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Peripheral blood lymphocyte proliferative responses in cattle infected with or vaccinated against *Anaplasma marginale*

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Abstract An assay was developed for measurement of the peripheral blood lymphocyte proliferative response (PBLPR) in cattle infected with or immunised against *Anaplasma marginale*. PBLPR was not evident in all cattle that had recovered from *A. marginale* infection. However, *A. marginale*-sensitised lymphocytes were detected in the spleens of all immune cattle tested in the absence of detectable PBLPR. During the course of initial infection, cattle exhibited detectable PBLPR for a period corresponding with and up to 2 weeks after patent parasitaemia, followed by a second, usually larger peak in PBLPR corresponding to the time of sub-clinical relapse of cattle. Analysis of the PBLPR of *A. marginale* chronically infected cattle demonstrated highly variable PBLPR between individuals and over time. A positive PBLPR was induced in cattle by vaccination using a crude *A. marginale* antigen preparation. The PBLPR of vaccinated cattle subsequently infected with *A. marginale* was markedly different from that of naive cattle, with reduced PBLPR being associated with the onset of parasitaemia. The antigen used in the PBLPR assay was inactivated by proteolysis. Proteolysis also abolished immunity that had been induced in cattle vaccinated using the antigen preparation. *A. marginale*-sensitised PBL did not proliferate in response to antigen from the heterologous species *A. centrale*. *A. centrale*-sensitised PBL, however, responded to *A. marginale* antigen. Interferon-γ (IFN-γ) was detected in PBLPR-assay supernatants and was associated with a strong PBLPR.

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Introduction

Anaplasma marginale, an intraerythrocytic rickettsia, is the causative agent of bovine anaplasmosis. Cattle that survive initial *A. marginale* parasitaemia usually remain sub-clinically infected carriers and are immune to subsequent re-infection for at least several years (Ristic and Nyindo 1973). This immunity is retained for at least 8 months after the elimination of latent parasitaemia by chemotherapy (Magonigle and Newby 1984). Protection against severe anaplasmosis may be induced by vaccination using non-living purified parasites (Montenegro-James et al. 1991) or by infection of cattle with the less pathogenic species *A. centrale* (Theiler 1912). Anti-*Anaplasma* antibodies are readily detected in cattle infected with *A. marginale* (Murphy et al. 1966).

We have previously shown that transfer of *Anaplasma*-immune sera from either previously infected or vaccinated cattle to susceptible bovines does not confer any protection against *A. marginale* infection (Gale et al. 1992). Further evidence that antibody per se is not protective against bovine anaplasmosis is provided by the observations that calves born to immune mothers are not protected by colostral antibody (Zaugg and Kuttler 1984) and immune carrier cattle relapse severely when splenectomised despite the continued presence of high levels of circulating anti-*Anaplasma* antibody (Ristic et al. 1958). Immunity against *A. marginale* is therefore likely to involve cell-mediated immune (CMI) responses, such as specific T-cell-mediated activation of macrophages, which operate during the resolution of many other rickettsial and intracellular bacterial infections (Kiderlen et al. 1984; Li et al. 1987; Zhan and Cheers 1993, and references therein). Circumstantial evidence exists for the involvement of CMI responses in immunity against bovine anaplasmosis. Non-specific resistance is induced against *A. centrale* and *A. marginale* by concurrent infection with the immunologically unrelated, benign haemo-

protozoan parasite *Theileria buffeli* (syn. *T. orientalis*; Stewart et al. 1990; Gale et al., manuscript in preparation). In addition, non-specific immunity against *A. marginale* infection has been induced by treatment of cattle with mycobacterial preparations (Sharma 1988). Buening (1976) has shown an association between a positive CMI response to *A. marginale* antigens as measured by the lymphocyte-migration inhibition test (LMIT) and immunity in cattle, although the absence of an LMIT response was not indicative of susceptibility.

In this paper we describe the development of an assay to measure in vitro the peripheral blood lymphocyte proliferative response (PBLPR) against *A. marginale* and the use of this assay to investigate the induction of responses during the course of initial *A. marginale* infection in naive and vaccinated cattle. Antigenic material utilised in the PBLPR assay was derived using an effective and simple method for the purification of *Anaplasma* from infected blood. We also report preliminary data on the nature of the *Anaplasma* antigens that induce proliferation, the distribution of sensitised lymphocytes between the spleen and blood and the species cross-reactivity of *Anaplasma*-sensitised lymphocytes.

Materials and methods

Medium and PBL preparation

Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) was supplemented with glucose (4 g/l), folate (6 mg/l), L-arginine (116 mg/l), L-asparagine (36 mg/l), HEPES (2.38 g/l) and $NaHCO^3$ (2 g/l). Prior to use the medium was also supplemented with fetal bovine serum (to 10% v/v, heat-treated at 56°C for 30 min prior to use), L-glutamine (to 2 m*M*), 2-mercaptoethanol (to 50 µ*M*) and penicillin/streptomycin (ICN Biomedicals, each to 200 IU/ml). Bovine PBL were prepared using an ice-cold water lysis method (Riding and Willadsen 1981). Briefly, blood (43 ml) was collected from the jugular vein of experimental cattle into ethylenediaminetetraacetic acid (EDTA) anticoagulant (EDTA 6 g/l, NaCl 7 g/l, vol. 7 ml) and centrifuged and the buffy coat was retained. After a washing step in phosphate-buffered saline (PBS; pH 7.3, Oxoid) to remove plasma, erythrocytes in the buffy-coat-material (e.g. 10 ml in PBS) were lysed by the addition of an equal volume of ice-cold water. After this had been briefly mixed, an equal volume of NaCl (0.46 *M*) was added, plus PBS to a final volume of 50 ml. Cells were pelleted by centrifugation and resuspended briefly in 10 ml of ice-cold water to which 10 ml of NaCl (0.46 *M*) and PBS (30 ml) were then added. PBL were pelleted by centrifugation and finally resuspended in medium to give a cell density of 10^7/ml. Cells were either used immediately or stored in the vapour phase of liquid nitrogen after resuspension in 90% fetal bovine serum/10% dimethylsulfoxide (DMSO) at a cell density of 6×10^7/ml.

PBLPR assay

Lymphocyte proliferation assays were performed as 3-well triplicates in flat-bottomed 96-well microtitre plates (Nunc, Denmark). To each well was added medium (100 µl) plus PBL suspension (50 µl/5×10^5 fresh cells or 10^6 freeze-thawed cells) plus either test antigen (50 µl in medium), medium only (50 µl) or concavalin A mitogen (Sigma, 50 µl of a 12.5 µg/ml solution in medium). Plates were incubated at 37°C in a humidified incubator containing 5% CO_2. Cell proliferation was measured by the addition of [methyl-^{3}H]-thymidine (185 GBq/mmol; Amersham, UK, 25 µl of PBS containing 0.023 MBq) at 18 h prior to cell harvest onto filter discs (Whatman GF/A). Incorporated thymidine was counted after the addition of 3 ml of scintillation fluid (Optiphase HiSafe, Wallac, Finland). The level of proliferation induced by a test antigen was expressed directly as counts per minute for test and control cells and, in some instances, as a stimulation index (SI), where SI = the mean counts per minute incorporated after the addition of antigen divided by the mean counts per minute incorporated in the absence of added antigen.

Measurement of interferon-γ levels in PBLPR-assay supernatants

Supernatants were stored at –120°C prior to assay in duplicate for bovine interferon-γ (IFN-γ) using a sandwich enzyme immunoassay technique essentially as described previously (Bovine γ Interferon Test, CSL Diagnostic Division, Australia; Rothel et al. 1990). Briefly, a monoclonal antibody adsorbed onto 96-well microtitre plates was used to capture bovine IFN-γ from culture supernatants. A second monoclonal antibody conjugated to horseradish peroxidase that was specific for a different bovine IFN-γ epitope was used as the detection reagent in conjunction with a tetramethyl-benzidine substrate. This assay detects levels of IFN-γ in the range of 0.025–1.6 ng/ml.

Purification of *Anaplasma marginale* from infected blood for use in the PBLPR assay

To obtain pure *A. marginale* antigen for use in lymphocyte proliferation assays, the method of Conrad et al. (1987) was used with some modifications for the removal of haemoglobin and white cell nuclei from infected blood. A density-gradient method was then developed for the purification of the intact rickettsia from host membrane material. All steps were performed aseptically using sterile reagents. High-parasitaemia blood (e.g. 5 l, 80% parasitaemia) was collected into EDTA anticoagulant. Cells were pelleted by centrifugation (1,000 *g*, 30 min) and the plasma and buffy coat were removed by aspiration. Erythrocytes were resuspended in 5 vol. of PBS and the centrifugation/aspiration steps were repeated. After a further two washes in PBS, packed erythrocytes were warmed to 37°C and an equal volume of saponin (1 mg/ml in water at 37°C) was added. After this had been mixed for 10 s, 4 vol. of PBS were added and the resulting lysed erythrocyte material was centrifuged (1,000 *g*, 20 min) to remove most intact host cells and leucocyte nuclei. The resulting supernatant was decanted and recentrifuged (14,000 *g*, 30 min). The dark red supernatant was removed by aspiration and the soft pellet (consisting of *A. marginale* initial bodies and erythrocyte membrane material) was resuspended back to the original volume in PBS. The resuspended soft pellet was transferred to a clean centrifuge bottle, leaving a darker, underlying pellet behind. The resuspended soft pellet was centrifuged (14,000 *g*, 30 min) and the PBS washing procedure was repeated three times further or until the supernatant was clear.

The free *A. marginale* initial bodies were purified from the large amount of contaminating host membrane material by sucrose/Percoll density centrifugation as follows. A gradient was prepared by mixing of 1 vol. of the washed parasite/erythrocyte membrane material with 1.1 vol. of 90% Percoll (Pharmacia)/0.25 *M* sucrose and 8 vol. of 0.25 *M* sucrose. The gradient was sonicated (30 s, 1 W/ml of gradient) to disrupt the rickettsia/host-cell-membrane association and centrifuged (25,000 *g*, 35 min, 4°C). The top two-thirds of the gradient containing most of the erythrocyte material was removed by aspiration. The remaining gradient was mixed, recentrifuged and aspirated as before. The remaining gradient was then added to 9 vol. of fresh gradient medium (10% Percoll/0.225 *M* sucrose/0.1× PBS), recentrifuged and aspirated two times further. The resulting pure rickettsia pellet in Percoll/sucrose was resuspended in PBS and centrifuged (10,000 *g*, 10 min) and the supernatant was discarded. Two further PBS washes were performed to remove all Percoll/sucrose and the pure parasites were

resuspended in PBS to give a dense suspension. The two-thirds of the gradient material that had been removed after the first centrifugation was resonicated and processed as described above to recover a further yield of pure rickettsia. Pure rickettsia [e.g. 5 ml of a 7-mg/ml concentration of total protein as determined by bicinchoic acid assay (BCA; Pierce)] were intact and essentially free of contaminating host material as judged by microscopy (Geimsa-stained thick films) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970) plus Coomassie staining, respectively. Purified *A. marginale* were disrupted by sonication (10 W/ml, 5 min, on ice) and stored frozen in the vapour phase of liquid nitrogen in small aliquots.

Experimental animals

Hereford-breed steers aged approximately 12 months and mixed-British-breed calves were obtained from an area of Western Queensland that is free of anaplasmosis, babesiosis, and *Boophilus microplus*, the tick vector of these diseases in Australia. Cattle were confirmed to be free of haemoparasites by examination of Geimsa-stained thick blood films as previously described (Gale et al. 1992). Cattle were kept under isolation conditions to exclude incidental infection. Following experimental infection of cattle with *A. marginale*, parasitaemia and packed red cell volume (PCV) data were collected daily as described elsewhere (Gale et al. 1992). Cattle that were judged on the basis of PCV and parasitaemia data to be at high risk of mortality were drug-treated and removed from further parasitological observations as previously described.

Vaccination against anaplasmosis using crude *A. marginale* antigen

A. marginale-infected blood was collected from a highly infected bovine (65% parasitaemia, 20% PCV). Erythrocytes were pelleted by centrifugation (1,000 *g*, 20 min, 4°C) and the plasma and buffy coat were removed by aspiration. Erythrocytes were then resuspended to the original volume in normal saline (0.85% NaCl) and the centrifugation/aspiration steps were repeated two further times. Washed erythrocytes were disrupted by sonication (10 W/ml, 2 min, on ice) and the sonicate was centrifuged (100,000 *g*, 60 min, 4°C). Four cattle were vaccinated subcutaneously using 2 ml per dose of the resulting supernatant containing 2 mg *Quillaja* saponin on days 0 and 28 prior to inoculation of these and four control non-vaccinated cattle with 10^8 *A. marginale*-infected erythrocytes on day 56.

Proteolysis of purified *A. marginale* antigen

Purified *A. marginale* antigen (total protein concentration 650 µg/ml) was digested using proteinase K (Boehringer Mannheim, 50 µg/ml) for 1 h at 37°C. The enzyme was then inactivated by the addition of phenylmethylsulfonyl fluoride (PMSF) to 0.1 m*M*. Antigen was also incubated exactly as described above, but in the absence of proteinase K (sham-incubated control). Incubated antigen preparations and untreated *A. marginale* antigen were analysed by SDS-PAGE and immunoblotting. The results (not shown) indicated that proteolysis abolished all Coomassie protein staining of *A. marginale* antigen and all reactivity with *A. marginale* hyperimmune serum as determined by immunoblotting. The staining properties of the sham-incubated antigen were not altered relative to those of the unincubated antigen.

Vaccination of cattle using intact or protease-treated pure *A. marginale* antigen

A total of 18 cattle aged approximately 12 months were randomly assigned to 3 groups of 6. Group 1 cattle were vaccinated using 100 µg per dose of pure *A. marginale* antigen as used in the LP assay. Group 2 cattle were vaccinated using the same amount of the material that had been protease-treated as described above. Group 3 cattle were vaccinated using adjuvant only. All vaccinations were formulated in 2 ml of PBS containing 2 mg of *Quillaja* saponin. Vaccinations were given subcutaneously on days 0 and 28 prior to challenge on day 56 using 10^8 *A. marginale*-infected erythrocytes from a splenectomised donor calf. Parasitaemia and PCV data were collected during the challenge period as described above.

Comparison of proliferative responses of PBL and splenocytes

Spleen cells were prepared from tissue taken by biopsy using a local anaesthetic (Kreier et al. 1964) or from whole spleens taken immediately after splenectomy of calves under deep anaesthesia. Spleen cells were isolated by passage of the tissue through sterile nylon gauze or metal tissue sieves. Cells were otherwise processed and stored as described for the preparation of PBL. PBL and splenocytes sampled concurrently were tested in the proliferation assay against concanavalin A (Con A) mitogen and *A. marginale* antigen.

One calf (animal 137) aged approximately 4 months was infected with *A. marginale* from an infected donor animal. Parasite levels were monitored daily until high-level parasitaemia was observed (16% parasitaemia, 13% PCV). At this time, blood was taken for the preparation of PBL and the spleen was removed for the preparation of splenocytes.

Three calves aged approximately 4 months were infected and monitored as described above. All three calves survived *A. marginale* infection without the need for drug treatment and tested microscopically negative for detectable *A. marginale* parasitaemia at 5 weeks after initial infection. Calf 467 and calf 469 were splenectomised and PBL and splenocytes were tested at 9 and 12 weeks after initial infection, respectively. Calf 468 was tested by spleen biopsy at 24 weeks after initial infection.

Testing of the species specificity of sensitised PBL

High-parasitaemia blood obtained from a splenectomised calf infected with *A. centrale* was used to prepare purified *A. centrale* antigen as described for the purification of *A. marginale* antigen. The calf was subsequently drug-treated to prevent death. PBL prepared from blood collected 6 days later and stored frozen were tested in the LP assay using either purified *A. centrale* (homologous) or *A. marginale* (heterologous) antigen. Similarly, PBL prepared from an *A. marginale*-infected splenectomised calf were tested against the homologous and heterologous antigen preparations.

Results

Purification of *Anaplasma marginale* from infected blood

Initial attempts to develop a PBLPR assay utilised crude *A. marginale* antigen prepared by saponin lysis of infected blood (Conrad et al. 1987). Crude antigen prepared from either *A. marginale*-infected or uninfected blood strongly inhibited proliferative responses of PBL to Con A mitogen (results not shown). Subsequently, a method was developed for the purification of *A. marginale* from infected blood using Percoll gradient centrifugation as described in Materials and methods. SDS-PAGE analysis of purified *A. marginale* before and after Percoll gradient centrifugation and in comparison with erythrocyte membrane material purified from uninfected blood is shown in Fig. 1.

Optimisation of the LP assay

PBL taken from two *A. marginale* carrier cattle at 3 months after initial *A. marginale* infection were tested in

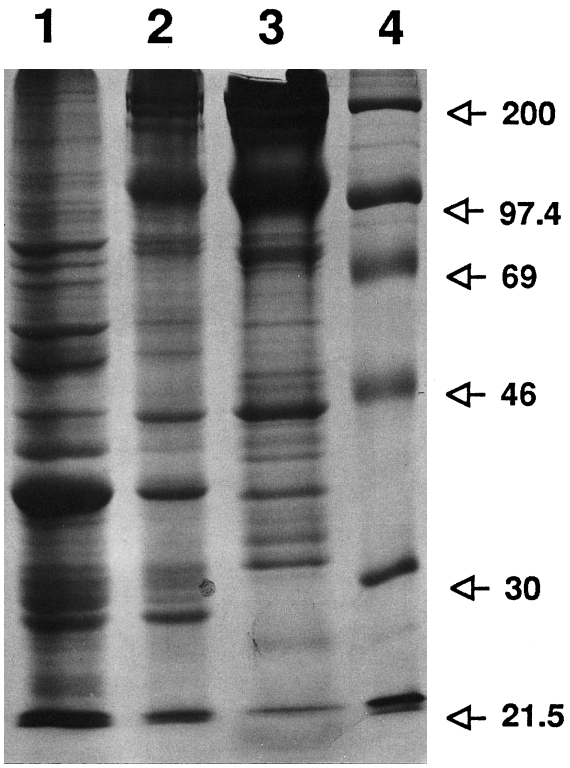

Fig. 1 SDS-PAGE analysis of purified *Anaplasma marginale* antigen as used in the PBLPR assay. Samples (25 μg/lane) were separated in 10% polyacrylamide and stained using Coomassie blue R250. (*Lane 1, A. marginale* Percoll-purified antigen; *lane 2, A. marginale* antigen before the Percoll-gradient purification step; *lane 3,* bovine cell-membrane material purified from uninfected blood according to the method used for the material in lane 2). Molecular weights of markers (Rainbow, Amersham) are given in kDa

the PBLPR assay using dilutions of *A. marginale* antigen purified as described in Materials and methods. The results (Table 1) show that PBL from one of the two immune cattle (animal 7381) showed a significant, dose-dependent proliferative response to the antigen at all antigen dilutions tested. PBL from the remaining immune bovine (animal 147) and the negative control animal (number 985) did not show significant stimulation in the assay at any of the antigen dilutions tested. Strong proliferative responses were obtained with Con A mitogen for all three cattle. In this assay, radiolabel was added after culture of cells with antigen for 4 days prior to counting on day 5. An antigen dilution of 10^{-2} was selected for use in all subsequent PBLPR assays.

PBL from three bovines were used to investigate the effect of culture time on PBLPR. Replicate assays were harvested on days 2, 4, 6 and 8 after the addition of radiolabel 18 h previously. The results (Fig. 2) show that no PBLPR was detected for animal 407 (haemoparasite-negative control) or 130 (*A. marginale*-infected splenectomised calf, 6 months after recovery from initial infection) at any time point. PBL from animal 384 (taken at the time of peak *A. marginale* infection) showed an elevated SI beginning on day 4, which increased with prolonged culture. A strong PBLPR was obtained using Con A mitogen for all three cattle. Cells were harvested after culture for 8 days in all subsequent assays.

Measurement of IFN-γ in PBLPR-assay supernatants

To investigate the production of IFN-γ in the PBLPR assay, culture supernatants (100 μl/well) were harvested on days 2, 4, 6 and 8 from replicate plates during the time-course experiment described above and were assayed as described in Materials and methods. The results obtained for PBL from the three cattle tested (Fig. 3) show that high levels of IFN-γ were detected in the supernatants of Con A-stimulated cells from all three cattle from day 2 to day 8 and in the supernatants of the antigen-stimulated cells from animal 384 from day 4 to day 8. Interestingly, the results obtained in animal 130 showed the production of high levels of IFN-γ in the unstimulated cells from day 4 to day 8 but not in the corresponding cells with added *A. marginale* antigen. Production of IFN-γ by PBL from this animal was not associated with a significantly elevated SI (as shown in Fig. 2), although the amount of radioactivity incorporated into the cells of this animal in the absence and presence of antigen (e.g. 2,486 ± 119 and 3,726 ± 442 cpm, respectively, on day 6) was significantly higher than that incorporated into the correspond-

Table 1 Effect of *Anaplasma marginale* antigen dilution on PBLPR of two *A. marginale* carrier cattle (animals 147 and 7381) and one negative control bovine (animal 985). PBLPR is expressed as counts per minute (*cpm*) and stimulation indices (*SI*) ± SD (*Con A* concanavalin A mitogen)

Antigen dilution	Animal 147		Animal 7381		Animal 985	
	cpm ± SD	SI ± SD	cpm ± SD	SI ± SD	cpm ± SD	SI ± SD
10-2	8964± 1204	2.03±0.27	9071±2217	10.80±2.64	17955±1244	2.76±0.19
10-3	9066± 2912	2.05±0.66	9230±2393	11.00±2.85	17330±2374	2.67±0.36
10-4	6400± 885	1.45±0.20	7446± 449	8.87±0.53	19461±2992	2.99±0.46
10-5	6008± 393	1.36±0.09	3747±1156	4.47±1.38	12859± 598	1.98±0.09
10-6	7178± 1043	1.62±0.24	2477± 487	2.95±0.58	15122±2777	2.33±0.43
10-7	7533± 1043	1.70±0.24	2294± 808	2.73±0.96	16109±3269	2.50±0.51
Cells only	4423± 373	1.00±0.08	839± 133	1.00±0.16	6501±1190	1.00±0.18
Con A	175527±11574	39.7 ±2.62	131136±9884	156±11.8	136836±8890	21.0±1.36

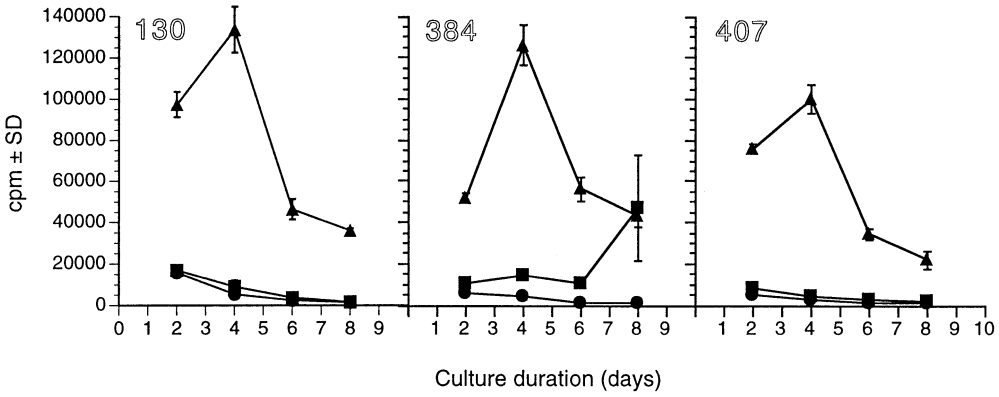

Fig. 2 Effect of the duration of cell culture on PBLPR of three cattle. Cells were either unstimulated (*black circles*) or stimulated with *A. marginale* antigen (*black squares*) or ConA mitogen (*black triangles*). Animal *407* was a negative control free of haemoparasites; animal *130*, a splenectomised calf at 6 months after initial *A. marginale* infection; and animal *384*, a bovine undergoing initial peak *A. marginale* parasitaemia

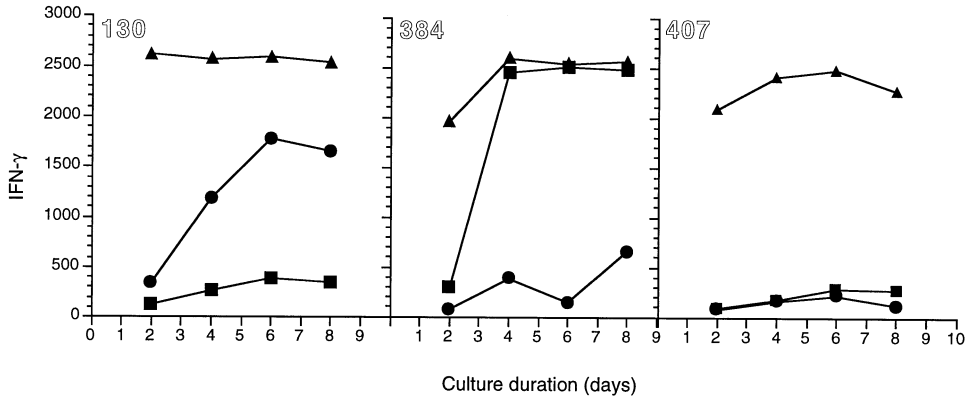

Fig. 3 IFN-γ level (ELISA absorbance units ×1,000) in PBLPR-assay supernatants plotted as a function of time (days). Cells were either unstimulated (*black circles*) or stimulated with *A. marginale* antigen (*black squares*) or ConA mitogen (*black triangles*). Animal *407* was a negative control free of haemoparasites; animal *130*, a splenectomised calf at 6 months after initial *A. marginale* infection; and animal *384*, a bovine undergoing initial peak *A. marginale* parasitaemia

ing cells from animal 407 (1,520 ± 408 and 2,441 ± 608 cpm, respectively) and that incorporated into the unstimulated cells of animal 384 (976 ± 129 cpm). Supernatants of cells from the negative control animal (number 407) were shown to contain low levels of IFN-γ throughout the course of the experiment.

Induction of LP responses in cattle following vaccination with non-living *A. marginale* and subsequent infection

To investigate the induction of PBLPR, cattle vaccinated against anaplasmosis using disrupted *A. marginale*-infected erythrocytes and control cattle were infected with *A. marginale* as described in Materials and methods. PBL from all cattle were tested in the LP assay as described above at weekly intervals from 1 week before infection to 4 weeks post-infection. An additional LP test was performed 8 weeks after the inoculation of parasites. Parasitaemia and PCV data were collected for the duration of patent *A. marginale* infection as described above. PBLPR and parasitaemia data for the four control animals and four vaccinated cattle are shown in Fig. 4A and Fig. 4B, respectively. Assays conducted 8 days before the infection of cattle showed significant proliferative responses in the two vaccinated cattle that were tested and no response in the four control animals. For this time point the PBLPR-assay results for two of the vaccinated cattle (animals 240 and 241) were not obtained due to a cell-harvester malfunction.

Following infection, PBLPR in all four vaccinated cattle varied considerably (Fig. 4B). Animals 240 and 241 showed a transient increase in PBLPR that was coincident with peak *A. marginale* parasitaemia. Animals 243 and 244 showed a decrease from a strong PBLPR that was coincident with rising *A. marginale* parasitaemia followed by a transient increase in PBLPR after the resolution of peak *A. marginale* infection. Following infection, all four cattle in the control group developed significant PBLPR (Fig. 4A) between day 14 and day 29, which coincided approximately with the time of peak *A. marginale* parasitaemia. All vaccinated and control cattle exhibited a low or negative PBLPR when retested at day 56. All four of the vaccinated cattle survived untreated. The mean maximal parasitaemia of this group was 4.1 ± 2.5%, with minimal PCV values being greater than 15% for all cattle. Three of the four control cattle required treatment (animals 233 and 239 on day 17 and animal 242 on day 20 post-infection) to prevent probable mortality. The mean maximal parasitaemia of this group was 12.8 ± 7.7%, with minimal PCV values being less than 15% for three of the cattle.

Further characterisation of the induction of LP responses in cattle following infection

Five cattle were infected by the intravenous inoculation of 10^8 *A. marginale*-infected erythrocytes taken from a

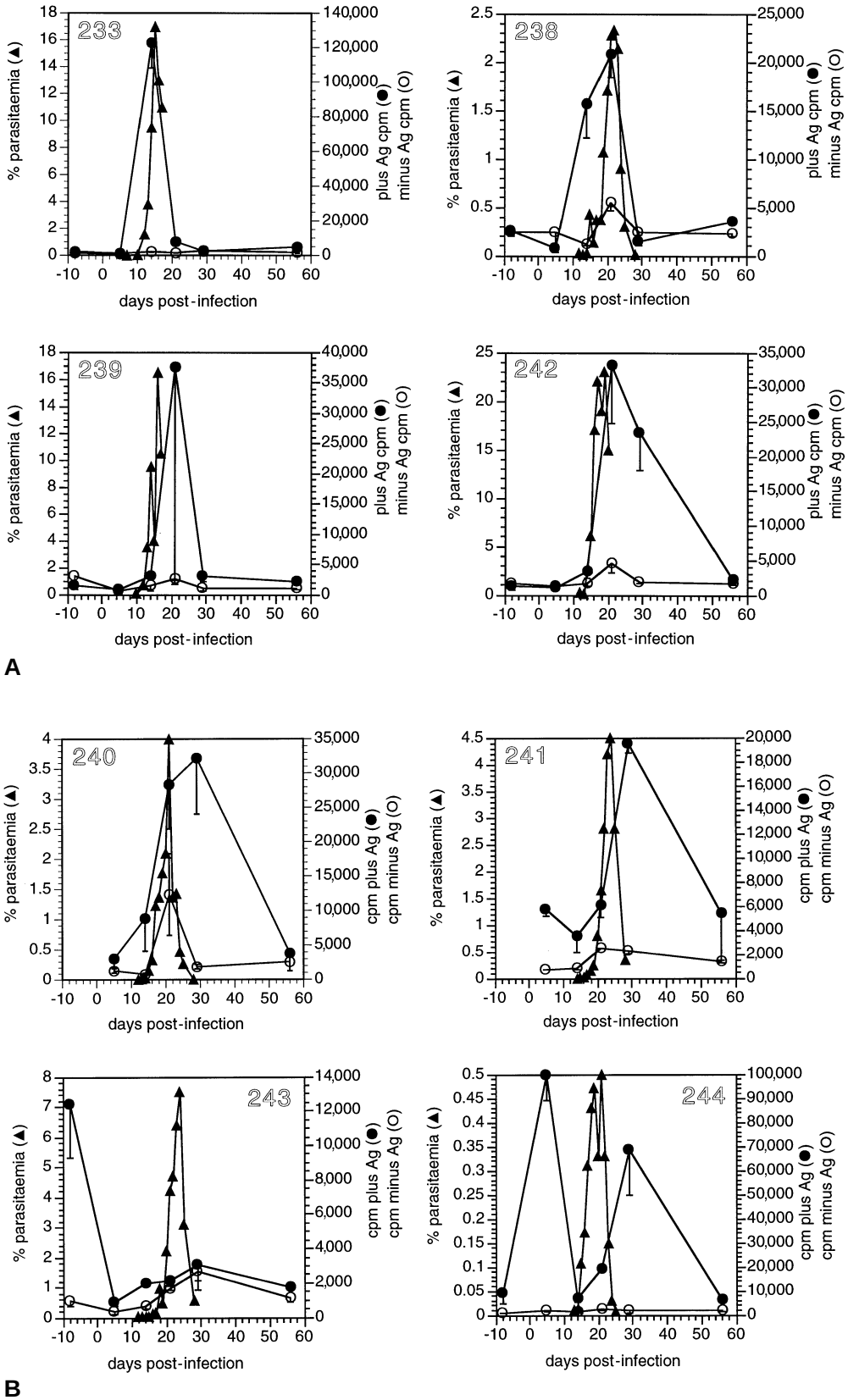

Fig. 4A, B PBLPR (cpm) and parasitaemia (% infected erythrocytes) plotted as a function of time for four control cattle (**A**) and four cattle vaccinated using a crude *A. marginale* antigen preparation (**B**), all undergoing initial *A. marginale* infection. Days indicated are relative to the day of infection (*day 0*) and represent the day of sampling of PBL from cattle. Y-axis scaling is varied

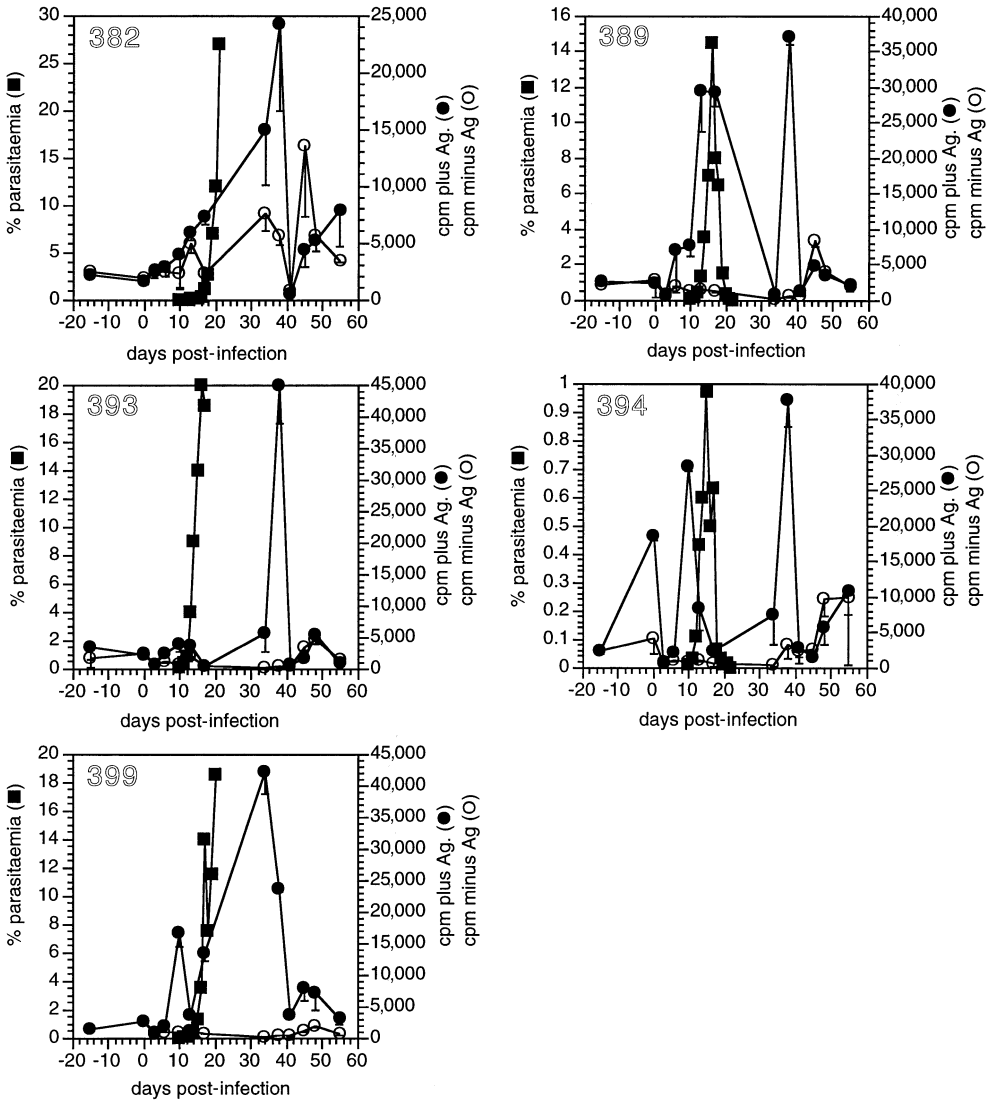

Fig. 5 PBLPR (cpm) and parasitaemia (% infected erythrocytes) plotted as a function of time for five previously unexposed cattle undergoing initial infection with *A. marginale*. Days indicated are relative to the day of infection (*day 0*) and represent the day of sampling of PBL from cattle. Y-axis scaling is varied

splenectomised donor calf on day 0. PBL from the five animals were tested in the PBLPR assay as described above on days –15 and 0 prior to infection and then at twice-weekly intervals until day 55 post-infection. The PBLPR results and parasitaemia data obtained for the five cattle are shown in Fig. 5. Three of the five cattle required drug treatment to prevent probable mortality (animal 393 on day 17 and animals 382 and 399 on day 21 post-infection). Four cattle developed high-level *A. marginale* parasitaemia (maxima of 14–30% of total erythrocytes parasitised), whereas the remaining animal (number 394) suppressed parasitaemia to below 1%. All five cattle developed a transient PBLPR from 10 to 40 days post-infection. Individual cattle exhibited a wide range in the magnitude of LP response (e.g. a maximal SI of 4.3 ± 1.36 in animal 382 up to 183 ± 14.5 in animal 399) that was not related to any variation in responsiveness to Con A mitogen (PBLPR for Con A-stimulated cells remained strong for all animals during the course of the experiment; results not shown). In all cattle a positive PBLPR was concurrent with or just preceded maximal parasitaemia and a second peak occurred at approximately 20 days after peak parasitaemia. The second peak of LP activity was of larger magnitude than the initial peak in the four cattle that developed high-level parasitaemias (>14%) but was lower than the initial peak in animal 394, which suppressed maximal parasitaemia (<1%).

Production of IFN-γ in PBLPR assays during the course of *A. marginale* infection

Assay supernatants from duplicate wells of the experiment described above were harvested at day 8 and stored at –120°C prior to testing using the IFN-γ immunoassay. The results obtained for *Anaplasma* antigen-stimulated and control cells for the five individual animals are shown against the corresponding PBLPR data in Fig. 6. High levels of IFN-γ were produced by PBL from all five cattle at the times corresponding to maximal proliferative responses to added *A. marginale* antigen. PBL from four of the five cattle also produced IFN-γ in the absence of

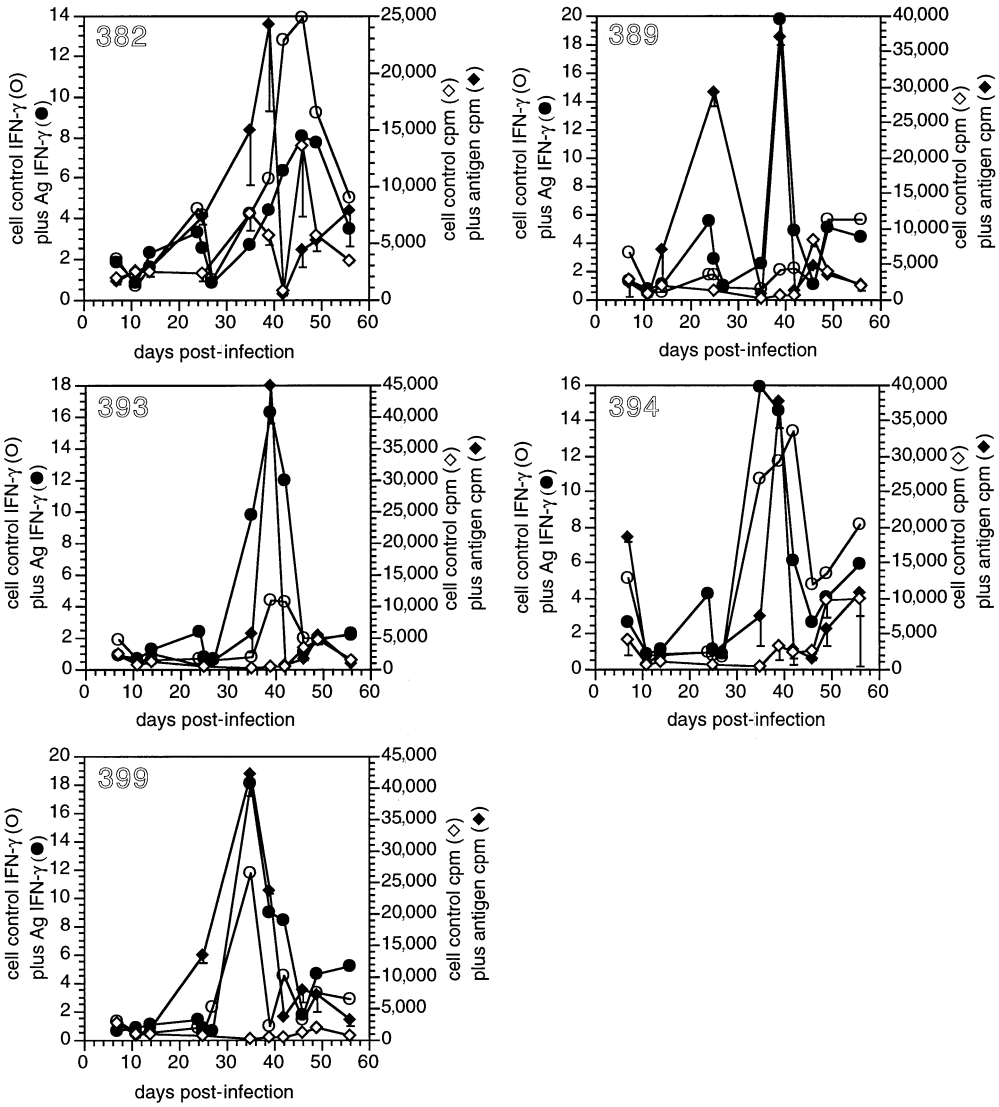

Fig. 6 IFN-γ level (ELISA absorbance units ×10) in PBLPR-assay supernatants and PBLPR (cpm) plotted as a function of time for five previously unexposed cattle undergoing initial *A. marginale* infection. Days indicated are relative to the day of infection (*day 0*) and represent the day of sampling of PBL from cattle. Y-axis scaling is varied.

added antigen at these time points. PBL from animal 382, which exhibited the lowest PBLPR to *A. marginale* antigen, produced higher levels of IFN-γ in the absence of added antigen. High IFN-γ production by cells in the absence of added antigen was associated with elevated incorporation of radiolabel in the cells at these time points, as was also observed in animal 130 previously (Figs. 2, 3).

LP responses in PBL from long-term *A. marginale* carrier cattle

The PBLPR of long-term *A. marginale* carrier cattle and the variation of these responses with time was investigated. PBL from four cattle aged approximately 18 months that had been experimentally infected with *A. marginale* 6 months previously and one negative control animal that was free of haemoparasites were tested in the PBLPR assay at 14-day intervals for a period of 12 weeks. The four carrier cattle were shown using a polymerase chain reaction (PCR)-based assay (Gale et al., manuscript in preparation) to be infected with microscopically undetectable levels of *A. marginale* (results not shown). All cattle were negative for *A. marginale* as determined by microscopy (approximately 30,000 erythrocytes were scanned per test) throughout the course of the experiment. The results obtained are shown in Fig. 7. The mean SI value of the negative control calf plus two standard deviations was used as the positive cut-off for the assay (SI 2.32 or higher). All four of the carrier cattle showed a positive SI at one or more of the time points tested. Large variations in both the magnitude and the frequency of positive LP responses were evident between the four carrier cattle. Animal 384 was positive at five of the six time points tested (mean SI 16.0 ± 13.2), animal 399 was positive at three of the six time points tested (mean SI 6.36 ± 7.78) and animals 393 and 398 were positive once (mean SI 1.54 ± 1.50 and 2.45 ± 2.46, respectively). The negative control animal (number 410) tested as slightly positive (SI 2.35 ± 0.28) on one of the six occasions sampled (the mean SI for this animal was 1.17 ± 0.57).

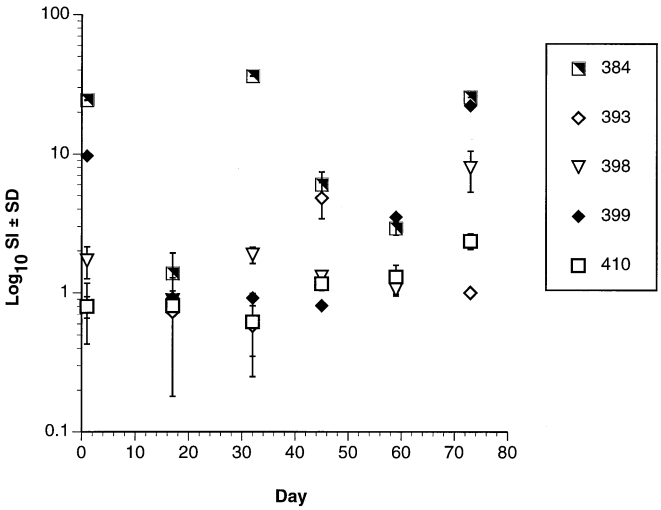

Fig. 7 Results of PBLPR assays performed at 14-day intervals on four *A. marginale* carrier cattle (animals *384*, *393*, *398* and *399*) at 6 months after initial infection and on one uninfected bovine (animal *410*). Results are expressed as stimulation indices (*SI*) ±SD

Effect of proteolysis on PBLPR to *A. marginale* antigen

To investigate the nature of the material that induced proliferation in the PBLPR assay, *A. marginale* antigen purified as described in Materials and methods was digested with proteinase K as described in Materials and methods. This material, sham-incubated antigen and unincubated antigen were then tested in the PBLPR assay using positive PBL as used in the antigen-dilution experiment described above (animal 7381). All antigens were tested at a final dilution of 1:100 (approximately 60 μg/ml of total protein before treatment). Antigens produced SI values as follows: unincubated antigen 10.6 ± 0.5, sham-incubated antigen 11.0 ± 0.7 and protease-treated antigen 2.8 ± 0.9. The SI values of the Con A and cell-only control wells were 40.8 ± 4.8 and 1.0 ± 0.3, respectively. Similar results were obtained when the assays were conducted two further times (results not shown).

Effect of proteolysis on protection induced by pure *A. marginale* antigen

An experiment was performed to determine whether the reduction of the PBLPR-inducing activity of the *A. marginale* antigen by proteolysis also effected the protection afforded by this material when used as an immunogen. Three groups of six cattle were vaccinated using either intact pure *A. marginale* antigen, proteolysed pure antigen or saponin adjuvant only as described in Materials and methods. Immunity to subsequent infection with *A. marginale* was evaluated as described above. Cattle that exhibited a fall in PCV to 15% or lower were drug-treated to prevent probable mortality. Protection against severe clinical anaplasmosis was induced in four of the six cattle vaccinated using pure *A. marginale* antigen. The mean maximal parasitaemia of this group was 1.63 ± 1.22% and two of the six cattle required drug treatment. In contrast, all six cattle vaccinated using protease-treated antigen required drug treatment, and the mean peak parasitaemia of this group was 6.39 ± 3.21%. Five of the six cattle in the unvaccinated control group required drug treatment, and the mean maximal parasitaemia of this group was 19.08 ± 8.04%.

Proliferative responses of splenocytes as compared with PBL

To compare the proliferative responses of splenocytes and PBL to *A. marginale*, both cell populations were sampled concurrently from calves at various times after infection and tested in proliferation assays. The results (Table 2) demonstrated that although PBL taken at these times were negative in the assay, splenocytes taken concurrently showed significant proliferative responses to the added *A. marginale* antigen for all time points tested (at peak parasitaemia and 9, 12 and 24 weeks after initial infection). No proliferative response was induced when spleen cells taken from a calf with no previous exposure to *A. marginale* were tested.

Table 2. Comparison of PBL and splenocyte proliferative responses against *A. marginale* antigen (*Ag.*) and concavalin A mitogen (*Con A*) in the LP assay. PBL and splenocytes were sampled concurrently either at peak *A. marginale* parasitaemia (calf 137, approximately 3 weeks after initial infection) or at 9, 12 or 24 weeks after initial infection (calves 467, 469 and 468, respectively). Results are expressed as counts per minute (*cpm*) ± 1 SD and as stimulation indices (*SI*) ± SD. Negative-control spleen cells (*Neg. spleen*) were prepared from a spleen removed from a calf with no previous exposure to *A. marginale*

Animal number/ cell type	Plus Ag. cpm ± SD	Minus Ag. cpm ± SD	Plus Ag. SI ± SD	Con A cpm ± SD	Con A SI ± SD
137/PBL	1189 ± 142	895 ± 142	1.33 ± 0.16	52969 ± 3801	59.18 ± 4.25
137/spleen	17400 ± 11380	1920 ± 803	9.06 ± 5.93	19174 ± 4587	9.99 ± 2.39
467/PBL	783 ± 138	461 ± 26	1.70 ± 0.30	12340 ± 3320	26.77 ± 7.20
467/spleen	2643 ± 1036	438 ± 201	6.90 ± 2.70	3024 ± 438	6.90 ± 1.00
468/PBL	81 ± 48	72 ± 7	1.12 ± 0.66	37938 ± 1061	519 ± 14
468/spleen	914 ± 578	103 ± 17	8.87 ± 5.61	24848 ± 1940	241 ± 19
469/PBL	457 ± 114	369 ± 54	1.24 ± 0.31	11436 ± 3579	30.99 ± 9.69
469/spleen	8612 ± 2784	709 ± 217	12.15 ± 3.93	16474	23.24
Neg./spleen	959 ± 307	1044 ± 6	0.92 ± 0.29	10776 ± 3079	10.32 ± 2.95

Table 3. PBLPR (expressed as SI ± SD) of positive PBL from one *A. marginale*-immune bovine, one *A. centrale*-immune bovine and a control animal against *A. marginale* (*A.m.*) and *A. centrale* (*A.c.*) antigen preparations and concavalin A (*Con A*) mitogen (*Neg*. Negative)

Treatment	A. m. PBL	A. c. PBL	Neg. PBL
Cell-only control	1.00 ± 0.48	1.00 ± 0.25	1.00 ± 0.25
A.m. antigen	6.38 ± 0.73	2.45 ± 0.81	2.74 ± 2.13
A.c. antigen	0.60 ± 0.37	3.46 ± 0.42	1.26 ± 0.41
Con A	5.16 ± 1.62	5.73 ± 0.26	4.68 ± 0.57

Species specificity of proliferative responses

An experiment was conducted to investigate the species specificity of the proliferative responses of *Anaplasma*-sensitised PBL against antigen from the two closely related species *A. marginale* and *A. centrale* as described in Materials and methods. The results (Table 3) showed that the *A. marginale*-sensitised PBL proliferated only in response to *A. marginale* antigen. *A. centrale*-sensitised PBL, however, proliferated in response to both *A. centrale* and *A. marginale* antigen preparations.

Discussion

The development of an assay to measure PBLPR against *Anaplasma marginale* required the use of a highly purified antigen preparation that was prepared aseptically and used as a sonicate containing disrupted whole rickettsia. Other methods for the rigorous purification of *A. marginale* from infected blood have been reported (McHardy and Gilson 1974; Budden and Dimopoullos 1977; Hart et al. 1981), but they were not utilised in this study due to the requirement for specialised affinity columns and extensive enzymatic digestions or due to the inadequate purification reported. The method developed in the current study is simple and effective and requires no specialised reagents or equipment to perform. The resulting material was visibly free from contamination with major host cell proteins as judged by SDS-PAGE (Fig. 1). A further requirement for the development of the PBLPR assay was the use of PBL from appropriate experimental animals, as peripheral blood from most long-term *A. marginale*-immune cattle was shown to lack detectable levels of *A. marginale*-sensitised lymphocytes (see below).

Titration of the pure *A. marginale* antigen used in the PBLPR assay demonstrated that significant stimulation was obtained down to a final total protein concentration of approximately 17.5 ng/ml (10^{-5} antigen dilution, Table 1), although a concentration of 17.5 μg/ml was routinely used in the assay. The sensitivity of the assay was shown to increase with increased duration of cell culture (Fig. 2) and, consequently, a culture period of 8 days was routinely used.

PBLPR was induced in cattle that had been protected against anaplasmosis by vaccination using crude *A. marginale* antigen and saponin adjuvant (Fig. 4B). When these cattle were infected, PBLPR decreased and were minimal at the time of peak parasitaemia. PBLPR transiently increased in three of the four cattle at the time of resolution of the patent infection. It is noteworthy that of the four vaccinated cattle, the individual that most effectively controlled parasitaemia and PCV fall (animal 244, maximal parasitaemia 0.50%, maximal PCV decrease –35.9%) also displayed a significantly stronger pre-peak parasitaemia PBLPR (99,609 cpm) than did the three other cattle (mean maximal parasitaemia 5.3 ± 1.5%, mean maximal PCV decrease –49.1 ± 5.4%, mean maximal pre-parasitaemia PBLPR 7,088 ± 3,952 cpm). Unvaccinated cattle were negative in the PBLPR assay before infection, and three of the four animals were subsequently treated to prevent mortality. Transient PBLPR coinciding approximately with the time of peak parasitaemia was induced by infection in all four cattle of this group. No correlation was evident between the magnitude of the PBLPR induced by infection and the susceptibility of individual cattle to disease as judged by the need for drug treatment. This observation indicates either that factors other than the induction of PBLPR are responsible for the resistance of previously unexposed cattle or that a PBLPR is not indicative of the overall immunological status of the cattle (see below).

Further investigation of the PBLPR of cattle undergoing initial *A. marginale* infection using increased sampling frequency revealed the presence of a second, significantly larger peak of PBLPR that occurred approximately 20 days after peak parasitaemia (Fig. 5). The magnitude of this second peak of PBLPR was extremely variable between individuals of the group and showed no correlation with the magnitude of the preceding parasitaemia. Cattle infected with *A. marginale* undergo a single sub-clinical relapse following recovery from the acute phase of infection (Lotze 1947). We have observed that cattle of similar breed and age, that have been infected with the same strain of *A. marginale* used in the current study have relapsed to a mean parasitaemia of approximately 1% at 15 days after initial peak parasitaemia (results not shown). The second peak of PBLPR is therefore coincident withe the expected time of this relapse in *A. marginale* parasitaenia.

Analysis of *A. marginale* carrier cattle at 6 months after recovery from clinical anaplasmosis demonstrated similar variation in PBLPR with time for individual cattle and large differences in the magnitude of PBLPR between individuals (Fig 7). As for the leucocyte-migration inhibition test (LMIT) and cell-mediated cytotoxic assay (CMCA) responses (Buening 1973, 1976), these results show that although a positive PBLPR is indicative of carrier status/immunity against *A. marginale*, a negative response is not necessarily indicative of carrier-free status/susceptibility.

IFN-γ has been shown to play a key role in immunity against a wide variety of pathogens, including intracellular bacteria (Kiderlen et al. 1984; Gould and Sonnenfeld 1987; Zhan and Cheers 1993) and rickettsia (Li et al.

1987), via pleiotropic effects, including activation of macrophages (Nathan et al. 1983) and neutrophils (Steinbeck et al. 1986). Due to the location of *A. marginale* within erythrocytes and the lack of protection afforded by antibody in immunity against this parasite, IFN-γ is a good candidate for effecting immunity against this pathogen. This may involve the stimulation of the production of tumor necrosis factor (TNF) and toxic reactive nitrogen and/or oxygen intermediates by monocytes, as has been shown for immunity against the intraerythrocytic stages of malaria (reviewed by Good and Miller 1989). This proposed non-specific effector mechanism is consistent with the observed non-specific immunity against *A. marginale* conferred by bacille Calmette-Guérin (BCG) vaccination (Sharma 1988) or concurrent *Theileria* infection (Stewart et al. 1990; Gale et al., manuscript in preparation). We demonstrated high levels of IFN-γ in PBLPR-assay supernatants from day 4 to day 8 and subsequently analysed day-8 supernatants in further experiments. The analysis of IFN-γ levels in the experiments described above (Figs. 3, 6) clearly demonstrates that PBL that exhibit strong proliferation in response to *A. marginale* result in the accumulation of high levels of IFN-γ in cell-culture supernatants. The association of high levels of IFN-γ with a strong PBLPR is consistent with the probable involvement of cell-mediated immune responses in immunity against *A. marginale*.

PBL from the five cattle analysed during the course of initial *A. marginale* infection (Fig. 6) also produced detectable IFN-γ and PBLPR in the absence of added *A. marginale* antigen at some time points where a strong PBLPR and high IFN-γ levels were evident in response to added antigen. At these time points a significant level of erythrocytes present in the circulation of the cattle were infected as a result of either initial peak parasitaemia or relapse parasitaemia. It is therefore possible that circulating *A. marginale* resulted in the carry-over of antigen (present in antigen-presenting cells) into the PBLPR assays at these time points. This may also be responsible for the IFN-γ detected in the absence of added antigen for the assays using PBL from the splenectomised carrier calf (animal 130, Fig. 3), as splenectomised cattle maintain relatively high levels of circulating *A. marginale*.

In this study a PBLPR was detected when *A. centrale*-sensitised PBL were cultured in the presence of *A. marginale* antigen, although no proliferative response was detected when *A. marginale*-sensitised PBL were cultured with *A. centrale* antigen. The use of live *A. centrale* as a vaccine to reduce the severity of subsequent infection with the more pathogenic species *A. marginale* has been practised for many years (Theiler 1912). Interestingly, no protection against clinical disease due to *A. centrale* infection was conferred by prior infection with *A. marginale* (Kuttler 1967a), although significant serological cross-reactivity between the two species has been reported (Kuttler 1967b). Thus, the one-way cross-reactivity of sensitised T-cells between the two *Anaplasma* species observed in this study correlated with the previously reported one-way cross-species protection.

The spleen plays a key role in immunity against *A. marginale* as demonstrated by the increased susceptibility of splenectomised bovines to infection and the severe relapse of carrier cattle after splenectomy (Ristic et al. 1958). The absence of a detectable PBLPR in some immune cattle and the variability of PBLPR in immune cattle over time (Fig. 7) may result from the homing of sensitised T-cells from the circulation to lymphoid tissues via the expression of specific cell-surface molecules (Stoolman 1989). In this study, *A. marginale*-sensitised T-cells were detected in the spleens of all four infected calves tested and at time points ranging from peak parasitaemia to 23 weeks post-infection. At these times, no *A. marginale*-sensitised T-cell was detected in the peripheral blood. These results are consistent with the variable and transient PBLPR responses observed in infected cattle in this study. Clearly, in *A. marginale*-immune cattle a spleen-derived LP response as an indicator of previous *A. marginale* exposure is far superior to a blood-derived lymphocyte response.

A preliminary study to elucidate the nature of the lymphocyte-stimulatory material present in the purified *A. marginale* preparation revealed this to be sensitive to inactivation by proteolysis. Proteolysis also abolished the protection against clinical anaplasmosis that had been obtained when purified *A. marginale* antigen was used to vaccinate cattle. The isolation of specific proteins that stimulate T-cell proliferation by various screening methods has been reported elsewhere (Hutchings et al. 1990; Brooks-Worrell and Splitter 1992). Such methodologies may now be applied to test the capacity of specific *A. marginale* antigens to induce proliferation of lymphocytes from immune cattle of various major histocompatibility complex (MHC) genotypes. Characterisation of some CD4+ T-cells as Th1 or Th2 is possible on the basis of the types and relative amounts of cytokines that are secreted (Mosman et al. 1986; Scott et al. 1989). Specific T-helper-cell subsets preferentially induce specific immune effector functions, with Th1 mediating primarily cellular responses and Th2, humoral responses (Stout and Bottomly 1989; Oswald et al. 1992; Romagnani 1992). The existing evidence regarding the likely effector mechanisms that confer immunity against *A. marginale* suggests a potential role for antigens that, preferentially induce Th1 responses as protective immunogens against *A. marginale* infection.

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