in the control of canine monocytic ehrlichiosis as tick vectors mainly become infected during the acute phase of the disease when rickettsaemia is relatively high (Lewis *et al*. 1978). Further studies are required to optimize the immunization regimen in a larger number of dogs and to determine the level and type of immune protection induced against virulent, heterologous *E. canis* challenge.

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