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USE OF T-LYMPHOCYTES TO IDENTIFY VACCINE ANTIGENS AGAINST HEARTWATER

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1. EXECUTIVE SUMMARY

Cowdria ruminantium is an obligate intracellular pathogen that causes heartwater in ruminants. Several findings suggest that T cells play an important role in protection against the disease. The aim of the project was to generate data which will lead to an effective vaccine against heartwater, one of the three major diseases of domestic ruminants in sub-Saharan Africa. In addition we need to identify proteins of the heartwater agent, *Cowdria*, which may be used as components of a recombinant vaccine. *Cowdria* proteins with low molecular masses of 11, 12, 14 to 17 and 19 to 23 kDa have subsequently been identified that induce proliferative responses by lymphocytes from immunized cattle. This data was published in Infection and Immunity. Further experiments, included in a paper to be submitted to 'Experimental Parasitology', indicated that proteins of molecular mass 11 to 21 kDa induced $CD4^+$ lymphocytes to proliferate from all the cattle tested, but only proteins of molecular masses from 13 to 18 and 31 kDa induced $CD4^+$ lymphocytes to produce IFN-γ. As IFN-γ is known to play an important role in immunity to heartwater this may suggest that the *Cowdria* proteins that induce both this cytokine and T helper lymphocyte proliferation are likely to be valuable as candidate vaccine antigens. Furthermore, collaboration between the grant partners has contributed to the establishment of an effective immunological team, experienced in cell-mediated immunology, at the OVI. In the process of achieving these goals the immunological capabilities of the OVI was simultaneously strengthened in the field of cell-mediated immunity; through effective cooperation among all the institutions the KVI experience was gained in molecular biology methodology, specifically, in handling

plasmid vectors for proteins derived from rickettsial organisms, specifically *Anaplasma*, causing anaplasmosis in cattle.

2. RESEARCH OBJECTIVES

Cowdria ruminantium (*Cowdria*) is an obligate intracellular pathogen that causes heartwater disease in ruminants. The disease is controlled primarily through immunization by infection with virulent blood and subsequent treatment with antibiotics to prevent a serious course of the disease. This is a cumbersome procedure that has numerous disadvantages including its unsuitability for use in countries where potential vectors are present, but which do not harbor the disease. There is thus a real need for an improved vaccine, especially in terms of increased efficacy and improved safety. Subunit vaccines are potentially safe, cheap, heat stable, easy to administer and can induce a broad immune response with life long memory (Liljeqvist & Stahl, 1999). Several findings suggest that cellular immunity plays an important role in protection against heartwater (Byrom *et al.,* 2000; Du Plessis, 1982; Du Plessis *et al.*, 1991; Du Plessis *et al.*, 1992; Martinez 1997; Mwangi *et al.*, 1998a-c; Totté *et al.*, 1997; Totté *et al.*, 1998). Cattle immunized against heartwater by infection or with inactivated organisms produce *Cowdria*-specific, IFN-γ producing $CD4^+$ T lymphocytes (Mwangi *et al.* 1998; Totté *et al.* 1997). Short term $CD4^+$ T cell lines generated using *Cowdria* lysates respond to soluble *Cowdria* proteins fractionated by fast performance liquid chromatography (FPLC), a single peak of proliferation was observed which included proteins between 20 and 32 kDa (Totté *et al.*, 1998). IFN-γ a cytokine associated with a Type 1 immune response has also been shown to be important in immunity to

heartwater. This cytokine is a very potent inhibitor of *Cowdria* growth in endothelial cells, *in vitro* (Totté *et al.* 1996) and T lymphocytes from immune cattle produce IFN-γ in the presence of total lysates of *Cowdria* (Totté *et al.* 1997).

Protective malarial parasite proteins have been identified by vaccination trials with fractions obtained by continuous flow electrophoresis of *Plasmodium chabaudi adami* schizont proteins (Kima *et al.*, 1992). Since T-cells are required for protective immunity in malaria (Grun & Weidance, 1981; Brake *et al.*, 1988) this finding suggested that potentially protective T-cell antigens could be identified by this technique. Using a similar procedure, Brown and co-workers (Brown *et al.*, 1995) successfully identified eight antigens of *B. bovis* merozoites that stimulated proliferation of Th-cell clones and IFN-γ production. The major surface protein 2 (MSP2) of *Anaplasma marginale* has been shown to induce protective immune responses in cattle characterized by a T helper lymphocyte response and high levels of IFN-γ production (Brown *et al.*, 1998). MSP2 epitopes have subsequently been identified that induce T helper responses and are thus targets of further vaccine development (Brown *et al.*, 2001).

From the current state of knowledge described above, it is clear that any rational attempt to develop a recombinant vaccine should therefore focus on components of the parasite that can elicit the appropriate immune response.

3. METHODS AND RESULTS

To identify the proteins involved in recall T-cell responses in immune cattle, *Cowdria* was therefore fractionated by continuous flow electrophoresis and each fraction tested for its ability to stimulate lymphocyte proliferation *in vitro*. Uninfected endothelial cells were similarly fractionated and served as controles in the ensuing assays. In an

attempt to simulate the natural infective process, the PBMC used in this study were obtained from animals rendered immune by infection and treatment. In a parallel study four cattle were immunized with inactivated *Cowdria* to determine whether their lymphocytes also responded to fractionated proteins.

Cowdria infected and uninfected endothelial cell lysates were fractionated between 11-38 kDa and 50-168 kDa on 15% and 7% acrylamide gels respectively. Proliferation assays after immunization by infection and treatment detected no *Cowdria* specific proliferation *in vitro* after one vaccination. Proliferation was however observed between one to four weeks after challenge. This was followed by a period of no detectable response after which the response reappeared. PBMC from animals immunized with inactivated organisms proliferated specifically in response to antigen soon after the first immunization. Only *Cowdria* proteins with low molecular masses of 11, 12, 14 to 17 and 19 to 23 kDa induced proliferative responses by lymphocytes from all six animals.

These results are published in: Van Kleef, M., N.J. Gunter, H. Macmillan, B.A. Allsopp, V. Shkap, and W.C. Brown. 2000. Identification of *Cowdria ruminantium* antigens that stimulate proliferation of lymphocytes from cattle immunized by infection and treatment or with inactivated organisms. Infection and Immunity. 68:603-614. **Please, see attached reprint**.

As the type I cytokine interferon-gamma has been implicated in protective immunity to heartwater a further study was undertaken to determine which T-cell subset responds to and whether IFN-γ is produced when stimulated with fractionated *Cowdria* proteins. *Cowdria* specific T-cell lines were established from cattle immunized by infection and treatment or with inactivated organisms. It was possible to enrich for $CD4^+$

T-cells over time by alternating stimulation with a rest cycle. This led to greater *Cowdria* antigen specific proliferation and IFN-γ production with concomitant lower background. Proteins of molecular mass 11 to 21 kDa induced $CD4^+$ lymphocytes to proliferate from all the cattle tested, but only proteins of molecular masses from 13 to 18 and 31 kDa induced $CD4^+$ lymphocytes to produce IFN-γ. Although the two groups of cattle were immunized differently, their lymphocytes responded similarly to the aforementioned fractions. The results extend our initial findings, by identifying responder cells as predominantly $CD4^+$. Furthermore, the low molecular mass proteins could induce the $CD4^+$ lymphocytes to produce IFN-γ.

These results are to be submitted to 'Experimental Parasitology' **(please see attached draft**). Van Kleef, M., H. Macmillan, N.J. Gunter, E. Zweygarth, B.A. Allsopp, V. Shkap, D.H. Du Plessis, and W.C. Brown. *Cowdria ruminantium*: low molecular mass proteins induce bovine helper T-cell lines to proliferate and produce interferon-gamma. Experimental Parasitology.

3.1. Additional unpublished results.

3.1.1. Immunoblot analysis to determine which fractions contain the MAP1 protein.

A western blot of fractions in the molecular mass range of MAP1 (ie 30-32 kDa) incubated with monospecific anti-MAP1 goat serum indicated that MAP1 appears to be dominant in fractions 123-125 coinciding with proteins with molecular mass of 31 kDa (Fig. 1). These fractions fall outside the area of common recognition and we can conclude that MAP1 does not induce PBMC or short term T-cell lines to proliferate in our assays. However MAP1 did induce T-cell lines to produce IFN-γ.

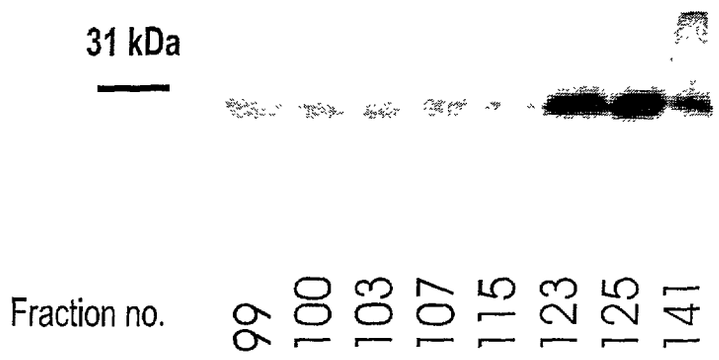


FIG. 1. Western blot analysis of selected CFE fractions probed with goat anti-serum raised against the MAP1 protein of *Cowdria.* A volume of 20 µl of every selected precipitated fraction was subjected to analytical SDS-PAGE, blotted onto a PVDF membrane and probed with goat anti-serum raised against the MAP1 protein of *Cowdria.*

3.1.2. Generation of T-cell Clones.

To further characterize in detail the $CD4^+$ T cell response to *Cowdria,* attempts were made at cloning. Numerous attempts at cloning by limiting dilution of enriched $CD4^+$ T-cell lines were however unsuccessful. Cloning T-cells from PBMC cultured for 7 days led to $\gamma\delta$ T-cell clones that did not proliferate to *Cowdria* antigen.

3.1.3. Protective capacity of identified proteins.

To determine the protective capacity of the common stimulatory proteins and for the preparation of serum, mice were immunized with pools of selected proteins. *Cowdria* proteins were fractionated by continuous flow electrophoresis on a 15% acrylamide gel (Fig. 2.). Selected fractions were precipitated and pooled into 7 groups (P1 - P7) that represent the following molecular masses: P1 (11-13); P2 (14-16); P3 (19-22); P4 (23-24); P5 (27-28); P6 (29-30) and P7 (34-35) kDa. Figure 3 illustrates the molecular masses of the precipitated fractions before they were pooled into the aforementioned groups.

Splenocytes from each mouse showed the highest proliferative response to the antigenic pools with which it was immunized (Fig. 4a.). The mice also showed proliferative responses to other antigenic pools. This can be explained by the complex composition of each pooled antigenic groups which can overlap or have cross reactive antigens in other pools. The proliferative reactions were nevertheless specific compared to the response of the untreated mouse and the response to uninfected BSV cell lysates. The low proliferative response to Welgvonden infected lysate may be an indication that the concentration of the relevant antigens were to low to elicit an adequate reaction (Fig 4b.).

To determine an adequate challenge dose, control mice were immunized with adjuvant alone and challenged in three groups of 5. Each group received 200μl of a 10^{-4}; 10^{-5} or 10^{-6} dilution of *Cowdria* infected endothelial cell stock. In the 10^{-4} and 10^{-5} groups all mice died at 12 ± 1 and 15 ± 2 days respectively. In the 10^{-6} group two out of five survived. This corresponded with previous challenges done with the same stock in a similar fashion, indicating that a 10^{-5} dilution of challenge material is sufficient to kill all control mice.

The results of the challenge experiment are shown in Table 1 for groups 1-3. All animals in the control group that were challenged with the same dilution as the experimental animals survived whereas none of the animals that were challenged with a ten times higher dilution or a ten times lower dilution survived. One of the animals in experimental group 1, two in group 2 and three in group three died. This result showed that the challenge dose was probably not adequate and highly variable. Groups 4-7 were challenged with a newly titered challenge dose that indicated an adequate challenge dilution to be 2×10^{-5}. At the time of the challenge three control groups were challenged with 10x, 1x and 0.1x challenge doses to evaluate the challenge dose. Results of the challenge are tabulated in Table 2. All the animals in experimental groups died as shown in Table 2. Inconsistent dying of animals receiving 10 times the challenge dose of the experimental animals makes sensible conclusions impossible. It seemed that 4/8 mice in group 5 and 3/8 mice in group 6 lived longer than day 16 compared to control animals that lived only till day 15 or survived. However these experiments again highlighted the problems that other researchers and we have experienced to obtain an accurate challenge dose. Consequently no conclusions can be made with current data. Serum collected from each group of mice during and after immunization failed to detect

any *Cowdria* proteins in western blots. The challenge material has subsequently been optimized and sheep have currently been immunized with various *Cowdria* fractions. These experiments are supported by a FRANCO/South African grant.

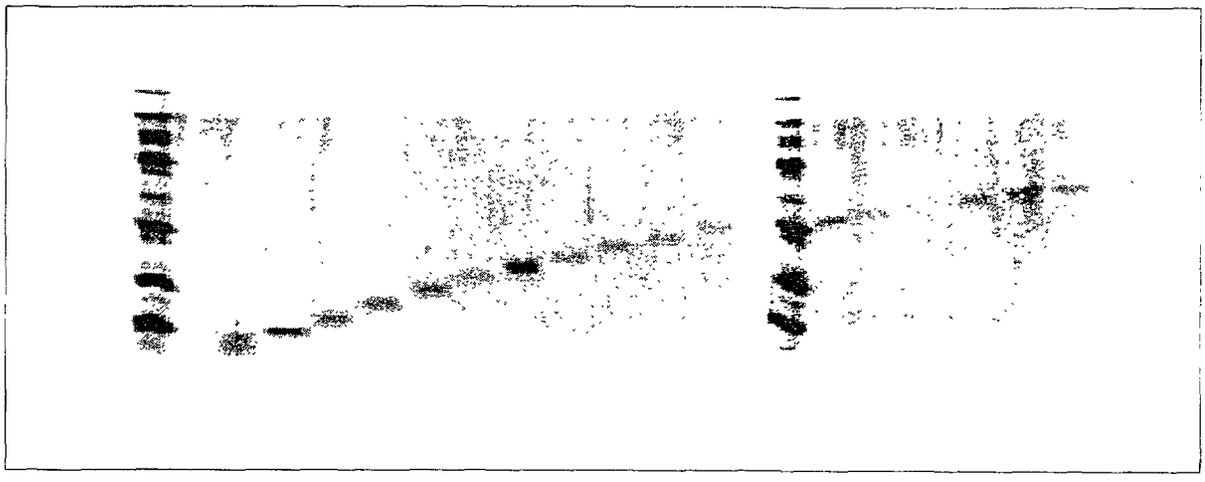


FIG. 2. SDS-PAGE and silver staining analysis of *Cowdria* proteins fractionated by CFE. Crude *Cowdria* infected BSV cell lysates were fractionated by CFE on a 15% acrylamide gel. Sixty percent of each fraction was precipitated with acetone and resuspended in 500 µl PBS and visualized on silver stained gels.

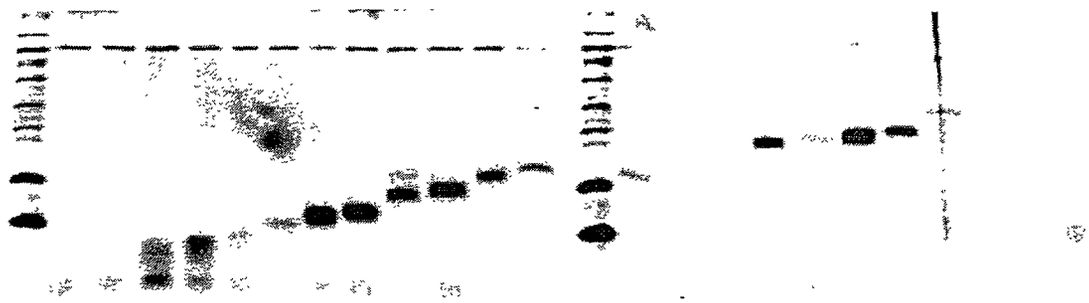
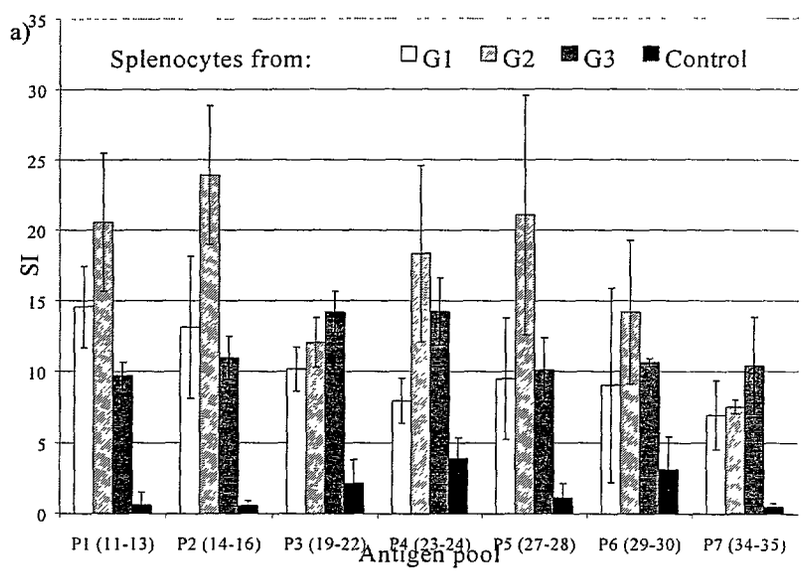


FIG. 3. SDS-PAGE and silver staining analysis of selected fractions of *Cowdria* proteins fractionated by CFE on a 15% acrylamide gel.



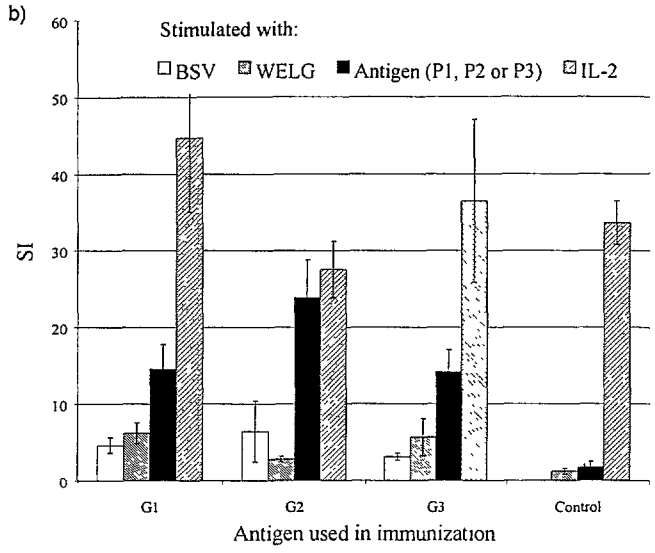


FIG. 4. Proliferative responses of splenocytes from a control mouse and mice from G1-G3. a) Splenocytes were assayed with antigenic pools (P1 - P7) to determine if the immunization was successful. The G1 mouse was immunized with antigenic group P1, the G2 mouse with P2 and G3 mouse with P3. Values between brackets indicate the MM range of antigens that are included in the antigenic pool. b) Specificity of proliferative responses was determined by assaying the splenocytes with uninfected BSV (BSV); Welgevonden infected BSV cells (Welg), human recombinant Interleukin-2 (IL-2) and the relative antigen pool that it was immunized with. The * indicates the average proliferative reaction of the splenocytes from the untreated mouse to all antigenic pools.

Table. 1.

Survival of mice immunized with pooled *Cowdria* fractions or PBS controls after challenge.

Mouse Group	Molecular mass (kDa) of fractions used to immunize mice	Dilution of challenge material[a]	Survival[b]	Number of Mice succumbing X days after challenge					
				13	14	15	16	17	23
1	11-13	1×10^{-5}	7/8				1		
2	14-16	1×10^{-5}	6/8			1	1		
3	19-22	1×10^{-5}	5/8	1			1		1
Control A	-	1×10^{-4}	0/5		2	2	1		
Control B	-	1×10^{-5}	5/5						
Control C	-	1×10^{-6}	0/5		1	2		2	

[a] The challenge material were diluted in RPMI 1640 medium.
[b] Number of mice survived/total number of mice in group.

Table. 2.
Survival of mice immunized with pooled *Cowdria* fractions or PBS controls after challenge.

Mouse Group	Molecular mass (kDa) of fractions used to immunize mice	Dilution of challenge material[a]	Survival[b]	Number of Mice succumbing X days after challenge						
				12	13	14	15	16	17	18
4	23-24	2×10^{-5}	0/6	1			4	1		
5	27-28	2×10^{-5}	0/8	1	1	1		2	2	1
6	29-30	2×10^{-5}	0/8	1	2	1			1	3
7	34-35	2×10^{-5}	0/8	2		3	3			
Control A	-	2×10^{-4}	3/5		2					
Control B	-	2×10^{-5}	0/4		2	1	1			
Control C	-	2×10^{-6}	5/5							

[a] The challenge material were diluted in RPMI 1640 medium.
[b] Number of mice survived/total number of mice in group.

3.1.4. Production of antiserum to *Cowdria* proteins.

In order to be able to identify the genes encoding the fractions of interest antiserum can be used to screen phage display libraries which could lead to the identification of the genes encoding these proteins. Their role in the protection against heartwater can then be determined. Goats, a chicken and rabbits were used for the preparation of serum specific to the identified proteins of *Cowdria*.

3.1.4.1. Antiserum production in Goats.

No antigen specific serum was obtained when two goats were immunized with either the 10 or 15 kDa protein of *Cowdria* (Table 1.). Due to the relative simplicity of preparing IgY antibodies in chickens it was then decided to immunize chickens.

3.1.4.2. Production of IgY in Chickens.

A chicken was immunized with the 15 kDa protein of *Cowdria*. This resulted in IgY that identified the 16; 14 and 12,5 kDa proteins of *Cowdria*. These proteins could only be detected with ECL at an antibody dilution of 1/20. This antibody preparation cross reacted with MAP1 (31 kDa) and bovine serum albumin (Fig. 5. Table 1.). Such a phenomenon has previously been observed when serum was prepared to the 27 kDa protein of *Cowdria* in rabbits. Due to the low titer and specificity of these antibodies serum preparation in rabbits was considered as an alternative.

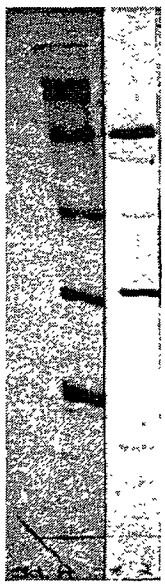


FIG. 5. Western blot analysis of *Cowdria* infected BSV cells probed with chicken IgY raised against the 15 kDa protein of *Cowdria*. *Cowdria* infected BSV cells were subjected to SDS-PAGE, blotted onto a PVDF membrane and probed with chicken IgY.

3.1.4.3. Antiserum production in rabbits.

Four rabbits were immunized with homogenized gel containing proteins ranging between 23 to 29; 18 to 22; 17 to 19 and ≤ 13 kDa respectively. Freunds complete followed by Freunds incomlpete adjuvant was used as it is known for its effectiveness in preparation of serum. Only two rabbits resulted in serum recognizing *Cowdria* specific proteins between 18 and 24 kDa (Fig. 6; Table 3.). Once again these proteins could only be detected with ECL at serum dilutions of 1/20. In addition to this the rabbit serum contained numerous non-specific antibodies to BSA amongst other proteins. Anti-serum form rabbits C and D were used to probe a *Cowdria* phage display library for peptide identification. Four *Cowdria* peptide sequenced were identified and are being further characterized (Personal communication Jeanni Fehrsen, Immunology Division, OVI).

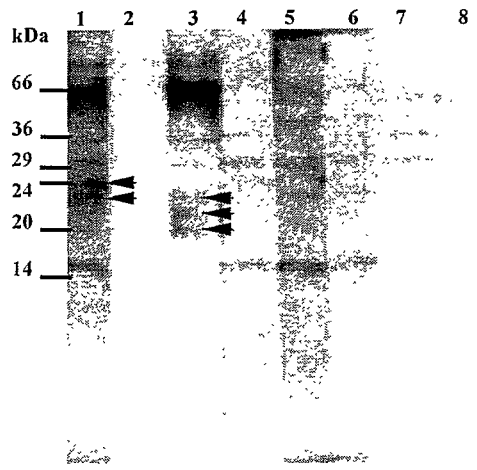


FIG. 6. Western blot analysis of *Cowdria* infected BSV cells probed with rabbit IgG raised against various proteins of *Cowdria*. *Cowdria* infected BSV cells were subjected to SDS-PAGE, blotted onto a PVDF membrane and probed with rabbit serum. Lanes 2; 4; 6 and 8 were probed with prebleed serum from each respective rabbit. Lane 1: probed with serum from rabbit immunized with 23 to 28 kDa proteins; Lane 3: probed with serum from rabbit immunized with 18 to 22 kDa proteins; Lane 5: probed with serum from rabbit immunized with 17 to 18 kDa proteins and Lane 1: probed with serum from rabbit immunized with ≤ 13 kDa proteins. The arrows indicate protein specific binding.

TABLE 3.

Summary of the results obtained after immunizing goats, a chicken and rabbits with various *Cowdria* proteins.

ANIMAL	IMMUNIZED WITH kDa	RESULT KDa
Goat A	10	X
Goat B	15	X
Chicken A	15	12; 14; 16
Rabbit A	≤13	X
Rabbit B	17-19	X
Rabbit C	18-22	18; 20; 22
Rabbit D	23-29	22; 24

3.1.5. Two-dimensional electrophoresis.

Considering that the antibodies obtained had low titers and specificity, even after numerous attempts in different animals, another approach for gene identification should be investigated. Alternatively, the partial amino acid sequence of identified proteins can be determined and probes developed for screening genomic libraries for identification of the genes coding the proteins. For this the specific proteins that induce lymphocytes to proliferate and produce IFN-γ must firstly be identified. Two-dimensional electrophoresis may be used to determine whether proteins are homogenous, when separated by SDS-PAGE. This method offers high resolution and sensitivity for the separation of proteins from a complex biological source since each dimension separates proteins according to independent parameters (O'Farrel, 1975). Results obtained after two-dimensional electrophoresis of crude organisms showed that there were more than one protein within a molecular weight region that differ in pI (Fig. 3.7.).

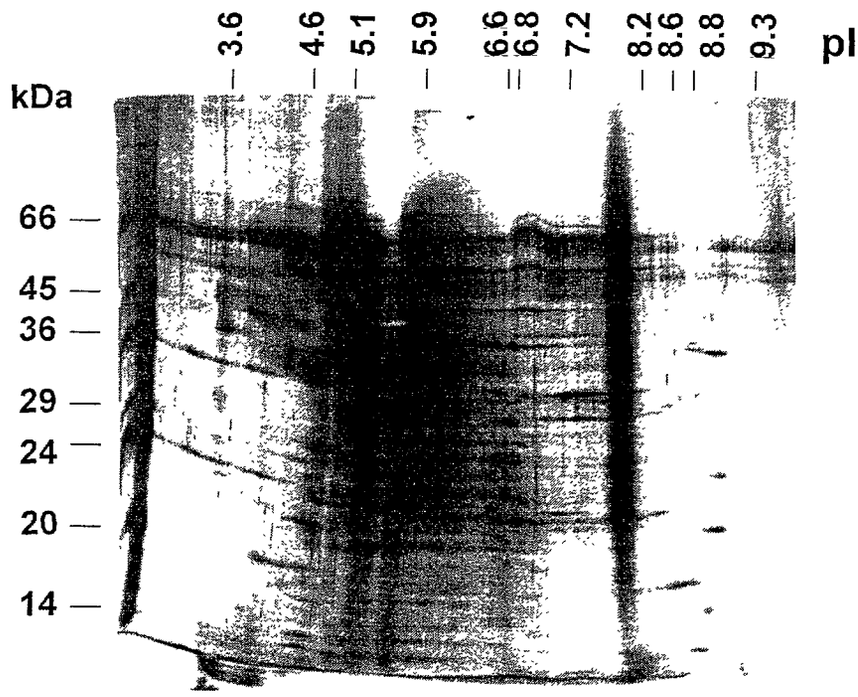


FIG 7. Two-dimensional electrophoretic analysis of crude *Cowdria* infected BSV cell lysates. First-dimension electrophoresis was performed with Ampholine PAGplate precast gels pH 3.5-9.5. The second-dimension was performed with a 12% SDS polyacrylamide gel run on a BIO-RAD vertical electrophoresis unit. The second-dimension gel was developed with Gelcode silverstain.

3.2. Methods.

3.2.1. Sodium dodecyl sylphate-polyacrylamide gel electrophoresis.

SDS-PAGE was performed with crude infected and uninfected cell culture extracts as described by Van Kleef *et al.*, 1993. The stacking gel contained 4 % acrylamide, 0.1 % bisacrylamide, 0.1 % SDS in 0.1 M Tris buffer (pH 6.8). The separating gel contained 7, 12 or 15 % acrylamide, 0.3 % bisacrylamide, 0.1 % SDS in 0.4 M Tris buffer (pH 8.8). Each gel was polymerized by the addition of ammonium persulphate and TEMED at final concentrations of 0.05 % and 0.1 % respectively in the stacking gel and 0.05 %

each in the separating gel. Large gels of 1.5 x 120 x 160 mm or mini gels of x x mm were prepared. The samples (40 μg/well, unless otherwise stated) and molecular mass standards were dissolved in 0.06 M Tris buffer (pH 6.8), 16 % glycerol, 2 % SDS, 2.5 % DTT and 0.001 % bromophenol blue by heating at 100°C for 10 min. Electrophoresis was performed in an electrode buffer containing 0.02 M Tris, 0.1 M glycine and 0.06 % SDS, pH 8.3. The large gels were electrophoresed for 2 h at a constant current of 45 mA whilst the mobile front was in the stacking gel. Once the mobile front entered the separating gel the electrophoresis was performed at a constant current of 65 mA for 3 h. Protein bands were visualized by staining the gel with Coomassie brilliant blue or Copper sulphate stain (Lee *et al.*, 1987).

3.2.2. Western blotting onto PVDF membranes.

Electrophoretic transfer of SDS-PAGE separated proteins was achieved as described by (Van Kleef *et al.*, 1993). Proteins were transferred to a PVDF membrane (Millipore) by electrophoresis in 10 mM CAPS buffer, pH 9.0 at 0.25 A for 1 h 45 min. The blotted sheet was air-dried and stored at –20°C until further use. The molecular mass standards and samples were visualized by staining the membrane with Coomassie brilliant blue. Immunostaining of PVDF membranes was performed as described previously (Van Kleef *et al.*, 1993). Proteins were detected by ECL.

3.2.3. Generation of T-cell Clones.

One week cultures of peripheral blood mononuclear cells or $CD4^+$ enriched T-cell lines (Van Kleef *et al.,* 2001, submitted) were subjected to cloning by limiting dilution. Cloning was performed in 96-well round-bottom plates as described elsewhere (Brown *et al.*,

1992). A statistical average of 1 or 0.3 cell per well was stimulated with 1 μg of *Cowdria* antigen per ml of complete RPMI 1640 medium containing 10% TCGF and irradiated autologous PBMC. Seven days after the last stimulation, cells were tested for antigen specific proliferation and phenotyped by flow cytometric analysis as described Van Kleef *et al* 2001, submitted).

3.1.3. Protective capacity of identified proteins.

Cowdria proteins were fractionated by continuous flow electrophoresis on a 15% acrylamide gel as described previously (Van Kleef *et al.,* 2000). Selected fractions were precipitated and pooled into 7 groups (P1 - P7) that represent the following molecular masses: P1 (11-13); P2 (14-16); P3 (19-22); P4 (23-24); P5 (27-28); P6 (29-30) and P7 (34-35) kDa. All material used in immunizations were prepared as follows: adequate volumes of each fraction were pooled and mixed 1:1 with Montanide ISA50 and made into an emulsion. Mice were divided into 7 groups (G1 - G7) of 9 mice each. Mice in G1 were immunized three times with P1 (11-13 kDa); G2 with P2 (14-16 kDa) etc. Six groups of control mice (five mice per group) were immunized with ISA-50 and PBS. Animals were immunized with the emulsion on days 1, 14 and 35. Each animal received a 200μl volume of emulsion subcutaneously at each immunization. Animals were challenged four weeks later with 200μl of live *C.ruminatium* infected endothelial cell stock diluted either 1 or 2 x 10^{-5} and administered by intravenous injection into the tail vain. Three groups of control mice (5 mice per group) were similarly challenged with dilutions of either 1 or 2 x 10^{-4}; 10^{-5} and 10^{-6} to verify the challenge dose. Mice were checked daily for deaths during challenge period. On the day of challenge one mouse each from G1, G2 and G3 were sacrificed together with a control mouse (before

challenge). The spleens were removed under sterile conditions, cut into fine pieces and then forced through a fine metal mesh. The resulting suspension was put through nylon wool to obtain a single cell suspension. The cells were Ficoll purified and prepared for lymphocyte proliferation assays as described (Van Kleef *et al.*, 2000).

3.2.4. Production of antiserum.

Preparative SDS-PAGE was performed with differentially centrifuged Welgevonden/ E5 antigen as described previously (Rossouw *et al.,* 1990). The bands of interest were excised and prepared for immunisation as described in Tables 2-4. Alternatively, indicated fractions from continuous flow electrophoresis (CFE) were used to immunize the animals as described in Tables 4-6. The sera collected were evaluated by immunoblotting using Welgevonden isolate infected and uninfected culture extracts as antigen in western blots as described previously (Rossouw *et al.,* 1990). Blots were developed by ECL.

Table 4.
Two goats were immunized with either the 10 or 15 kDa proteins of *Cowdria* according to the following schedule:

Goat	KDa	Week	Immunized with
A	10	0	2 gels + ISA50
		4	200 µl CFE fraction no 4
B	15	0	400 µl CFE fraction no 28
		4	200 µl CFE fraction no 31

Table 5.
A chicken was immunized with the 15 kDA protein of *Cowdria* according to the following schedule:

Week	Immunized with:
0	½ gel + ISA50
3	¼ gel + ISA50
4	¼ gel + ISA50

Table 6.
Four rabbits were immunized with the following proteins: A) ≤ 13 kDa; B) 17 to 19 kDa; C) 18 to 22 kDa and D) 23 to 28 kDa according to the following schedule:

Week	Immunized with
0	Gel + FCA
4	Gel + FIA
8	Gel only
12	Gel only
17	Gel only

3.2.5. Two dimensional electrophoresis.

Two dimensional electrophoresis was performed as described by O'Farrel (1975). First dimension Isoelectric focusing: Crude Welgevonden infected and uninfected cell culture extracts and standard proteins with known isoelectric points were dissolved 1:1 in a sample buffer containing 8 M urea, 2 % CHAPS, 2 % ampholines, DTT (7 mg/2.5 ml stock) and bromophenol blue. Isoelectric focusing was performed using an Ampholine PAG plate (Pharmacia Biotech) and 1 M NaOH at the cathode and 1 M H_3PO_4 at the anode at 1500V, 50 mA, 30 W for 1.5 h. The gel was either Coomassie stained or subjected to SDS-PAGE second dimension. Second dimension SDS-PAGE: The IEF gel was equilibrated in SDS-PAGE sample buffer for 30 min. The top of the stacking gel was covered with 1 ml molten 1 % (w/v) agarose. The equilibrated first dimension gel was immediately positioned on top of the molten agarose and imbeded with more agarose. A low molecular weight standard sample was loaded into a precast well. Electrophoresis was performed in a 12 % polyacrylamide separating gel and 4 % stacking gel. Protein bands were visualized by staining the second dimension SDS-PAGE gel with Gelcode^R colour silver stain kit (Pierce).

4. IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

4.1. Scientific Impact and Relevance.

The primary scientific goal is to identify individual *Cowdria ruminantium* proteins, which may be used as components of a recombinant vaccine. *Cowdria* protein fractions were identified which induced proliferation of lymphocytes from animals immunized by infection and treatment and from animals immunized with inactivated organisms. We have confirmed these results, with added controls, and extended these findings to the

identification of the phenotype of the responder cells ($CD4^+$ T-cells) and those fractions that induced IFN-γ production in addition to proliferation. The combination of infection and treatment resembles natural infection, and although the dose administered is higher than that acquired in the field, the proteins identifies by PBMCs from the immunized cattle led to identification of potential candidate antigens to be considered in vaccine design. We are thus now able to focus on a narrow range of proteins that may be useful as vaccine candidates. The next stage would be to identify individual proteins from these fractions, and to locate the genes coding for them. The need for cell mediated immunity expertise at the Onderstepoort Veterinary Institute, in order to facilitate our heartwater research, was the goal of the 'Institutional Strengthening' portion of this project. This goal has been effectively achieved. The financial support from the project was a major contributor to a decision, made in 1995, to establish a Division of Immunology.

4.2. Indigenous Research Strengthening

Each of the two young immunologists employed for training under this project, Nico Gunter and Henriette Macmillan, became core members of the Immunology Department of the Institute. Under the supervision of Mirinda van Kleef there is now at the OVI an effective immunological team experienced in the vital area of cell-mediated immunology. In addition this expertise has lead to new Grants being awarded to OVI: 1) Franco/SA Co-operative research grant: Development of a recombinant vaccine against heartwater based on genes of the pathogen (*Cowdria*) that code for protective antigens. 2) INCO-DEV grant ICFP 599A4PR01: Development of an improved vaccine against contagious bovine pleuropneumonia.

5. PROJECT ACTIVITIES/OUTPUTS

5.1. Meetings attended.

The collaboration, travel and training components of the project during the grant consisted of:The PI from Israel, Dr. Varda Shkap, and the co-PI from the USA, Dr. Wendy Brown visited OVI during February 1988 and October 1999. During each visit the progress of the project was thoroughly reviewed and plans for the remainder of the project drawn up. Parts of the research were presented at STVM Meetings in France, 1997, Montpellier and USA, 1999, Key West.

5.2. Training.

Mr. Nico Gunter visited Dr. Wendy Brown's lab in WSU during November 1997 to May 1998: flow cytometry, cytokine RT-PCR, general cellular immunology and molecular techniques. Ms. H. Macmillan visited Dr. Wendy Brown's lab in WSU during June to October 1997 and September to October 1999: she gained experience in T-cell cloning and analysis of cellular immune response, RT-PCR detection of cytokine levels, multiple FACS staining and analysis. Ms. H. Macmillan also met with Dr. T.C. McGuire (a cytotoxic T-cell expert) and made contact with people in his lab working with cytotoxic T-cell assays. Valuable information was gained on the possibility of doing cytotoxic T-cell assays in our own lab. Dr. W.C. Brown also kindly provided OVI with IFN-γ; IL-2; IL-4; IL-10 and β-actin competitor plasmids which were grown up and tested at the OVI. All the relevant reagents are now in place at the OVI for RT-PCR. The initial aim of the second visit was to gain experience in starting T-cell clones from lymph nodes that are removed

from immunized animals. These animals were immunized with the N-terminal portion of RAP-1 (*Babesia bovis*). Before the lymph nodes could be pulled it was important to show that the cattle had seroconverted. Western blotting experiments indicated that the cattle had not sero-converted and it was decided to immunize the animals again. Thus the cattle were not ready for lymph node experiments during her visit. Valuable information and experience was however gained on macrophage isolation, culturing and use in a Griess assay to measure the production of nitric oxide. In addition techniques were mastered to isolate dendritic cells from blood. The collaboration between OVI and WSU led to Dr B. Davis (Washinton State University, Pullman, WA) kindly providing monoclonal antibodies at cost price specific for bovine CD2 (mAb BAQ95), CD3 (mAb MM1A), B cell (mAb BAQ44A), CD4 (mAb GC50A1), CD8 (mAb CACT80C) and $\gamma\delta$ TcR1 (mAb GB21A). These monoclonal antibodies were used at the OVI in flow cytometric analysis of the responding lymphocytes. Furthermore, Dr. W.C. Brown kindly provided OVI with IFN-γ; IL-2; IL-4; IL-10 and β-actin competitor plasmids which were grown up and tested at the OVI. All the relevant reagents are now in place at the OVI for RT-PCR.

From Israel, Thea Molad and Lea Fish obtained intensive training in molecular biology technology, cellular immunology of rickettsial diseases in the laboratories of Prof. W. Brown and Guy Palmer at the Washington State University. Ms. Thea Molad from KVI visited Prof. G. Palmers laboratory at WSU where she gained experience in cloning of one of the genes of *A. marginale*, taxonomically related to *Cowdia ruminantuim* rickettsial pathogen of cattle. Specifically, she was trained in cloning and expression of two regions of one of the genes encoding the major surface proteins (MSP-2) of *Anaplasma*.

5.3. Publications.

Van Kleef, M., N.J. Gunter, H. Macmillan, B.A. Allsopp, V. Shkap, and W.C. Brown. 2000. Identification of *Cowdria ruminantium* antigens that stimulate proliferation of lymphocytes from cattle immunized by infection and treatment or with inactivated organisms. Infection and Immunity. 68:603-614.

Van Kleef, M., H. Macmillan, N.J. Gunter, E. Zweygarth, B.A. Allsopp, V. Shkap, D.H. Du Plessis, and W.C. Brown. *Cowdria ruminantium*: low molecular mass proteins induce bovine helper T-cell lines to proliferate and produce interferon-gamma. Experimental Parasitology (Submitted).

6. PROJECT PRODUCTIVITY

The project accomplished its most important goals in that proteins, of the heartwater agent *Cowdria ruminantium*, were identified that induce T helper lymphocytes to proliferate and produce IFN-γ. These proteins may now be further investigated for use as components of a recombinant vaccine. Furthermore the immunological capabilities of the Onderstepoort Veterinary Institute (OVI), in the field of cell-mediated immunity, has been substantially strengthened. The "Institutional Strengthening " capacity as one of the major goals of the project has **been thoroughly effectively achieved.**

Experience was gained in WSU in cytokine analysis by RT-PCR detection. The technique and reagents are established at the OVI. This technique was however not pursued due to the coastlines and our difficulties of establishing antigen specific T-cell

clones. Mrs. Mirinda Van Kleef from OVI revealed an outstanding capability in applying the most advanced techniques in immunochemistry and cellular immunology (first author in the two scientific papers). Under her leadership the OVI has now an effective immunology team.

7. FUTURE WORK

To fully accomplish the scientific goal of the project additional research is required to identify genes encoding for the proteins which have been identified as capable to induce profound cell-mediated immune responses. Further studies will characterize the *Cowdria* proteins, of molecular mass 11, 12, 14 to 17 and 19 to 23 kDa. SDS-PAGE separated proteins of the indicated molecular masses will each be further fractionated by isoelectric focusing into their respective pI points. These will be tested in short term T-cell proliferation and IFN-γ production assays and cytokine profile determinations. Proteins of interest will be partially sequenced, oligonucleotide probes designed and genomic libraries screened in search of their encoding genes. This type of work is too important to be abandoned, therefore the research will continue using available national funds. Further collaboration between the OVI and Dr. B. Davis WSU is underway for possible testing of monoclonal antibodies for use with buffalo lymphocyte phenotyping.

A preproposal for a cooperative research project between Onderstepoort Veterinary Institute, Kimron Veterinary Institute and Oklahoma State University has been submitted to CDR on the development of a culture-derived vaccine against anaplasmosis in cattle, caused by a rickettsial pathogen, *Anaplasma mariginale* and *A. centrale*. The expertise gained by OVI and KVI through the present CDR will greatly contribute to accomplish the goals of the research programs submitted.

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Identification of *Cowdria ruminantium* Antigens That Stimulate Proliferation of Lymphocytes from Cattle Immunized by Infection and Treatment or with Inactivated Organisms

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***Cowdria ruminantium* is an obligate intracellular pathogen that causes heartwater in ruminants. Several findings suggest that T cells play an important role in protection against the disease. In order to identify which proteins are involved in T-cell immunity, *C. ruminantium* proteins were fractionated by continuous-flow electrophoresis and tested for their ability to stimulate lymphocyte proliferation in vitro. *C. ruminantium*-infected endothelial cell lysates were fractionated at between 11 and 38 kDa and 50 and 168 kDa on 15 and 7% acrylamide gels, respectively. In an attempt to stimulate the natural infective process, peripheral blood mononuclear cells (PBMC) were obtained from two cattle rendered immune by infection and treatment and assayed in proliferation assays with fractionated proteins. In a parallel study, four cattle were immunized with inactivated *C. ruminantium* to determine whether their lymphocytes also responded to fractionated proteins. Proliferation assays after immunization by infection and treatment detected no *C. ruminantium*-specific proliferation in vitro after one vaccination. Proliferation was observed, however, between 1 and 4 weeks after challenge. This was followed by a period of no detectable response, after which the response reappeared. PBMC from animals immunized with inactivated organisms proliferated specifically in response to antigen soon after the first immunization. Only *C. ruminantium* proteins with low molecular masses of 11, 12, 14 to 17, and 19 to 23 kDa induced proliferative responses by lymphocytes from all six animals. These protein fractions may have potential as vaccine antigens.**

Heartwater is caused by the rickettsia *Cowdria ruminantium* (7). The pathogen is transmitted exclusively by ticks of the genus *Amblyomma* to wild and domestic ruminants in sub-Saharan Africa and the Caribbean (31). Heartwater is also a threat to livestock on the American mainland, where potential vectors are present but where the disease is currently absent (1). Cowdriosis is controlled primarily through immunization by infection with virulent blood and subsequent treatment with antibiotics to prevent a serious course of the disease (38–40). This procedure has obvious drawbacks, including the possibility of unwanted transmission of other pathogens and its unsuitability for use in countries where potential vectors are present but which do not harbor the disease (30). There is thus a real need for an improved vaccine.

Studies have demonstrated that animals can be protectively immunized with culture-attenuated (18) or inactivated (23, 26) organisms, suggesting that the development of a subunit vaccine may be feasible. Vaccination with culture-attenuated organisms nevertheless has disadvantages in that cross-protection against different isolates is not complete. It would therefore be necessary to attenuate several strains to cover the entire antigenic repertoire. In addition, only certain strains can be attenuated by in vitro passage, which limits the use of this method in vaccination (18). On the other hand, immunization with inactivated *C. ruminantium* in adjuvant could allow many different strains to be incorporated into a vaccine.

It has been shown that peripheral blood mononuclear cells (PBMC) from animals rendered resistant to challenge by vaccination with inactivated organisms contain *C. ruminantium*-specific, major histocompatibility complex class II-restricted, gamma interferon-producing, $CD4^+$ T lymphocytes (36). Short term $CD4^+$-T-cell lines generated by using *C. ruminantium* lysates respond strongly to whole lysates but not to the recombinant 32-kDa protein (major antigenic protein 1 [MAP1]) or the 21-kDa protein (MAP2) (35). When these cell lines were stimulated with soluble *C. ruminantium* proteins fractionated by fast-performance liquid chromatography, a single peak of proliferation, which included proteins of between 20 and 32 kDa, was observed (37). Flow cytometric analysis of PBMC showed no significant change in the immune cell population after vaccination and boosting with inactivated organisms. Nevertheless, significant changes occurred after challenge, including an initial progressive depletion of $CD4^+$, $CD8^+$, and $\gamma\delta$ T-cell subsets and a rise in numbers of monocytes together with strong activation. This was followed by an increase in $CD8^+$ T lymphocytes (25). The last finding is in accordance with previous studies with a murine model which led the authors to suggest that $CD8^+$ T cells play a major role in immunity to heartwater (10–12).

Recently, Mwangi and coworkers (28) demonstrated that cattle immunized against heartwater by infection and treatment generated T-cell responses specific for two immunodominant recombinant antigens of *C. ruminantium*, namely, MAP1 and MAP2. Proliferation of PBMC was also elicited in vitro by

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TABLE 1. Proliferative responses of PBMC from a naive animal, two immune animals immunized by infection and treatment (B9191 and B1359), and three animals immunized with inactivated organisms (B821, B1351, and B775) to various *C. ruminantium* preparations[a]

Animal	Mean cpm ± SD				
	PBMC	DC (neg)[b]	DC (pos)[c]	PDGC[d]	PSIAC[e]
Naive	2,208 ± 801	4,348 ± 1,945	2,661 ± 1,765	2,389 ± 977	3,877 ± 1,869
B9191	813 ± 163	792 ± 250	7,902 ± 1,809	10,641 ± 1,654	9,990 ± 436
B1359	1,152 ± 418	2,124 ± 832	14,341 ± 4,784	3,857 ± 756	14,229 ± 3,139
B821	1,079 ± 876	54,612 ± 4,876	128,080 ± 7,748	102,501 ± 9,016	120,260 ± 4,750
B1351	1,065 ± 145	73,391 ± 7,215	144,438 ± 10,721	130,012 ± 6,481	140,391 ± 8,600
B775	1,002 ± 140	40,312 ± 6,175	66,292 ± 13,756	99,371 ± 4,101	106,392 ± 3,352

[a] PBMC were collected from the immunized animals at a time when they proliferated specifically to *C. ruminantium* antigen.
[b] DC of uninfected BSV cell lysates.
[c] DC of *C. ruminantium*-infected BSV cell lysates.
[d] PDGC of *C. ruminantium*-infected BSV cell lysates.
[e] PSIAC of *C. ruminantium*-infected BSV cell lysates.

either autologous infected endothelial cells or infected monocytes but not by elementary bodies. The endothelial cells required pretreatment with T-cell growth factors to induce class II major histocompatibility complex expression prior to infection and their subsequent use as stimulators of PBMC. These proliferative responses were characterized by a mixture of $CD4^+$, $CD8^+$, and $\gamma\delta$ T cells and strong expression of gamma interferon, tumor necrosis factors alpha and beta, and interleukin-2 (IL-2) (27).

In another approach, a naked-DNA vaccine containing the *map*1 gene of *C. ruminantium* was shown to be able to protect between 23 and 88% of immunized mice (29). The best-characterized proteins of *C. ruminantium* are the above-mentioned MAP1 (19, 33) and MAP2 (24) proteins, as well as the GroEL heat shock protein (22), the genes of which have been cloned (4, 22, 24, 42). No studies to date have reported on additional antigens being involved in stimulating protective immunity. For the development of a subunit vaccine, it may nonetheless be important to identify additional *C. ruminantium* proteins which stimulate T lymphocytes and, in turn, to relate these to protective responses. Protective malarial parasite proteins have been identified by vaccination trials with fractions obtained by continuous-flow electrophoresis (CFE) of *Plasmodium chabaudi adami* shizont proteins (21). Since T cells are required for protective immunity in malaria (3, 15), this finding suggested that potentially protective T-cell antigens could be identified by this technique. Using a similar procedure, Brown and coworkers (5, 6, 34) have successfully identified several antigens of *Babesia bovis* merozoites that stimulated proliferation of T-cell lines and clones. To identify the proteins involved in recall T-cell responses in immune cattle, *C. ruminantium* was therefore fractionated by CFE, and each fraction was tested for its ability to stimulate lymphocyte proliferation in vitro. In an attempt to stimulate the natural infective process, the PBMC used in this study were obtained from animals rendered immune by infection and treatment. In a parallel study, four cattle were immunized with inactivated *C. ruminantium* to determine whether their PBMC recognize similar proteins in proliferation assays.

MATERIALS AND METHODS

In vitro cultivation of *C. ruminantium*. The Welgevonden isolate of *C. ruminantium* was cultured either in bovine saphenic vein (BSV) endothelial cells (prepared from animal B9191) or in a calf endothelial cell line (E_5), as described previously (2). The Welgevonden isolate was chosen because the pathological patterns caused by this isolate resemble those caused by the Ball3 isolate presently used in the live vaccine (32). Additionally, unlike the Ball3 isolate, the Welgevonden isolate is pathogenic to mice, permitting viability tests to be done with these animals. The Welgevonden isolate elicits total immunity against more isolates than does the Ball3 isolate (13).

Preparation of crude *C. ruminantium* infected or uninfected BSV cell culture lysates. Crude extracts of Welgevonden isolate-infected or uninfected cell cultures were prepared as described previously (33). Briefly, uninfected or infected (harvested when maximally infected with organisms) BSV cell lysates were centrifuged for 30 min at 10,000 × *g*. The resulting pellet was suspended in phosphate-buffered saline (PBS) (0.14 M NaCl, 1 mM KH_2PO_4, 8 mM $Na_2HPO_4 \cdot 12H_2O$, and 3 mM KCl, pH 7.4). The lysates were stored at −20°C and used for fractionation of antigens by CFE.

Preparation of semipure inactivated *C. ruminantium* lysates from cell cultures. Semipure inactivated organisms were prepared as described previously (36). Differential centrifugation (DC) was done with either maximally infected E_5 or BSV cell cultures by centrifuging first at 1,000 × *g* for 10 min. The resultant supernatant was centrifuged at 30,000 × *g* for 30 min. The pellet was resuspended in PBS containing sodium benzylpenicillin (0.12 mg/ml) and streptomycin sulfate (0.198 mg/ml). The suspension was subjected to five freeze-thaw cycles performed with liquid nitrogen and stored at −20°C. In order to confirm that the preparation contained inactivated *C. ruminantum*, mice were inoculated intravenously with 0.2 ml of the same lysate per mouse. The inactivated *C. ruminantium* lysate prepared from infected E_5 cell cultures was used for immunization of cattle, and the inactivated *C. ruminantium* lysate prepared from infected BSV cell cultures was used as antigen in proliferation assays.

Semipure *C. ruminantium* lysates were prepared from BSV cell cultures by DC, positive-selection immunoaffinity chromatography (PSIAC), and Percoll density gradient centrifugation (PDGC). DC was done with maximally infected BSV cell cultures as described above.

PSIAC was performed with purified goat anti-MAP1 immunoglobulin G (41) coupled to CNBr-activated Sepharose 6MB (Pharmacia). A crude *C. ruminantium*-infected BSV cell culture lysate was applied to the column, and after a 30-min incubation period, the unbound material was washed off with PBS. The bound organisms were desorbed with 3 M KSCN–50 mM Tris–0.02% NaN_3, pH 9. The desorbed organisms were centrifuged at 30,000 × *g* for 30 min, suspended in PBS, and stored at −20°C (4).

PDGC was performed with *C. ruminantium*-infected BSV cell cultures by centrifuging at 1,500 × *g* for 30 min and then centrifuging the resulting supernatant at 30,000 × *g* for 30 min. The organisms were resuspended in 1 ml of PBS and loaded onto a step gradient of 0, 10, 20, 30, and 40% Percoll (Pharmacia) prepared in PBS. The gradients were centrifuged at 400 × *g* for 30 min, and the purified organisms were harvested from the 0% layer and washed twice in PBS at 30,000 × *g* for 30 min. The resultant pellet was resuspended in PBS and stored at −20°C (23). These semipure preparations were used as antigen in proliferation assays.

Protein determination. Protein concentrations were determined by the Bio-Rad (Hercules, Calif.) protein microassay with bovine serum albumin as a standard.

Experimental cattle. All animals were initially seronegative to *C. ruminantium*, *B. bovis*, and *Theileria mutans* as determined by indirect fluorescent-antibody assays and to *Anaplasma* as determined by competition inhibition enzyme-linked immunoassay.

(i) Immunization by infection and treatment. Animals B9191 (Fresian) and B1359 (Nguni) were inoculated intravenously with 5 ml of a Welgevonden isolate infective sheep blood stabilate. The animals were monitored daily and treated on the third day of a rising febrile reaction by intramuscular injection with long-acting oxytetracycline (Liquamycin LA; Pfizer) at a dose of 20 mg/kg of body weight. The animals were challenged with the same batch of homologous stabilate at the following intervals: B9191 at 1 month and 3 years and B1359 at 6 months and 8 months after immunization. The animals were monitored daily for temperature. As a positive control to verify the viability and virulence of the stabilate, mice were inoculated intravenously with 0.2 ml of the same stabilate per mouse. Postmortem examinations were done on the mice to determine the cause of death. The presence of *C. ruminantium* in the mice was confirmed by

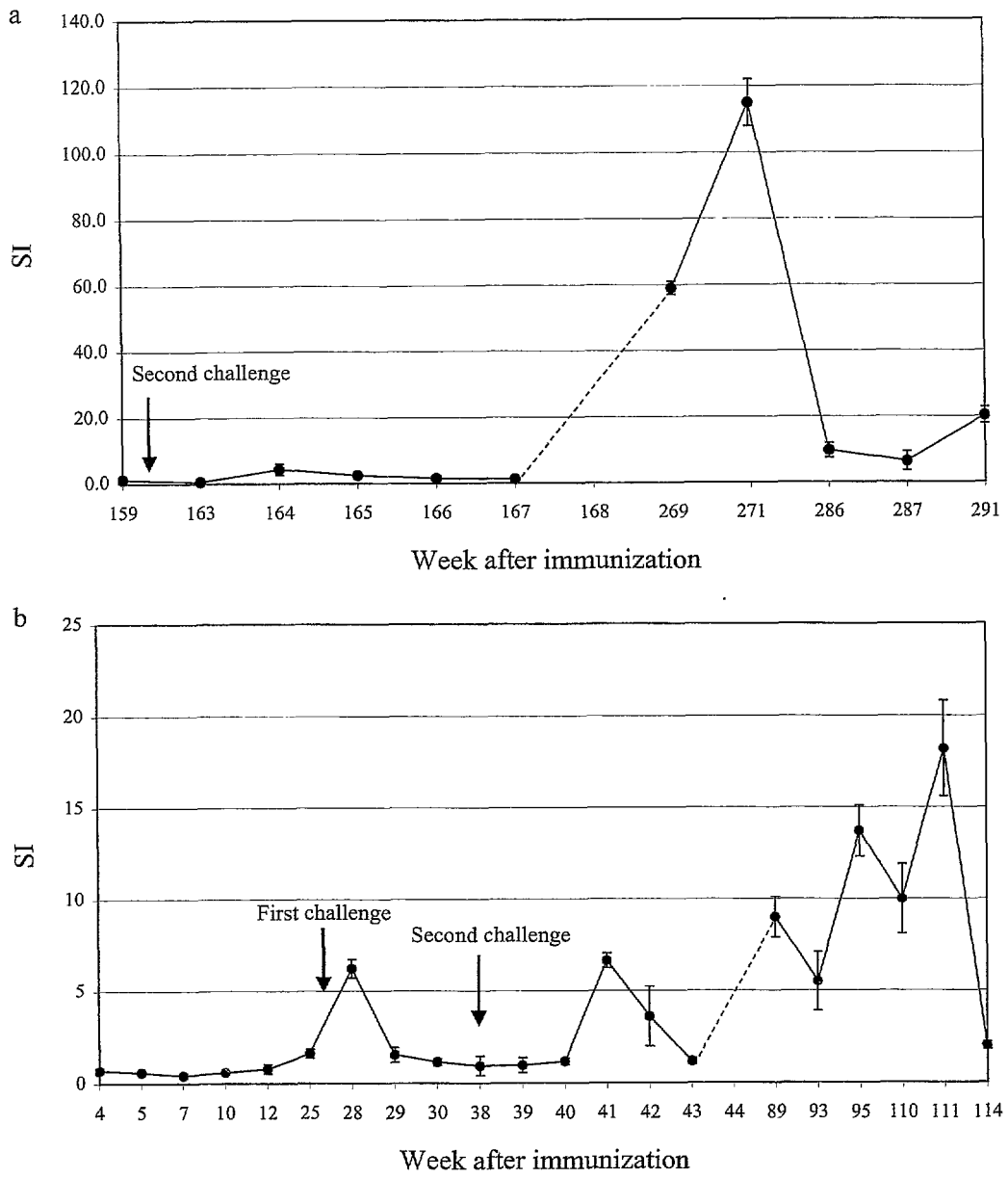

FIG. 1. Proliferative responses of PBMC from animals B9191 (a) and B1359 (b) at various intervals after immunization by infection and treatment. The PBMC were cultured for 6 days with differentially centrifuged lysates of *C. ruminantium*-infected BSV cells at a concentration of 1 μg/ml in either duplicate or triplicate wells. The mean counts per minute for the PBMC controls were 2,065 ± 2,194 (B9191) and 3,943 ± 3,090 (B1359). The arrows indicate the times of challenge. Results are presented as SI ± SD.

immunohistochemical identification of the organism in formalin-fixed tissue sections (17).

(ii) Immunizations with inactivated *C. ruminantium*. Four Nguni cattle (B809, B821, B1351, and B775) were injected with 15 μg of inactivated *C. ruminantium* per ml in Montanide ISA50 adjuvant (Seppic, Paris, France). One animal (B816) received only adjuvant as a control. The animals were injected twice at an interval between 14 and 16 weeks.

Fractionation of crude *C. ruminantium*-infected and uninfected BSV cell lysates by CFE. CFE of crude *C. ruminantium*-infected or uninfected BSV cell lysates was performed with a Prep-Cell Apparatus (Bio-Rad) as described previously (5) with the following modifications. Approximately 10 mg of protein was solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, boiled, and electrophoresed under reducing conditions on 28-mm (internal diameter) cylindrical gels consisting of either 7 or 15% acrylamide. Fractions of 2.5 ml were eluted and collected over the course of 15 h (7% acrylamide gels; 180 fractions) or 7.5 h (15% acrylamide gels; 175 fractions). The fractions were stored at −70°C. The fractions were precipitated by adding 8 times the volume of ice-cold acetone, incubated at −20°C for 16 h, and centrifuged at 10,000 × *g* for 10 min. The acetone was aspirated, and the precipitates were resuspended in 70% cold ethanol and centrifuged at 10,000 × *g* for 30 min. After the ethanol was aspirated, the pellets were air dried, suspended in PBS containing antibiotics, and stored at −70°C. To examine the potential toxicity of the fractions on T-cell proliferation, the effect of a randomly selected fraction, at concentrations ranging from 3 to 24% (vol/vol), on either IL-2- or concanavalin A-induced PBMC proliferation was tested.

SDS-PAGE analysis of fractionated *C. ruminantium* antigens. A volume of 20 μl of every 10th precipitated fraction was analyzed by SDS-PAGE. A minigel system with acrylamide gels of either 7% (7% Prep-Cell fractions) or 12% (15% Prep-Cell fractions) was used. Protein bands were visualized by silver staining.

Lymphocyte proliferation assays. Proliferation assays were carried out in duplicate or triplicate wells of half-area flat-bottom 96-well plates (Costar) at 37°C in a humidified atmosphere containing 5% CO_2 for 5 days (3 days with concanavalin

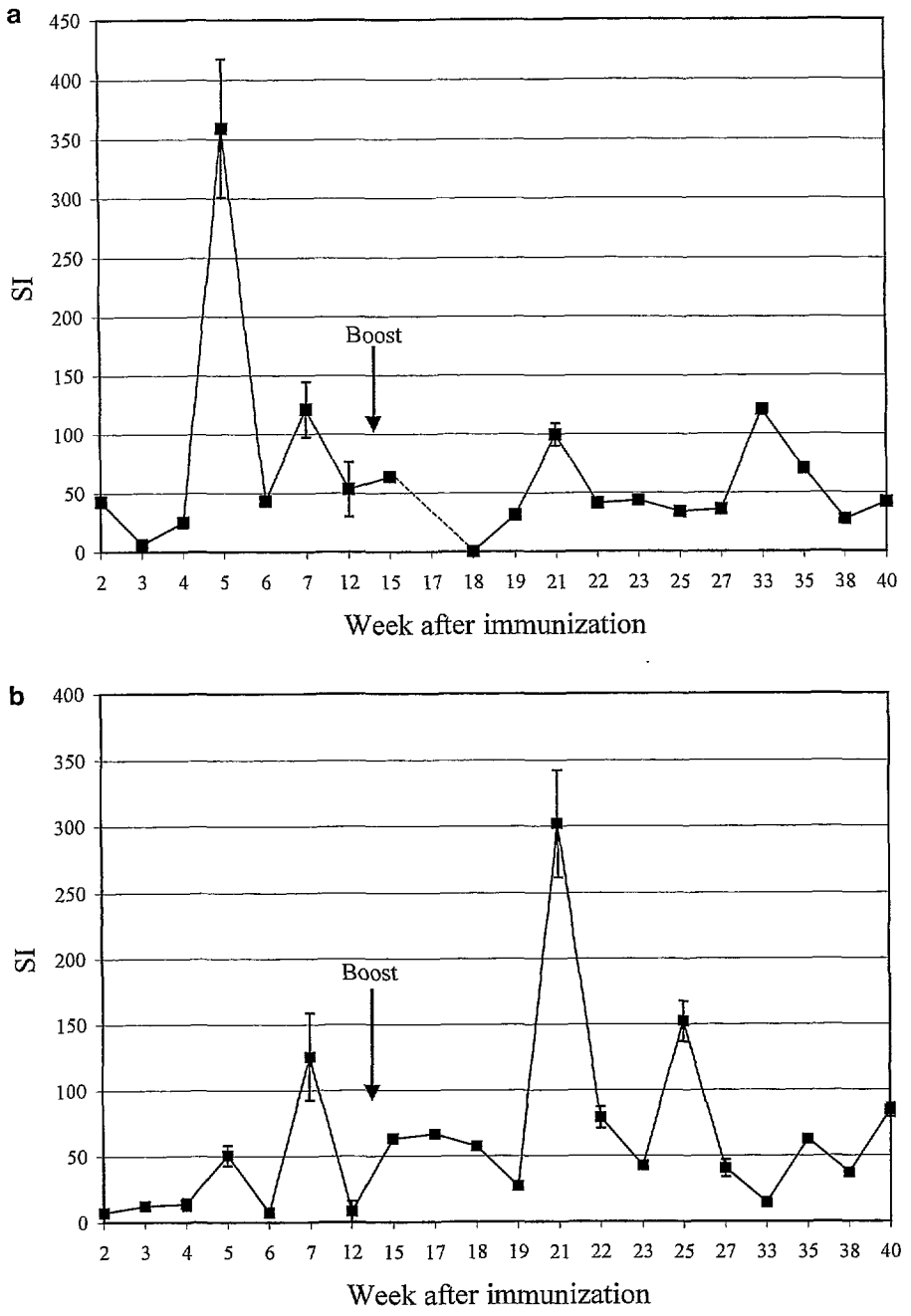

FIG. 2. Proliferative responses of PBMC from animals B809 (a), B821 (b), and B816, B1351, and B775 (c; ●, ■, and ◇, respectively) at various intervals after immunizing with inactivated *C. ruminantium*. The PBMC were cultured for 6 days with differentially centrifuged lysates of *C. ruminantium*-infected BSV cells at a concentration of 1 μg/ml in either duplicate or triplicate wells. The mean counts per minute for the PBMC controls were 15,707 ± 18,666 (B816), 4,120 ± 6,930 (B809), 2,529 ± 2,710 (B821), 8.948 ± 11,612 (B1351), and 6,724 ± 6,063 (B775). The arrows indicate the times of boosting. Results are presented as SI ± SD.

A) as described previously (5). Each well (total volume, 100 μl) contained complete RPMI 1640 medium, responder cells added at a final concentration of 4×10^6 PBMC/ml, and various concentrations of *C. ruminantium*-infected or uninfected BSV cell antigens (0.03 to 10 μg of antigen/ml). Fractionated antigens were included at final concentrations of 5% (vol/vol). Proliferation was determined by measuring the incorporation of 1 μCi of [*methyl*-^{3}H]thymidine added during the final 18 h of the assay. The cells were harvested, and the radioactivity was counted in a scintillation counter. Results are presented as a stimulation index (SI) ± standard deviation (SD), where SI = mean counts per minute of test sample/mean counts per minute of unstimulated control. The unstimulated control was PBMC in medium. Unless otherwise stated, an SI of ≥2 was considered to be an indication of antigen-specific proliferation. The one-tailed Student *t* test was used to determine the levels of significance between the uninfected and infected BSV cell CFE fractions.

RESULTS

Proliferative responses elicited by different rickettsial preparations. Different *C. ruminantium* preparations were tested for their ability to elicit an optimum proliferative response in the PBMC obtained from a naive animal, two immune animals

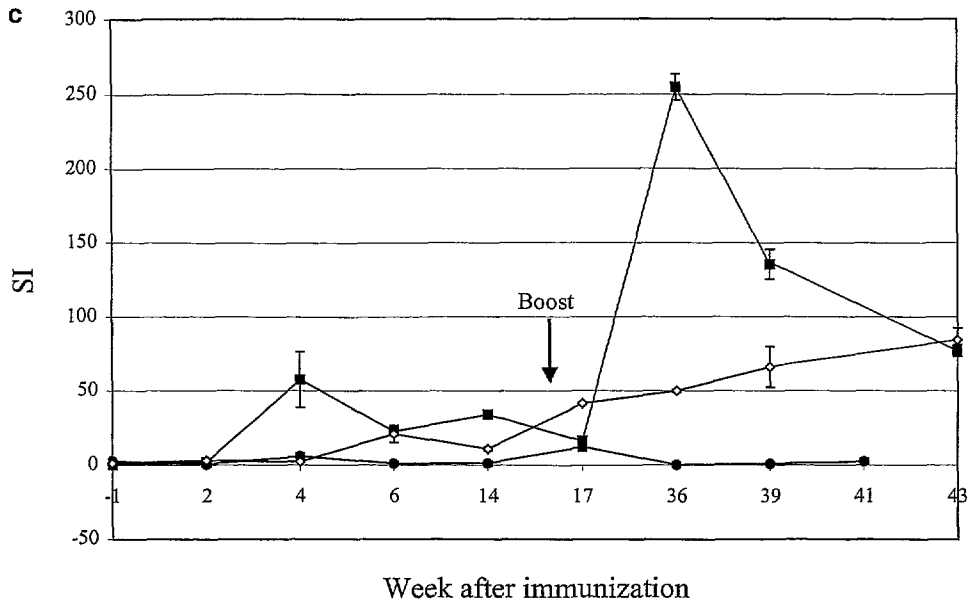

FIG. 2—*Continued.*

(B9191 and B1359), and three animals immunized with inactivated organisms (B821, B775, and B1351). *C. ruminantium* was partially purified from BSV cells by (i) DC, (ii) PSIAC, and (iii) PDGC. All of these antigen preparations were assayed with PBMC in triplicate wells on the same day. The highest proliferative response was obtained with antigen prepared by DC followed by PSIAC and PDGC (Table 1). DC was therefore subsequently routinely used to purify *C. ruminantium*. When differentially centrifuged uninfected BSV cell lysates were used as antigen in proliferation assays with PBMC from immunized animals, proliferation was consistently observed to be lower than that obtained with the infected preparations (Table 1). However, higher proliferation values were obtained with PBMC from cattle immunized with inactivated organisms and assayed with differentially centrifuged uninfected BSV cell lysates. This may be due to the presence of endothelial cell debris in the differentially centrifuged preparations used for immunizing these cattle. When differentially centrifuged uninfected BSV cell lysates were used as antigen in proliferation assays with PBMC from nine naive cattle, no proliferation was detected (SI ≤ 1.7) (results not shown). These results showed that uninfected BSV cell antigen did not induce alloreactive responses in PBMC from unrelated immunized or naive cattle.

Proliferative responses during the course of immunization. Following immunization by infection and treatment, both cattle (B9191 and B1359) were found to be immune to challenge as determined by the lack of a febrile response and symptoms of the disease. All mice injected with this live blood stabilate used for cattle challenge died. The symptoms before death, the time to death (±12 days), and the postmortem findings all indicated that the mice were infected with *C. ruminantium*. In addition, the presence of *C. ruminantium* in tissue sections of the mice was confirmed histopathologically. These findings indicated that the stabilate was indeed virulent. Proliferative responses during the course of immunization are illustrated in Fig. 1. PBMC tested prior to immunization or challenge did not respond to *C. ruminantium* antigens when tested at a range of protein concentrations varying from 0.03 to 10 μg/ml. In contrast, PBMC from both immune animals responded specifically to *C. ruminantium* antigens after challenge. The proliferative response peaked at 1 μg/ml (results not shown). Antigen concentrations of ≥2.5 μg/ml inhibited both concanavalin A- and IL-2-induced responses (results not shown). After B9191 was challenged for the second time, *C. ruminantium*-specific lymphocyte proliferation peaked 4 weeks after challenge (31,764 ± 8,702 cpm; 164 weeks after immunization) but was not measurable 2 weeks later (Fig. 1a). By comparison, proliferation assays with PBMC from B1359 indicated an antigen-specific response 1 week after the first challenge (52,099 ± 3,899 cpm; 28 weeks after immunization) and 3 weeks after the second challenge (48,626 ± 4,992 cpm; 41 weeks after immunization) and was not measurable a week later (Fig. 1b). When PBMC from these cattle were assayed again 1 to 2 years later, they showed antigen-specific proliferation with an SI of ≥5.5. The duration of the PBMC response is not known.

Following immunization of cattle with inactivated organisms, PBMC from B809, B821, B1351, and B775 continued to proliferate antigen specifically (Fig. 2). PBMC collected from the control animal B816 did not proliferate to *C. ruminantium* antigen before or after immunization with adjuvant alone (mean SI of <2) (Fig. 2).

Fractionation of *C. ruminantium*-infected and uninfected BSV proteins by CFE and the proliferative responses they stimulate. In an attempt to identify which of the *C. ruminantium* proteins were responsible for the T-cell responses described above, Welgevonden-infected and uninfected BSV cell culture lysates were fractionated under reducing conditions by CFE. Welgevonden-infected BSV cell culture proteins were fractionated at between 50 and 168 kDa on a 7% gel and at between 11 and 38 kDa on a 15% gel (Fig. 3). In a similar profile BSV cell culture proteins were fractionated at between 50 and 123 kDa on a 7% gel and at between 11 and 34 kDa on a 15% gel (results not shown). There were artifacts caused by silver staining at approximately 66 kDa on both the 7 and 15% gels. The other protein bands at fraction 150 on the 7% gel and fractions 5, 15, 25, 35, 65, and 75 on the 15% gel may be due to breakdown products. A total of 700 fractions were collected

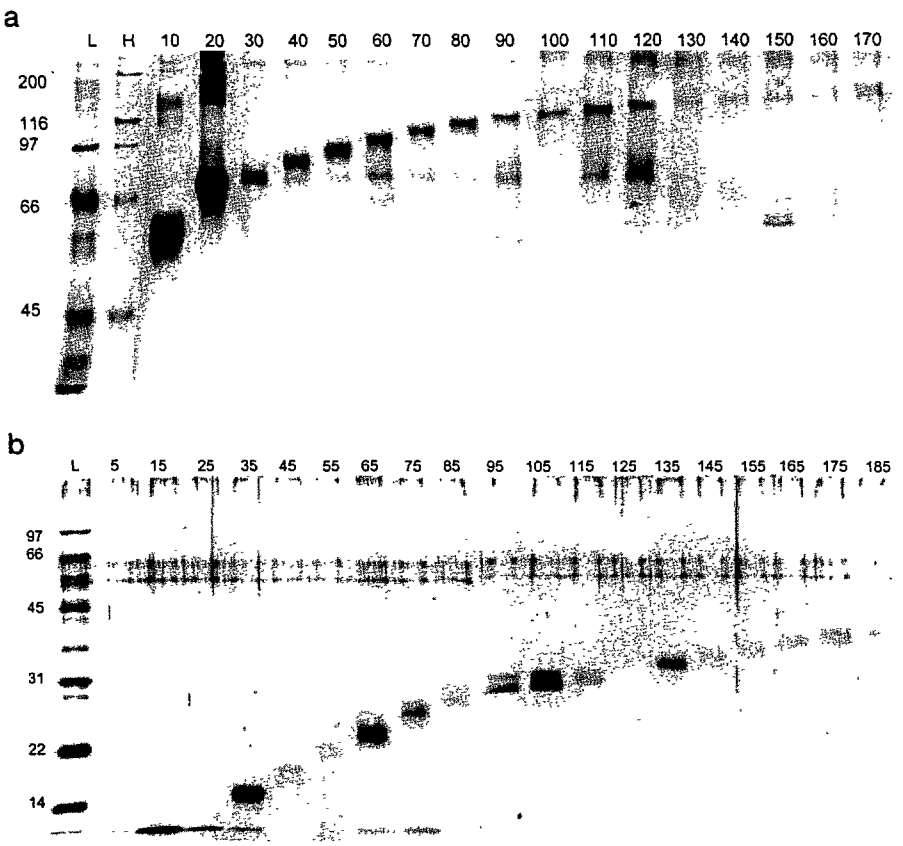

FIG. 3. SDS-PAGE and silver staining analysis of *C. ruminantium*-infected BSV cell lysates fractionated by CFE. Crude *C. ruminantium*-infected BSV cell lysates were fractionated by CFE on either 7% (a) or 15% (b) acrylamide gels. The fractions were precipitated with acetone and resuspended in 500 μl of PBS. A volume of 20 μl of every 10th precipitated fraction was subjected to analytical SDS-PAGE (7% acrylamide for the 7% CFE fractions and 12% acrylamide for the 15% CFE fractions) and visualized on the silver-stained gels. The relative mobilities of the low-molecular-mass standards (lanes L) and high-molecular-mass standards (lanes H) are indicated on the left of each panel in kilodaltons.

and prepared for lymphocyte proliferation assays by acetone precipitation. A randomly chosen Prep-Cell fraction (with a molecular mass of approximately 100 kDa), when tested at concentrations of 3 to 24% (vol/vol), did not inhibit a concanavalin A-induced proliferative response (results not shown). Based on these findings together with previous results (5), a final concentration of 5% (vol/vol) was selected for testing with *C. ruminantium*-immune PBMC.

Day-to-day variations in proliferative responses obtained with PBMC from each animal were observed. To address this variation, unstimulated PBMC controls were assayed within the same assay plates on the same day and used to determine the SI. The SI for PBMC proliferation induced by CFE fractions prepared from either infected or uninfected cultures was determined for each animal. A mean SI baseline (SI_{neg}) for CFE fractions prepared from uninfected BSV cultures was then determined for each animal. The one-tailed Student *t* test was used to determine whether there was a statistically significant difference between the SI obtained from each CFE fraction prepared from infected BSV cell cultures and the SI_{neg}.

(i) Proliferative responses of PBMC from animal B9191 induced by CFE fractions. Fractions from *C. ruminantium*-infected and uninfected BSV cells were assayed with PBMC collected from animal B9191 at week 291 after immunization. The SI_{neg} was determined to be 1 ± 0.1 and 2 ± 0.1 for the 7 and 15% polyacrylamide CFE fractions, respectively. An examination of the fractions derived from the CFE with a 7% polyacrylamide gel revealed that those inducing *C. ruminantium*-specific proliferation were localized to the first 24 fractions. These had molecular masses of ≤74 kDa. In addition, a further three discrete pools of higher-molecular-mass proteins (50 to 67, 81 to 111, 115 to 140, and 151 to 162 kDa) also induced proliferation (Fig. 4a). When a similar preparation was fractionated on a 15% polyacrylamide gel to resolve the low-molecular-mass proteins, the stimulatory fractions ranged from 11 to 38 kDa (Fig. 4b). Due to the high SI_{neg} of 2 obtained for the 15% polyacrylamide CFE, an SI ≥4 was considered as antigen specific for this preparation. Ten groups of fractions had SIs of ≥4. The molecular masses in these groups ranged from 11 to 24 kDa and from 26 to 38 kDa.

(ii) Proliferative responses of PBMC from animal B1359 induced by CFE fractions. Fractions from *C. ruminantium*-infected BSV cells were assayed with cryopreserved PBMC collected 41 weeks after immunization of B1359. In a similar way, lymphocytes from this animal responded specifically to *C. ruminantium* fractions with relatively low molecular masses of approximately 11 to 23 kDa and 26 to 27 kDa (Fig. 5b). In addition, only one pool of high-molecular-mass proteins of approximately 85 to 90 kDa also induced lymphocyte proliferation (Fig. 5a). In contrast, the SI_{neg} was determined to be 0.4 ± 0.1 and 0.7 ± 0.2 for the 7 and 15% CFE fractions, respectively. The low SI_{neg} values obtained indicated that no alloreactive responses were induced by the BSV cells in CFE fractions.

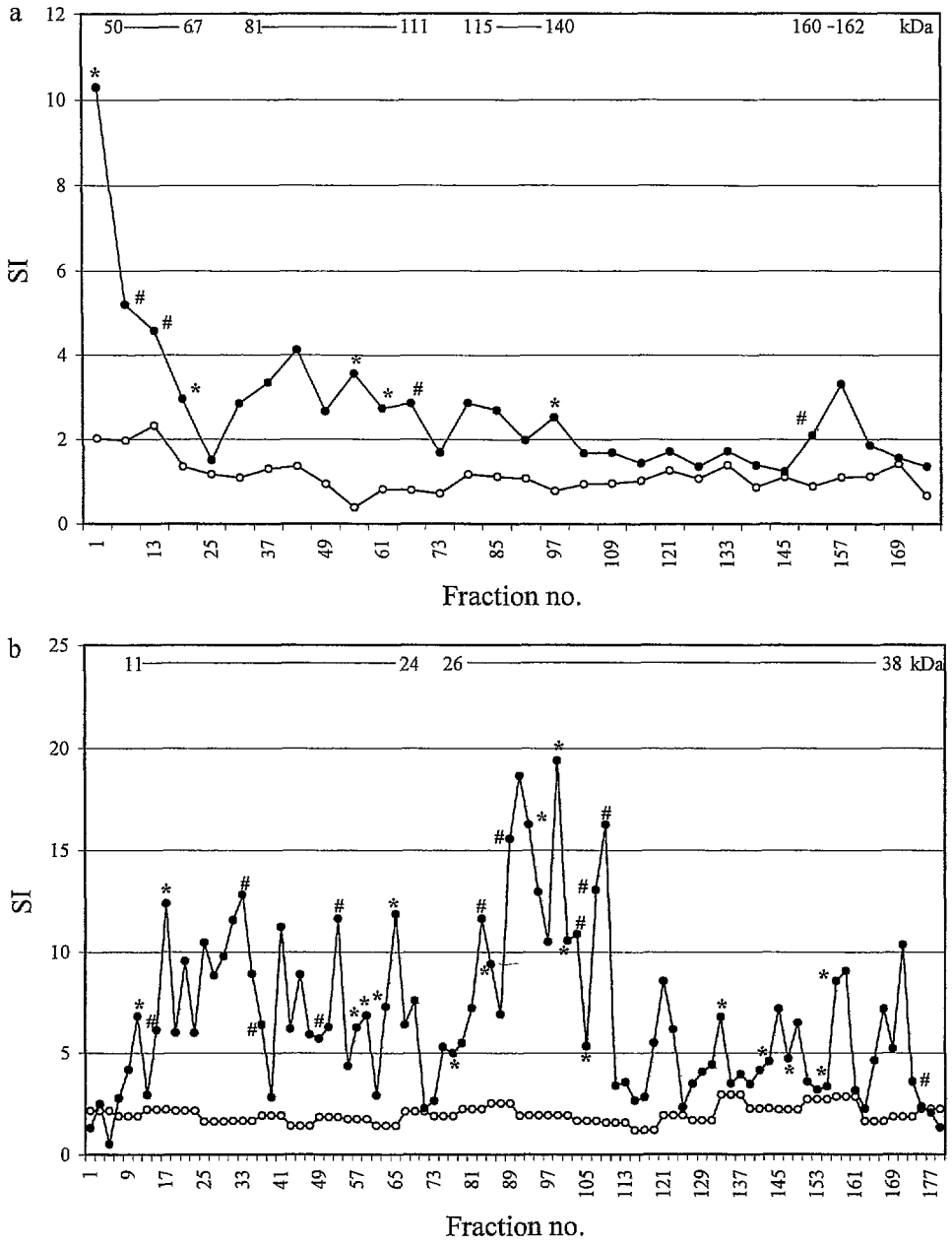

FIG. 4. Proliferative response of PBMC from animal B9191 to *C. ruminantium*-infected and uninfected BSV cell lysates fractionated by CFE. *C. ruminantium*-infected or uninfected BSV cells were electrophoresed on either 7% (a) or 15% (b) acrylamide gels. Proteins were eluted from the gels, collected as 2.5-ml fractions, precipitated with acetone, and resuspended in PBS. Open circles, fractions from control uninfected BSV cell lysates were pooled at six per sample and assayed in triplicate wells for stimulation of PBMC collected 291 weeks after immunization. Closed circles, fractions from infected BSV cell lysates were pooled at two per sample and assayed in triplicate wells for stimulation of PBMC collected 291 weeks after immunization. The mean counts per minute for the PBMC controls were 1,378 ± 361. The results are presented as SI. *, $P \leq 0.05$; #, $P \leq 0.1$. The approximate molecular masses of the fractions inducing *C. ruminantium*-specific proliferation are shown above the charts.

A comparison of the results obtained with animals B9191 and B1359 showed that stimulatory regions of 11 to 23 kDa and 26 to 27 kDa were commonly recognized by lymphocytes from both immune cattle.

(iii) Proliferative responses of PBMC from animals B809, B821, B1351, B775, and B816 induced by CFE fractions. Fractions from *C. ruminantium*-infected BSV cells were assayed with PBMC collected 1 week before and between 18 and 25 weeks after immunizations commenced. Fractions from control uninfected BSV cells were assayed with PBMC collected between 41 and 57 weeks after immunizations commenced. The proliferative responses of PBMC from ox B809 induced by CFE fractions are represented in Fig. 6 to illustrate the type of responses obtained. The proliferative responses of PBMC from animals B821, B1351, B775, and B816 induced by CFE fractions of *C. ruminantium*-infected BSV cells are summarized in Table 2. No antigen-specific proliferation was detected with PBMC from naive animals (before immunization with

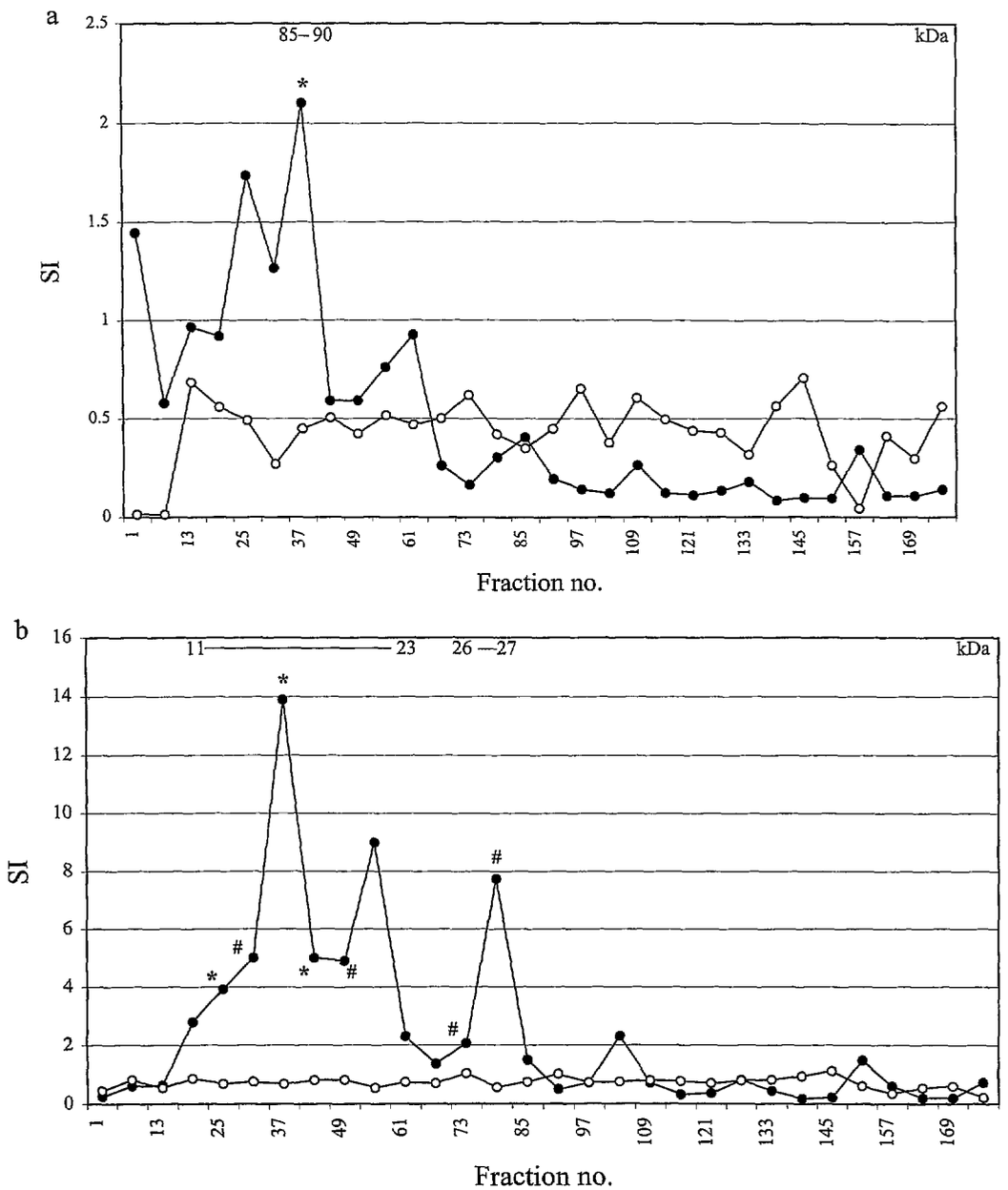

FIG. 5. Proliferative response of PBMC from animal B1359 to *C. ruminantium*-infected and uninfected BSV cell lysates fractionated by CFE. *C. ruminantium*-infected and uninfected BSV cell lysates were electrophoresed on either 7% (a) or 15% (b) acrylamide gels. Proteins were eluted from the gels, collected as 2.5-ml fractions, precipitated with acetone, and resuspended in PBS. Open circles, fractions from control uninfected BSV cell lysates were pooled at six per sample and assayed in triplicate wells for stimulation of PBMC collected 111 weeks after immunization. Closed circles, fractions from *C. ruminantium*-infected BSV cell lysates were pooled at six per sample and assayed in triplicate wells for stimulation of PBMC collected 41 weeks after immunization. The mean counts per minute for the PBMC controls were 2,434 ± 2,134. The results are presented as SI. *, $P \leq 0.05$; #, $P \leq 0.1$. The approximate molecular masses of the fractions inducing *C. ruminantium*-specific proliferation are shown above the charts.

inactivated organisms) and mean SIs of ≤2 were obtained (results for B809 are shown in Fig. 6; results for the remaining cattle are not shown). An SI_{neg} of <1.0 was observed for all cattle, except the control animal, when PBMC were tested with 7 and 15% polyacrylamide CFE fractions from uninfected endothelial cells (results for B809 are shown in Fig. 6; results for the remaining cattle are not shown). The low SI_{neg} values obtained indicated that no alloreactive responses were induced by the BSV cells in CFE fractions. PBMC from cattle immunized with inactivated organisms responded specifically and significantly to antigen fractions containing the following proteins: for B809, 11 to 31, 60 to 80, 85 to 134, and 145 to 148 kDa; for B821, 11 to 26 and 60 to 74 kDa; for B1351, 11 to 38 and 55 to 167 kDa; and for B775, 11 to 38, 55 to 152, and 157 to 167 kDa (Fig. 6 and Table 2). The SI_{neg} for the control animal B816 was determined to be 3.6 ± 0.9 and 4 ± 0.5 for the 7 and 15% polyacrylamide CFE fractions respectively. The high SI_{neg} of 4 obtained was taken into consideration, and an

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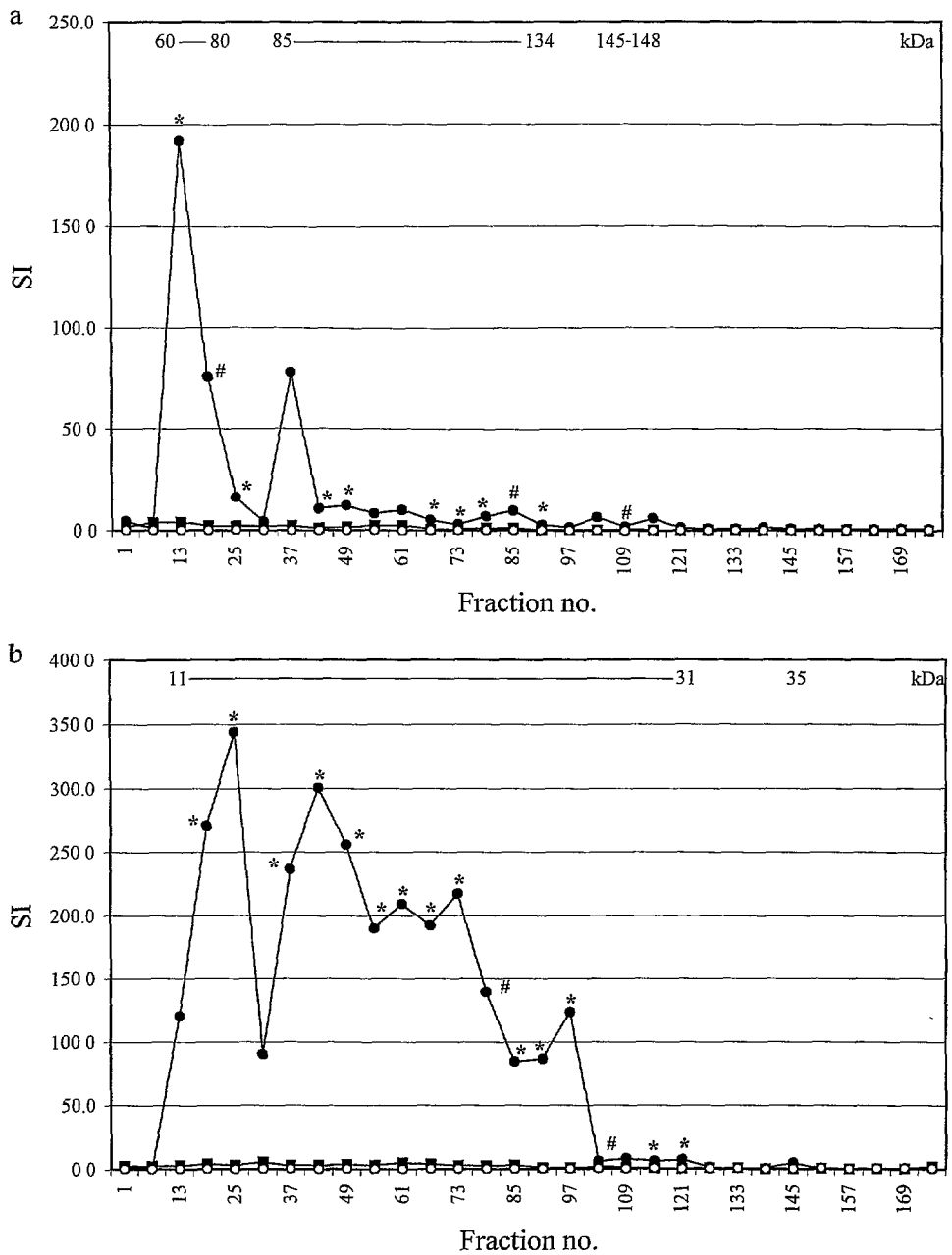

FIG. 6. Proliferative response of PBMC from animal B809 to *C. ruminantium*-infected and uninfected BSV cell lysates fractionated by CFE. *C. ruminantium*-infected or uninfected BSV cell lysates were electrophoresed on either 7% (a) or 15% (b) acrylamide gels. Proteins were eluted from the gels, collected as 2.5-ml fractions, precipitated with acetone, and resuspended in PBS. Squares, fractions from infected BSV cell lysates were pooled at six per sample and assayed in triplicate wells for stimulation of PBMC collected 1 week before immunization (naive animal). Open circles, fractions from control uninfected BSV cell lysates were pooled at six per sample and assayed in triplicate wells for stimulation of PBMC collected 57 weeks after immunization. Closed circles, fractions from infected BSV cell lysates were pooled at six per sample and assayed in triplicate wells for stimulation of PBMC collected 18 weeks after immunization. The mean counts per minute for the PBMC controls were 6,505 ± 11,797. The results are presented as SI. *, $P \leq 0.05$; #, $P \leq 0.1$. The approximate molecular masses of the fractions inducing *C. ruminantium*-specific proliferation are shown above the charts.

SI of ≥8 was considered to be antigen specific for this preparation. The high SI values obtained for this control animal (immunized with adjuvant alone) may have been a result of an adjuvant effect. However, six groups of fractions which induced significant proliferation were identified and corresponded to 13, 18, 26, 27, 29, and 30 kDa (Table 2).

When protein fractions that induce PBMC proliferation were compared between animals immunized by infection and treatment or with inactivated *C. ruminantium*, proteins with molecular masses of approximately 11, 12, 14 to 17, and 19 to 23 kDa were found to be common to both sets of animals.

DISCUSSION

This is the first report of fractionation of *C. ruminantium* proteins by CFE, as well as of the identification and charac-

TABLE 2. Summary of the proliferative responses of PBMC from three cattle immunized with inactivated *C. ruminantium* (B821, B1351, and B775) and one control ox immunized with adjuvant only (B816) to *C. ruminantium*-infected BSV cell lysates fractionated by CFE.

Fraction no[a]	7% CFE[b]					15% CFE[c]				
	SI[d] for animal:				kDa	SI[d] for animal:				kDa
	B821	B1351	B775	B816		B821	B1351	B775	B816	
1	2	**31***	**87***	0	<55	1	0	**3***	0	11
7	1	**17***	**54***	1	55–59	1	2	**31***	0	11
13	**6**	**18***	**53***	1	60–66	**31#**	**11***	**50***	0	11
19	3	**9***	**26***	5	67–74	**196***	**11***	**42***	4	11–12
25	1	**8***	**8***	4	75–80	**129***	**13***	**50***	**10#**	13
31	1	**12***	**18***	3	81–84	**41***	**17***	**45***	3	14–16
37	1	**9***	**19***	3	85–90	**154***	**14***	**52***	7	16–17
43	2	**7***	**11***	4	91–96	**64***	**8***	**46***	**10***	18
49	1	**7***	**13***	4	97–101	**56***	**8***	**44***	6	19–21
55	1	**9***	**21***	4	102–105	**26#**	**14***	**42***	7	22–23
61	1	**8***	**17***	3	106–108	**47**	**12***	**52***	3	23
67	1	**5***	**5***	2	109–111	**4***	**9***	**39***	7	24–25
73	1	**5***	**3***	2	112–114	**3***	**7***	**33***	**8***	26
79	1	**9***	**12***	2	115–119	**3**	**14***	**34***	**11***	27
85	1	**11***	**21***	3	120–125	**3**	**13***	**34***	**10**	28
91	1	**8***	**5***	1	126–134	1	**7***	**38***	6	29
97	1	**8***	**12***	2	135–140	2	**7***	**24***	**11***	29
103	1	**7***	**4***	2	141–144	1	**12***	**22***	**10***	29–30
109	1	**7***	**6***	4	145–148	1	**12***	**33***	7	30
115	1	**7***	**5***	4	149–152	1	**8***	**23***	6	31
121	1	**6***	1	2	153–156	1	**8***	**26***	6	31
127	1	**5***	**2***	2	157–158	1	**12***	**24***	7	32–33
133	1	**5***	**4***	3	158	1	**13***	**36***	6	33–34
139	1	**6***	**4***	2	158	1	**10***	**24***	7	34
145	1	**7***	**5***	4	158	2	**10***	**31***	7	35
151	1	**11***	**14***	2	160–163	1	**12***	**30***	7	35–36
157	1	**11***	**15***	2	164–166	1	**15***	**37***	5	36–37
163	1	**12***	**16***	1	166–167	1	**12***	**28***	7	37–38
169	1	**8***	**6***	2	168	1	**11***	**24***	3	38
175	1	**12***	**20***	1	168	1	1	1	5	38

[a] Fractions from *C. ruminantium*-infected BSV cells were pooled at six per sample and assayed in triplicate wells for stimulation of PBMC collected between 18 and 25 weeks after immunization.
[b] Proteins were fractionated on a 7% polyacrylamide gel. The mean counts per minute of the PBMC controls were 5,769 ± 6,841.
[c] Proteins were fractionated on a 15% polyacrylamide gel. The mean counts per minute of the PBMC controls were 3,869 ± 3,466.
[d] Boldface numbers indicate an SI greater than twice the background proliferation. *, $P \leq 0.05$; #, $P \leq 0.1$.

terization of the molecular masses of antigens that induce proliferation of PBMC obtained from cattle rendered immune by infection and treatment or immunized with inactivated organisms. The high resolution afforded by CFE allowed relatively fine discrimination of the immunostimulatory proteins. Seven outbred cattle were used in this study, resulting in the identification of a common region (11 to 23 kDa) that induced proliferation of their PBMC. Proteins with molecular masses of 20 to 23 kDa identified in our assays fall within a range of proteins (fractionated by fast-performance liquid chromatography) that had previously been identified to induce proliferation of a T-cell line derived from cattle immunized with killed *C. ruminantium* (37). It is interesting that fractions containing molecular masses of the major immunodominant proteins of *C. ruminantium*, of approximately 21 kDa (MAP2) (24), 27 kDa (33), and 32 kDa (MAP1) (19, 33), stimulated PBMC proliferation in our assays. Similar T-cell responses were also recently observed for recombinant forms of the 21-kDa (MAP2) and the 32-kDa (MAP1) proteins (28). The 32-kDa (MAP1) protein has also been implicated in protection in an immunization trial in mice (29). Our results confirm previous reports (37) that low-molecular-mass proteins of ≤32 kDa may be important in stimulating the cellular immune response, since they predominated in our stimulatory fractions.

The results presented here were obtained by studies undertaken with PBMC. As the animals B9191 and B1359 were immunized with live *C. ruminantium*, both replicating and circulating organisms should thus be present in these animals. In addition the organism has been shown to occur in various cell types, including macrophages, monocytes, Küpfer cells, reticulum cells of the lymph nodes, fibroblasts, and connective tissues, as well as cells of the spleen, brain, pancreas, and heart (7–9, 16). Therefore, the immune responses may be taking place locally (e.g., in the lymph nodes and/or spleen) during the periods when no detectable proliferative responses were obtained with circulating peripheral lymphocytes. On the other hand, animals that have been immunized with inactivated *C. ruminantium* always had responsive circulating lymphocytes present in the blood (36). Gale and coworkers (14) observed a highly variable PBMC proliferative response in cattle immune to *Anaplasma marginale*, but sensitized T cells were readily detected in their spleens. It has been suggested that sensitized T cells home into lymphoid tissue from the circulation via the expression of specific cell surface molecules. Therefore, a study of the responses in other immune compartments such as the lymph nodes or the spleen may give a more defined picture of the immune response to *C. ruminantium* during these periods. Furthermore, antigen-specific proliferation was later detected in PBMC from our cattle, and this may be explained by the return of such lymphocytes into circulation. Mwangi and co-

workers (27) similarly failed to detect a proliferative response before challenge, with PBMC collected from animals rendered immune by infection and treatment. Proliferation was detected only when autologous endothelial cells were pretreated with T-cell growth factors prior to infection with live organisms, fixed, and then used as stimulators. Their results suggest that antigen processing and presentation by infected endothelial cells or monocytes may be essential for the induction of specific T-cell responses during infection. This may be an alternative explanation for the periods during which no proliferation was observed in our assays.

An important consideration in vaccine design is whether a single parasite antigen will elicit the appropriate protective humoral and cell-mediated responses or whether these responses will need to be activated by many distinct antigens (21). Further studies should therefore not only investigate common proteins recognized by memory T cells from genetically different cattle but also characterize epitopes conserved between isolates. Different cross-protection patterns (13, 20) and antigenic diversity between isolates (20, 33) indicate the potential for polymorphism of proteins and T- and B-cell epitopes. The Welgevonden isolate was used in this study since it induces cross-protective immunity against more isolates than any other and is also highly virulent (13). Comparative studies performed with isolates against which the Welgevonden isolate is not cross-protective may identify additional proteins of immunological importance. The combination of infection and treatment resembles natural infection, but the dose administered is likely to be higher than that acquired in the field. Nevertheless, in this study identification of proteins by PBMC from cattle immunized in this way led to the identification of candidate vaccine antigens.

In summary, our results indicate that, as with *P. chabaudi adami* and *B. bovis*, fractionation of organisms by CFE provides a way to identify potential vaccine antigens of *C. ruminantium*. The use of sensitized primary polyclonal lymphocytes permits rapid and simple screening of CFE fractions for the proteins that stimulate specific immune responses (43). In this way, *C. ruminantium* proteins with molecular masses of 11, 12, 14 to 17, and 19 to 23 kDa with the potential to play a role in protection were identified, and these proteins will be further characterized.

ACKNOWLEDGMENTS

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Cowdria ruminantium: LOW MOLECULAR MASS PROTEINS INDUCE BOVINE HELPER T-CELL LINES TO PROLIFERATE AND PRODUCE INTERFERON-GAMMA.

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Short Title: Protein recognition by *Cowdria ruminantium* specific $CD4^+$ lymphocytes.

ABSTRACT

An important objective in vaccination strategies is the activation of lymphocytes with particular effector functions. Cellular immunity and the type I cytokine interferon-gamma has been implicated in protective immunity to heartwater. Furthermore, low molecular weight proteins of *Cowdria ruminantium* have been identified to induce peripheral blood mononuclear cells to proliferate. A study was undertaken to determine which T-cell subset responds to and induces interferon gamma production when stimulated with fractionated *C. ruminantium* proteins. *C. ruminantium* specific T-cell lines were established from cattle immunized by infection and treatment or with inactivated organisms. It was possible to enrich for $CD4^+$ T-cells over time by alternating stimulation with a rest cycle. This led to greater *C. ruminantium* antigen specific proliferation and interferon-gamma production with concomitant lower background. Proteins of molecular mass 11 to 21 kDa induced $CD4^+$ lymphocytes to proliferate from all the cattle tested, but only proteins of molecular masses from 13 to 18 and 31 kDa induced $CD4^+$ lymphocytes to produce IFN-γ. Although the two groups of cattle were immunized differently, their lymphocytes responded similarly to the fractions. The results extend previous findings, by identifying responder cells as predominantly $CD4^+$. Furthermore, the low molecular mass proteins could induce the $CD4^+$ lymphocytes to produce IFN-γ.

INDEX DESCRIPTORS AND ABBREVIATIONS: Cowdriosis, bovine; *Cowdria ruminantium*; T-cell lines; antigen presenting cells (APC); counts per minute (cpm); Interferon-γ (IFN-γ); IFN-γ production index (IPI); monoclonal antibodies (mAb); not done (nd); peripheral blood mononuclear cells (PBMC); Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE); stimulation index (SI).

INTRODUCTION

Cowdria ruminantium, a tick-borne parasite, is the causative agent of heartwater in domestic ruminants. As currently classified *C. ruminantium* is the only named species in the genus *Cowdria* in the family Rickettsiaceae, order Rickettsiales (Ristic & Huxoll, 1984). Phylogenetically it is one of the Genogroup III *Ehrlichia* species, and is particularly closely related to *E. ewingi* (Allsopp *et al.,* 1997). C. *ruminantium* is pleomorphic and varies in size between 0.49 and 2.7 μm in diameter. It develops within membrane-bound cytoplasmic vacuoles of endothelial cells where it multiplies by binary fission, multiple budding and endosporulation (Pienaar, 1970).

Heartwater affects all of sub-Saharan Africa, including Madagascar and other offshore islands, where it is transmitted by ticks of the genus *Amblyomma*, most importantly *A. hebraeum* and *A. variegatum* (Provost & Bezuidenhout, 1987). The disease also occurs in the New World. Heartwater-infected *A. variegatum* ticks were introduced from Senegal into the Caribbean, probably in the 18th century (Maillard and Maillard, 1998), and the disease is now established on three islands. The ticks are carried from island to island by migrating cattle egrets (Uilenburg, 1990) and the disease therefore threatens to invade the American mainland, where large areas are climatically suitable for the vector. In addition, three American *Amblyomma* species have been shown to be capable of transmitting the disease (Barré *et al.*, 1987). In southern Africa control is effected by an infection with viable *C. ruminantium* organisms in sheep blood followed by tetracycline treatment. This cumbersome procedure cannot be used in non-endemic areas, and also

requires the maintenance of a cold chain, so the development of a recombinant sub-unit vaccine which could be stored at ambient temperature is an important priority.

Deaths due to heartwater usually occur when susceptible animals are moved to, or are raised in endemic areas, when tick control methods fail and when tick vectors spread. The disease is characterized by the development of petechiae on the conjunctiva of the eye, high fever, severe nervous symptoms, hydrothorax and hydropericardium. The morbidity and mortality rates are largely influenced by the species, breed and age of the animal, the virulence of the *C. ruminantium* isolate, immunisation and tick control programs, specific chemotherapy and the season (Van De Pypekamp & Prozesky, 1987). Once neurological signs have developed, treatment becomes more difficult as not enough is known about the pathophysiology to make supportive treatment really effective (Van Amstel & Oberem, 1987).

Immunity to members of the genus Rickettsia (*Rickettsia tsutsugamushi, R. typhi, R. conorii, R. akari, R. prowazekii*) is thought to be mediated primarily by cellular mechanisms (Jerrells *et al.*, 1986; Osterman, 1985). This has been suggested on the basis of well established *in vitro* parameters of cell mediated immunity including lymphocyte proliferation in response to specific antigenic stimulation. Antigen specific helper T-cells (producing IFN-γ) were indicated to play an important role in protection against rickettsial infection (Kodama *et al.*, 1987). IFN-γ has moreover, been shown to inhibit the growth of intracellular rickettsiae (Wisseman & Waddell, 1983; Turco & Winkler, 1983). Production of this type 1 cytokine is important for optimal defense against intracellular pathogens, especially those living in mononuclear phagocytes (Billiau *et al.*, 1998). Of particular relevance is the fact that IFN-γ has been shown to play an important role in

protection against almost all of the tick borne diseases (Brown *et al.*, 1999; Brown *et al.*, 1996; Preston *et al.*, 1992; Kodama *et al.*, 1987; Totté *et al.*, 1996 & 1999). Accordingly, work on anti-tick borne disease vaccines is targeted at vaccines that stimulate IFN-γ producing $CD4^+$ T-cells. *Babesia* proteins, fractionated by continuous flow electrophoresis, with apparent molecular masses of 20-23, 40, 51-52 and 58-60 kDa were identified that stimulate IFN-γ secreting $CD4^+$ T-cells isolated from immune cattle (Brown *et al.*, 1995). The major surface protein 2 (MSP2) of *Anaplasma marginale* has been shown induce protective immune responses in cattle characterized by a T helper lymphocyte response and high levels of IFN-γ production (Brown *et al.*, 1998). MSP2 epitopes have subsequently been identified that induce T helper responses and are thus targets of vaccine development (Brown *et al.*, 2001).

Several findings suggest that cellular immunity plays an important role in protection against heartwater (Du Plessis, 1982; Du Plessis *et al.*, 1991; Du Plessis *et al.*, 1992; Martinez 1997; Mwangi *et al.*, 1998a-c; Totté *et al.*, 1997; Totté *et al.*, 1998). Antigens that are capable of inducing the production of Th1 cytokines including IL-12, IFN-α and IFN-γ therefore have potential in the development of a recombinant vaccine. For example cattle immunized against heartwater by infection and treatment generate lymphocyte responses characterized by a mixture of $CD4^+$, $CD8^+$ and γδ T-cells and strong expression of IFN-γ, TNF-α, TNF-β and IL-2. IFN-γ has been shown to be a potent inhibitor of *C. ruminantium* growth in endothelial cells (Totté, *et al.*, 1993 & 1996). Similar results were obtained with Concanavalin A stimulated bovine lymphocyte supernatants (Mahan *et al.*, 1994). This inhibitory activity was abolished by incubation with an anti-IFN-γ mAb, whereas an anti-TNFα mAb had no effect (Mahan *et al.*, 1996). Daily intraperitoneal

injection of mice with IFN-γ but not TNFα protected them against *C. ruminantium* infection (Totté, *et al.*, 1994). The mechanism whereby IFN-γ inhibits the growth of *C. ruminantium* still remains to be determined (Totté, *et al.*, 1999).

IFN-γ has potent immunomodulatory effects on a variety of immune cells, exerting its multiple biological activities by controlling the expression of many genes. This leads to macrophage activation and T-cell differentiation towards a Th-1 type of immune response. Production of IFN-γ by either T-cells or NK cells requires co-operation of accessory cells, mostly mononuclear phagocytes, which also need to be in some state of activation (Gessani & Belardelli, 1998). The anti-parasitic activities of parasite-activated $CD4^+$ T-cells and macrophages are effected primarily through the production of the inflammatory cytokines TNF-α, TNF-β and IFN-γ. These cytokines activate neutrophils and macrophages, resulting in enhanced phagocytosis (Kumaritalake *et al.*, 1991; Langhorne *et al.*, 1989) and production of reactive nitrogen and oxygen intermediates which are toxic for intracellular parasites (Langhorne *et al.*, 1989; Liew & Cox, 1991; Rockett *et al.*, 1991; Shoda *et al.*, 2000). It is important to focus on components of the parasite that can potentially elicit the appropriate cellular immune responses, for efficient vaccine development. Therefore, antigens that induce strong cell mediated immune responses characterized by T type 1 $CD4^+$ and $CD8^+$ T-cell responses are those most likely to be useful as vaccine antigens against heartwater.

Previous work showed that low molecular weight proteins of *C. ruminantium*, between 11 and 23 kDa, induce PBMC from cattle immunized by infection and treatment or with inactivated organisms to proliferate (Van Kleef *et al.*, 2000). This paper describes the phenotype of lymphocytes stimulated by the *C. ruminantium* and confirms their

importance in vaccine development by showing that they stimulate primed lymphocytes to produce IFN-γ.

MATERIALS AND METHODS

The Welgevonden isolate of *C. ruminantium* was cultured either in bovine saphenic vein (BSV) endothelial cells (prepared from B9191) or a calf endothelial cell line (E_5), as described previously (Bezuidenhout *et al.*, 1985).

Crude extracts of Welgevonden isolate infected or non-infected cell cultures were prepared as described previously (Rossouw *et al.*, 1990). Briefly, non-infected or infected (harvested when maximally infected with organisms) BSV cell lysates were centrifuged for 30 min at 10,000 × g. The resulting pellet was suspended in PBS (PBS: 0,14 M NaCl; 1 mM KH_2PO_4; 8 mM $Na_2HPO_4.12H_2O$ and 3 mM KCl, pH 7,4). The lysates were stored at -20°C and used for fractionation of proteins by continuous flow electrophoresis.

Semi-pure inactivated organisms were prepared as described previously (Van Kleef *et al.*, 2000). Differential centrifugation was done with either maximally infected E_5 or BSV cell cultures by centrifuging firstly at 1000 × g for 10 min. The resultant supernatant was centrifuged at 30,000 × g for 30 min. The pellet was resuspended in PBS containing sodium benzyl penicillin (0,12 mg/ml) and streptomycin sulphate (0,198 mg/ml). The suspension was subjected to five freeze-thaw cycles performed with liquid nitrogen and stored at -20°C. The inactivated *C. ruminantium* lysate prepared from infected E_5 cell cultures was used for immunisation of cattle and the inactivated *C. ruminantium* lysate prepared from infected BSV cell cultures was used as antigen in proliferation assays.

Protein concentrations were determined by the Bio-Rad protein micro assay (Hercules, California) using bovine serum albumin as a standard.

The details of experimental infection of the cattle by infection and treatment or with inactivated organisms are described previously (Van Kleef *et al.*, 2000). Briefly, animals B9191 (Fresian) and B1359 (Nguni) were inoculated intravenously with 5 ml of a Welgevonden isolate infective sheep blood stabilate. The animals were monitored daily and treated on the third day of a rising febrile reaction by intramuscular injection with long-acting oxytetracycline (Liquamycin LA, Pfizer) at 20 mg/kg body weight. The animals were challenged with the same batch of homologous stabilate at the following intervals: B9191 at one month and three years; and B1359 at six months and eight months after immunisation. The animals were monitored daily for temperature. Immunisations with inactivated *C. ruminantium*: In addition two Nguni cattle (B809 and B821) were injected with 50 μg/ml inactivated *C. ruminantium* in Montanide ISA50 adjuvant (SEPPIC). The animals were injected twice at an interval between 14 and 16 weeks.

Continuous flow electrophoresis of crude *C. ruminantium* infected or non-infected BSV cell lysates was performed with a Prep-Cell Apparatus (Bio-Rad) as described previously (Van Kleef *et al.*, 2000). Briefly, 10 mg of protein was solubilized in SDS-PAGE sample buffer, boiled and electrophoresed under reducing conditions on 28-mm (internal diameter) cylindrical gels consisting of either 7% or 15% acrylamide. Fractions of 2.5 ml were eluted, collected and stored at -70°C. The fractions were precipitated by adding 8 times the volume of ice-cold acetone, incubated at -20°C for 16 h and centrifuged at 10,000 × g for 10 min. The acetone was aspirated, the precipitates

resuspended in 70% cold ethanol and centrifuged at 10,000 × g for 30 min. After the ethanol was aspirated the pellets were air dried, suspended in PBS containing antibiotics and stored at -70°C.

T-cell lines specific for *C. ruminantium* were established from PBMC of animals B9191, B1359, B809 and B821 as described previously with the following modifications (Brown & Logan, 1992). Four million PBMC were cultured per well in a volume of 1.5 ml in 24-well plates (Costar) with 1 μg/ml *C. ruminantium* antigen. After 7 days the cells were subcultured to a density of 1 x 10^6 lymphocytes per ml and restimulated with 4 x 10^6 irradiated (3000 rad) autologous PBMC per ml as a source of APC (rest cycle). After 7 days the cells were subcultured to a density of 1 x 10^6 lymphocytes per ml and restimulated with 1 μg/ml *C. ruminantium* antigen and 4 x 10^6 irradiated autologous PBMC per ml (stimulation cycle). The T-cell lines were maintained by alternating the rest and restimulation cycles. Lymphocyte proliferation assays were performed after a rest cycle and flow cytometry analysis performed after each cycle.

Complement mediated lysis of γδ T-cells was performed on the T-cell lines after the fifth week in culture. Ficoll purified T-cells were resuspended with diluted mAb specific for bovine γδ T-cell (mAb GB21A and GB54A; 1:1000 dilution) and incubated for 30 min at 4°C. The cells were washed twice by centrifugation at 250 x g for 10 min. Sterile rabbit serum (SIGMA) was added at a dilution of 1:5 and incubated at 37°C for 45 min. The resultant cells were Ficoll purified to obtain viable cells. A sample was analysed by flow cytometry and in proliferation assays.

Proliferation assays were carried out as described previously (Van Kleef *et al.* 2000). Each well (total volume 100 μl) contained complete RPMI-1640 medium, responder cells

added at a final concentration of 4 x 10^6 PBMC per ml, or 2.5 x 10^5 T-cells per ml and 2 x 10^6 irradiated PBMC per ml, and an optimum concentration of 1 μg/ml *C. ruminantium* infected or non-infected BSV cell antigens. Fractionated antigens were included at final concentrations of 5% (v/v). Proliferation was determined by measuring the incorporation of 1 μCi of [methyl-^{3}H]thymidine added on day 5, during the final 18 h of the assay. The cells were harvested, and the radioactivity was counted in a scintillation counter. Results are presented as SI ± SD. SI = mean cpm of test sample/mean cpm of unstimulated control. The unstimulated control was PBMC or T-cell line with APC cultured without antigen. Unless otherwise stated a SI ≥ 2 was considered to be an indication of antigen specific proliferation.

To determine whether crude antigen and continuous flow electrophoresis fractions induce IFN-γ production supernatants were collected on day five of the proliferation assay. The levels of bovine IFN-γ in these supernatants (diluted 1:5) were determined using the BOVIGAM™ ELISA (CSL, Ltd., Australia). Results of IFN-γ ELISA are presented as IPI. IPI = mean absorbance at 450 nm (Abs_{450nm}) of test sample/mean Abs_{450nm} of unstimulated control (PBMC or T-cell line with APC). Index's greater than twice unstimulated control were considered positive. The one-tailed Student *t* test was used to determine the levels of significance between the IPI of the non-infected and infected BSV cell continuous flow electrophoresis fractions.

The surface phenotype composition of the T-cell lines was determined by indirect immunofluorescence staining using specific mAb. The samples were analysed by flow cytometry using a Coulter XL-4 flow cytometer. The mAbs used were specific for bovine CD2 (mAb BAQ95), CD3 (mAb MM1A), B cell surface marker B-B2 (mAb BAQ44A),

CD4 (mAb GC50A1), CD8α (mAb CACT80C) and γδ TcR-N24γ (mAb GB21A). These mAbs were kindly provided by Dr. William C. Davis (Washinton State University, Pullman, WA). The secondary antibody was FITC goat anti-mouse polyvalent (IgA, IgG and IgM) (SIGMA).

RESULTS

T-cell lines were established from *C. ruminantium* immune cattle to further characterize the lymphocyte response to fractionated *C. ruminantium* proteins. By alternatively stimulating T-cell lines for 7 days with antigen and resting for 7 days, T-cell lines were repeatedly and successfully established. These lines could be maintained in culture for up to six weeks (Table I). Weekly FACS analyses were performed to determine which T-cell subpopulations were present over time. After the first stimulation cycle of 7 days the cells from B809 contained 5.6% B cells, 13.1% $CD8^+$, 31.2% γδ and 31.5% $CD4^+$ T-cells (Table I). When these lymphocytes were tested in a proliferation assay a SI of 2 was obtained (Table I). By alternatively stimulating T-cell lines with antigen and resting without antigen, the cultures were enriched 43% for $CD4^+$ T-cells and depleted 27% for γδ T-cells. This led to greater antigen specificity as can be seen by the greater SI obtained at four and six weeks (Table I). Similar results were obtained for the other cell lines from three animals B821, B1359 and B9191 (Table I). The cell lines showed consistently lower background proliferation after exposure to non-infected endothelial cell antigen as indicated by a SI of 1. In contrast, PBMC assayed at the same time showed higher non-specific proliferation in response to non-infected endothelial cell

antigen (Table II). The cell lines were further enriched for $CD4^+$ cells by complement mediated lysis of γδ T-cells which led to yet greater antigen specificity (Table III), providing evidence that the responder cells are $CD4^+$ αβ T-cells.

C. ruminantium antigen was fractionated by continuous flow electrophoresis to determine the size of the proteins which were immunostimulatory for $CD4^+$ T-cells. The $CD4^+$ enriched T-cells responded specifically by proliferation, to *C. ruminantium* fractions containing the following proteins of molecular masses: B9191: 11 to 25, 27, 32, 33 and <55 kDa; B1359: 11 to 21 kDa; B809: 11 to 30, <55 to 80 and 85 to 96 kDa and B821: 11 to 25, 28, 35, 36 and <55 to 66 kDa (Tables IV & V). *C. ruminantium* proteins between 97 and 168 kDa did not induce lymphocytes from any of the cattle to proliferate (data not shown).

C. ruminantium proteins with molecular masses of 11, 12, 14 to 17 and 19 to 23 kDa were identified previously to induce antigen specific proliferation of PBMC from these cattle (Van Kleef *et al.,* 2000). The T-cell lines therefore also responded to similar *C. ruminantium* fractions. To ascertain whether the continuous flow electrophoresis fractions also induce IFN-γ production, supernatants were collected from proliferation assays performed with PBMC and T-cell lines exposed to continuous flow electrophoresis fractions and crude antigen. Partially purified *C. ruminantium* organisms induced PBMC to produce IFN-γ (Table II). In addition PBMC stimulated with non-infected endothelial cell antigen produced low levels of IFN-γ. Once again the T-cell lines were higher responders than PBMC, producing more IFN-γ in response to *C. ruminantium* antigen than to non-infected endothelial cell antigen (Table II).

The $CD4^+$ enriched T-cells obtained after γδ T-cell lysis at week four, responded specifically by producing IFN-γ after stimulation with antigen fractions containing proteins of indicated molecular masses: B9191 - 11, 13 to 23, 26, 28 to 31, 33 to 38 kDa; B1359 - 11 to 18, 24, 25, 27, 29 to 31, 33 to 36 and 38 kDa; B809 - 11 to 23 and 31 kDa and B821 - 11 to 25, 28, and 30 to 33 kDa (Table IV). The production of IFN-γ correlated positively with proliferation for animals B809 ($r^2 = 0.88$) and B821 ($r^2 = 0.96$). However, the correlation between IFN-γ production and proliferation was less for B9191 ($r^2 = 0.12$) and B1359 ($r^2 = 0.18$). The following proteins both induce proliferation and IFN-γ production: B9191 – 11, 13 to 23 and 33 kDa; B1359 - 11 to 18 kDa; B809 - 11 to 23 kDa and B821 - 11 to 25 and 28 kDa. Some individual fractions however showed good proliferation inducing ability but failed to induce IFN-γ production: B9191 – 12, 24, 25, 27, 32 and 33 kDa; B1359 – 19 to 21; B809 – 24 to 30; and B821 – 35 and 36 kDa. Some fractions induced IFN-γ production but failed to induce proliferation: B9191 – 26, 28, 29, 30, 31, 34, 35 and 38 kDa; B1359 – 24, 25, 27, 29 to 31, 33 to 36 and 38; and B821 – 30 to 33 kDa. PBMC from animal B9191 responded similarly to *C. ruminantium* protein fractions containing proteins of molecular masses in the ranges 11 to 13, 16 to 17, 19 to 21, 26, 29 to 38 kDa (results not shown). Non-infected endothelial cell fractions did not induce PBMC to proliferate or produce IFN-γ (results not shown). It was thus observed that the continuous flow electrophoresis fractions of molecular mass 13 to 18 kDa that were able to induce lymphocyte proliferation correlated with those that induced IFN-γ production (Table IV). This correlation was detected with PBMC and T-cell lines from animals immunized either by infection and treatment or T-cell lines from animals immunized with inactivated organisms.

DISCUSSION

The cattle used for these studies were immunized either by infection and treatment or with inactivated *C. ruminantium*. Our earlier previous study showed that PBMC derived from both groups of cattle proliferated specifically in response to *C. ruminantium* proteins with molecular masses between 11 and 23 kDa (Van Kleef *et al.,* 2000). The use of PBMC thus permitted rapid and simple screening of continuous flow electrophoresis fractions for the proteins that stimulate specific immune responses. However, the nature of the responding cell population was not determined. The present study identifies $CD4^+$ T-cells as the responder population.

One week after initiation of T-cell lines with *C. ruminantium* antigen $CD4^+$ lymphocytes were the predominant cells in the cultures. It was possible to enrich for $CD4^+$ T-cells over time by alternating stimulation with a rest cycle. This led to greater specificity towards *C. ruminantium* antigen with a concomitant lower background proliferation and production of IFN-γ. Enrichment of $CD4^+$ T-cells over time was similarly observed for *B. bovis* where a rest/stimulation cycle was also used (Brown and Logan, 1992). Helper T cells are central to both humoral and cellular immunity and identification of immunogens that induce these cells to respond in the majority of animals in an outbred population is important for vaccine development. $CD4^+$ T-cells act through the production of IFN-γ as both helper cells for immunoglobulin secretion and as effector cells to activate macrophages.

Proteins of molecular mass 11 to 21 kDa induced $CD4^+$ lymphocytes from all the cattle tested to proliferate, but only proteins of molecular masses from 13 to 18 and 31

kDa induced lymphocytes from all the cattle to produce IFN-γ. Proteins between 11 to 12 and 17 to 23 kDa induced lymphocytes from three of four cattle to produce IFN-γ. Although the two groups of cattle were immunized differently, their lymphocytes responded similarly to the fractions.

Interestingly, the fractions (no. 121 to 126; 31 kDa) containing the serologically immunodominant protein MAP1 (major antigenic protein 1, MAP1; Jongejan & Thielemans, 1989; Rossouw *et al.*, 1990) induced $CD4^+$ T-cell lines from all four cattle to produce IFN-γ in the absence of proliferation. The MAP1 protein can vary in molecular mass between 31 and 33 kDa (Barbet *et al.*, 1994) and immunoblot analysis showed that the MAP1 protein is in fractions corresponding to 31 kDa (unpublished data). The fact that some proteins induced IFN-γ production but not lymphocyte proliferation may indicate that these proteins induce macrophages to produce IFN-γ, a possibility that needs further investigation. The fractions (no. 49-54; 21 kDa) containing the serologically immunodominant protein MAP2 (Mahan *et al.*, 1994) induced $CD4^+$ T-cell lines from all four cattle to proliferate and three out of four cattle to produce IFN-γ. Because lymphocytes from each of the immunisation groups produced IFN-γ it may not be a phenomenon resulting from the immunisation method. Thus, although these two proteins are serologically immunodominant they do not appear to be immunodominant T-cell antigens for all cattle in our study. The MAP1 protein has been investigated for its protective ability. Immunisation with denatured MAP1, excised from SDS-PAGE gels and emulsified in complete Freund's adjuvant, did not protect goats and sheep despite high antibody titres before challenge (Van Kleef *et al.*, 1993). A naked DNA vaccine

containing the MAP1 gene of *C. ruminantium* was however able to protect between 23-88% of immunized mice (Nyika *et al.*, 1998).

PBMC from cattle rendered resistant to challenge by vaccination with inactivated organisms contain *C. ruminantium*-specific, MHC class II-restricted, IFN-γ-producing, $CD4^+$ T lymphocytes (Totté *et al.*, 1997). $CD4^+$ T-cell lines generated using *C. ruminantium* lysates responded strongly to whole lysates but not to recombinant MAP1 or MAP2 proteins (Totté *et al.*, 1998). When these cell lines were stimulated with soluble *C. ruminantium* proteins fractionated by fast performance liquid chromatography (FPLC), a single peak which included proteins between 20 and 32 kDa induced proliferation (Totté *et al.*, 1999). In contrast, $CD4^+$ T-cell responses specific for the MAP1 recombinant antigen of *C. ruminantium* was found to be induced when cattle were immunized by infection and treatment. Both proteins induced a T helper type 1 immune response (Mwangi *et al.*, 1998c).

IFN-γ has repeatedly been implicated to play a role in protective immunity to heartwater as seen above. Furthermore, it has been suggested that IFN-γ may have a direct effect on endothelial cells making them unsuitable for *C. ruminantium* growth. The effect appears to depend on the dose since low doses of IFN-γ inhibited the growth of the organism without inducing nitric oxide or cytotoxicity (Totté *et al.*, 1996). On the other hand, high doses inhibited growth, increased nitric oxide production and induced apoptosis of infected and non-infected endothelial cells (Mutunga et al 1998). Another important role of IFN-γ is the upregulation of MHC class I and II expression on various cells (Coutinho *et al.*, 1991). By analogy, this effect on monocytes should lead to increased presentation of *C. ruminantium* antigens to the immune system. IFN-γ also

leads to increased phagocytosis by monocytes and the release of reactive oxygen intermediates, NO and lysosomal enzymes such as myeloperoxidase (MPO) by monocytes. Recombinant MPO has been shown to confer protection in a murine model of cowdriosis possibly through a direct toxic effect on extracellular orgainsms (Tournay *et al.*, 1996).

Low molecular weight proteins of C. *ruminantium* consistently induce proliferation and IFN-γ production of PBMC and T-cell lines from animals immunized either by infection and treatment or with inactivated organisms. This may therefore be an indication that these proteins play an important role in protection against heartwater. Studies need to be conducted to determine the degree of conservation of these proteins amongst different isolates of *C. ruminantium.* This can however only be done once the individual proteins causing proliferation and/or IFN-γ production have been identified. Furthermore, whether they induce protective responses in animals with different MHC compositions needs to be determined. Cross-protection studies show that there is full-, non- or partial cross-protection between isolates (Jongejan *et al.,* 1988; Du Plessis *et al.,* 1989; Stewart, 1989), suggesting that the antigens responsible for protection are polymorphic. Polymorphic immunodominant antigens ranging from 23 to 29 kDa were identified using goat antiserum raised against the Gardel isolate and adsorbed on antigen of the Senegal isolate, and vice versa, to probe western blots. As the Senegal and Gardel isolates differ genetically and do not cross-protect these antigens may have potential for use in vaccine development (Perez *et al.*, 1998). In our study proteins in this range were, however, poor inducers of proliferation and IFN-γ production.

The results of this study confirm previous findings that low molecular mass proteins of *C. ruminantium* induce specific lymphocyte proliferation in cattle immunized with the organism. The results extend previous findings, by identifying responder cells as predominantly $CD4^+$. Furthermore, the low molecular mass proteins could induce the $CD4^+$ lymphocytes to produce IFN-γ. Since IFN-γ is likely to play an important role in protective immunity to heartwater, *C. ruminantium* proteins that induce both this cytokine and lymphocyte proliferation will be further investigated as candidate vaccine antigens.

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TABLE I

Proliferative responses and phenotype analyses of T-cell lines.

Animal no.	Week[a]	Cycle completed	% lymphocytes in T-cell line[b]				SI[c]	
			B-cells	$CD8^+$	$\gamma\delta$	$CD4^+$	Non-infected cells	Infected cells
B809	1	Stimulate	5.6	13.1	31.2	31.5	nd	2
	2	Rest	3	5.3	18.7	35.9	1	4
	3	Stimulate	1.2	2.7	8.7	50.2	nd	nd
	4	Rest	1.2	3.8	8	69.2	1	11
	5	Stimulate	1	8.4	7.1	57.8	nd	nd
	6	Rest	0.6	3.3	3.8	74.2	2	20
B821	1	Stimulate	4.4	8.5	29.2	24.5	nd	2
	2	Rest	3	5.5	15.8	57.3	1	20
	3	Stimulate	0	2.1	13.5	59.2	nd	nd
	4	Rest	0	1.1	8.6	70.9	1	59
	5	Stimulate	4	2.2	4.9	52.6	nd	nd
	6	Rest	16.2	3.9	12.2	58.5	1	180

[a]The cell lines were initiated from PBMC at the beginning of week 1.

[b] FACS analysis were performed at the end of each cycle

[c] Proliferation assays were performed at the end of each cycle

Non-infected cells - 1 μg/ml of non-infected endothelial cells

Infected cells - 1 μg/ml of *C. ruminantium* infected endothelial cells

TABLE II

Proliferation and IFN-γ production by PBMC and T-cell lines after stimulation with *C. ruminantium* infected and non-infected BSV cell lysates.

Animal no.	Sample	SI		IPI	
		Non-infected cells	Infected cells	Non-infected cells	Infected cells
B821	PBMC	24	73	2	5
	T-cell line	1	73	1	14
B809	PBMC	1	2	3	7
	PBMC	6	12	2	4
	T-cell line	2	15	1	2
B1359	PBMC	1	2	1	2
	T-cell line	1	10	1	2
	PBMC	4	14	3	4
	T-cell line[a]	1	30	1	2
	T-cell line[b]	1	151	1	3
B9191	PBMC	nd	nd	1	3
	PBMC	nd	nd	7	6
	T-cell line	1	18	3	7
	PBMC	6	128	1	3
	T-cell line[a]	2	143	1	16
	T-cell line[b]	2	240	0.3	6

[a] Week 4, before γδ T-cell lysis

[b] Week 4, after γδ T-cell lysis

Non-infected cells - Non-infected endothelial cells

Infected cells - *C. ruminantium* infected endothelial cells

TABLE III

Proliferative responses and phenotype analyses of T-cell lines.

Animal no.	Week[a]	Cycle completed	% lymphocytes[b]		SI[c]	
			γδ	$CD4^+$	Non-infected cells	Infected cells
B809	1	Stimulate	0.2	30.6	nd	nd
	2	Rest	0.9	50.9	1	2
	3	Stimulate	1.5	55.8	nd	nd
	4	Rest	4.7	55.4	2	24
	4*		8.4	85.5	1	37
B821	1	Stimulate	28.2	45	nd	nd
	2	Rest	12.2	66.3	1	11
	3	Stimulate	8.4	67.1	nd	nd
	4	Rest	7.4	35.7	1	62
	4*		2.6	85.5	1	108
B1359	1	Stimulate	28.8	34.4	nd	nd
	2	Rest	22.1	35.6	3	51
	3	Stimulate	19.2	48	nd	nd
	4	Rest	15.2	47.9	1	30
	4*		19	56.3	1	151
B9191	1	Stimulate	43.8	26.2	nd	nd
	2	Rest	28.1	49.6	1	38
	3	Stimulate	14.4	75.7	nd	nd
	4	Rest	13.3	64.5	2	143
	4*		5.1	88	2	240

[a]The cell lines were initiated from PBMC at the beginning of week 1.

[b] FACS analysis were performed at the end of each cycle

[c] Proliferation assays were performed at the end of each cycle

Non-infected cells - 1μg/ml of non-infected endothelial cells

Infected cells - 1μg/ml of *C. ruminantium* infected endothelial cells

* - after complement mediated γδ T-cell lysis

Table IV
Proliferative responses and IFN-γ production by $CD4^+$ enriched T-cell lines to *C. ruminantium* infected BSV cell lysates fractionated by 15% continuous flow electrophoresis.

Fraction no.	kDa	Animal no.							
		B9191		B1359		B809		B821	
		SI	IPI	SI	IPI	SI	IPI	SI	IPI
1	≤11	1	1	1	1	**4**	1	1	1
7	≤11	1	**4**	1	1	**13**	**5**	**5**	**4**
13	11	**8**	**2**	1	**2**	**32**	**6**	**67**	**12**
19	11-12	**10**	1	**4**	**3**	**41**	**8**	**61**	**11**
25	13	**14**	**2**	**3**	**3**	**53**	**11**	**66**	**10**
31	14-16	**9**	**4**	**2**	**2**	**45**	**11**	**116**	**14**
37	16-17	**7**	**2**	**4**	**3**	**33**	**10**	**107**	**15**
43	18	**9**	**3**	**3**	**2**	**25**	**5**	**16**	**5**
49	19-21	**4**	**5**	**3**	1	**24**	**4**	**7**	**2**
55	22-23	**4**	**4**	1	1	**24**	**5**	**4**	**2**
61	23	**6**	**2**	1	1	**7**	1	**4**	**3**
67	24-25	**2**	1	1	**2**	**13**	1	**3**	**2**
73	26	1	**2**	1	1	**17**	0.3	1	1
79	27	**2**	1	1	**2**	**9**	0.3	1	1
85	28	1	**2**	1	1	**2**	1	**3**	**2**
91	29	1	**2**	1	1	**4**	1	1	1
97	29	1	**2**	1	1	**2**	1	1	1
103	29-30	1	1	1	**2**	**3**	1	1	1
109	30	1	**2**	1	1	1	1	1	**3**
115	31	1	**2**	1	1	0.2	1	1	**3**
121	31	1	**2**	1	**2**	0.2	**2**	1	**3**
127	32-33	**2**	1	1	1	1	1	1	**2**
133	33-34	1	**3**	1	**2**	0.2	1	1	1
139	34	1	**2**	1	1	0.2	1	1	1
145	35	1	1	1	**2**	0.4	1	1	1
151	35-36	1	**2**	1	**2**	0.2	1	**4**	1
157	36-37	1	**2**	1	1	0.4	1	1	1
163	37-38	1	**2**	1	1	0.2	1	1	1
169	38	1	1	1	**5**	1	1	1	1
175	38	1	1	1	1	1	1	1	1

Boldface numbers: SI and IPI greater than twice background

TABLE V

Proliferative responses of $CD4^+$ enriched T-cell lines from animal no B9191; B1359; B809 and B821 to *C. ruminantium* proteins fractionated by continuous flow electrophoresis on a 7% acrylamide gel.

Fraction no.	kDa	SI of animal no:			
		B9191	B1359	B809	B821
1	<55	**7**	1	**36**	**85**
7	55-59	1	1	**2**	**2**
13	60-66	1	1	**8**	**2**
19	67-74	1	1	**4**	1
25	75-80	1	1	**2**	1
31	81-84	1	1	1	1
37	85-90	1	1	**2**	1
43	91-96	1	1	**2**	1

Boldface numbers: SI greater than twice background proliferation