Isotyping of Immunoglobulin G Responses of Ruminants and Mice to Live and Inactivated Antigens of *Cowdria ruminantium* the Causative Agent of Cowdriosis in Ruminants

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ABSTRACT : The Immunoglobulin IgG_1 and IgG_2 isotype immune responses of domestic ruminants and mice to *Cowdria. ruminantium* live infection or by immunization with inactivated organisms were determined by the enzyme linked immunosorbent assay and Western blotting. Immunization of goats with inactivated elementary bodies (IEBs) led to a predominant IgG_1 isotype response. This indicated that a Th2 response was induced. After challenge, the IgG isotype responses were mixed whereby both IgG_1 and IgG_2 antibodies were detected. Two goats that survived virulent challenge had a predominant IgG_2 isotype response. In cattle live infection by natural challenge or experiment led to a predominant IgG_1 isotype response. Immunization of cattle with IEBs however led to mixed IgG responses characterized by similar IgG_1 and IgG_2 ratios. In the mouse live infection led to a predominant IgG_2 isotype response. This indicated the mouse developed a true Th1 type cell mediated immune response when inoculated with live organisms. Immunization with inactivated organisms on the other hand led to a dominant IgG_1 response. It is evident from this work that the immune responses of ruminants and mice to *C. ruminantium* are different and that using mice as the experimental model for immune responses to *Cowdria ruminantium*. is not the appropriate. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 4 : 541-548)***

Key Words : Immunoglobulin G-isotypes, Ruminants, Mice, *Cowdria Ruminantium*, Antigens

INTRODUCTION

Heartwater (Cowdriosis) is an infectious virulent tick-borne disease of domestic and wild ruminants caused by the rickettsial organism *Cowdria ruminantium*. Animals recovering from natural or experimental infection develop antibodies to this organism (Neitz et al., 1986; Semu et al., 1992). Detection of the antibody responses of ruminants naturally infected with live *C. ruminantium* or those inoculated with inactivated elementary bodies (EBs) are carried out by a number of serological tests. These include the indirect fluorescent antibody test (IFAT) (Du Plessis et al., 1987). The enzyme-linked immunosorbent assay (ELISA) either as an indirect assay (Soldan et al., 1993) or as a competitive assay (Jongejan et al., 1991). Western blotting (Rossouw et al., 1990) has been used to identify antigenic components of the organism.

Four immunoglobulin classes occur in the ruminant, i.e IgG, IgM, IgA and IgE. Within the IgG class, are two major subclasses IgG_1 and IgG_2 of which IgG_1 is the predominant in sheep (McQuire, et al., 1979). Immunization of sheep with protein antigens generates antibody responses of both isotypes (Bird et al., 1995). Immunization of mice with soluble protein antigens leads to two types of $CD4^+$ T helper lymphocyte responses referred to as T helper1 (Th1) and T helper2 (Th2) that are differentiated by the cytokines they produced (Mosmann and Coffman. 1989). In Th1 type responses $CD4^+$ T cells produce interferon gamma (IFN-γ), interleukin-2 (IL-2) and tumor necrosis factor alpha (TNF-α). Their effect is to promote cell-mediated immunity and support production of IgG_2 antibodies. Immunity to intracellular bacteria is thought to be mediated principally by cell-mediated immunity. The IgG isotype antibody, which is associated with these infections, would therefore be mainly IgG_2.

C. ruminantium is an intracellular rickettsial organism, which is found in the cytoplasm of endothelial cells. Protective immune response to this organism has been suggested to be predominantly cell-mediated since passive transfer of antibodies do not confer protection against challenge. The objective of this study was to examine the IgG_1 and IgG_2 responses of cattle, sheep, goats and mice to *C. ruminatium* elemenntary body antigens following experimental infection by infection and treatment (I/T), field challenge, and immunization with inactivated elementary bodies (IEBs) or recombinant antigens. This was to establish the predominant IgG isotype produced after natural or experimental challenge and by immunization with inactivated organisms. This would indicate the type of immune response induced in the host and further elucidate if the immune response to *C. ruminantium* is principally cell mediated or mixed type response.

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MATERIALS AND METHODS

Antigens

The antigens used for Western blotting and ELISA tests were from elementary bodies (EBs) of *C. ruminantium* (Welgevonden stock). The organisms were propagated in bovine endothelial cell as described by Pow et al. (1993). Two recombinant antigens. (Ags) a 58 kDa Heat shock protein (58 kDa Hsp) and its subclone a 35 kDa protein were also used. The 35 kDa recombinant protein was expressed in *E. coli* and purified by a standard method then used for ELISA tests to detect IgG_1 and IgG_2 responses of mice and sheep immunized with the 58kDa Hsp recombinant Ag.

Sera : Sera from five groups of goats, two groups of sheep. three groups of cattle and three groups of mice were examined for *Cowdria* specific IgG antibodies by Western blotting and ELISA.

Caprine sera : Sera from five groups of goats (C1. to C5 with six goats each) were tested. The goats in the first group (C1) received primary inoculations of inactivated elementary bodies (IEBs) only and those in the second group (C2) were inoculated 2 times with IEBs 21 days apart. The antigens used for primary immunisation were mixed with equal volumes of Freund complete adjuvant (FCA) and inoculated intramuscularly. Antigens used for booster inoculations were mixed with equal volumes of Freund incomplete adjuvant (FIA) or Phosphate buffered saline (PBS) and inoculated 21 days later. Goats in the third group (C3) received detergent extracted elementary body (EB) antigens for both primary and booster inoculations.

Sera were collected sequentially from each group at days 0. 7. 14. 21, 28, and day 34 post inoculation (p.i.). All the goats in the above groups were challenged by intravenous inoculation of 5 ml of virulent blood stabilate of the Welgevonden stock at 36 days pi. Then detection of *Cowdria* specific IgG_1 and IgG_2 antibodies was carried out on sera collected after immunization (day 34 pi) and sera collected at day 64 pi (day 28 post challenge) from 4 survivors two each from group C1 (G614. G668) and C2 (G476, G601).

Sera in the fourth group (group C4) were collected from two infection treatment (I/T) goats. Two serum samples collected on day 28 and day 80 after infection were examined for *Cowdria* specific IgG_1 and IgG_2 antibodies. Sera in the fifth group (group C5) were from 2 goats (G107. G108) immunized with IEB but not challenged.

Ovine sera : Two groups of sheep sera were tested. In the first group (S1) 6 sera were tested. Three sera each originated from three sheep infected with the Mara and Nonile stock of C. ruminantium respectively. They were obtained at day 0, 14 and 28 after infection. In the second group (S2) were sequential sera obtained on day 0, 14, 21. 28, 64. and day 143 pi from 6 sheep immunized with 58 kDa Heat shock protein (Hsp) recombinant antigen. These sera were examined for Cowdria specific IgG_1 and IgG_2 responses to recombinant 35 kDa sub-clone antigen of the 58 kDa Hsp of C.ruminantium with their respective controls.

Bovine sera : Sera from three groups of cattle were examined for Cowdria specific IgG_1 and IgG_2 antibodies as follows: Group B1. These sera were raised by experimental infection using blood stabilates. Five cattle from which the sera were obtained were immunised by I/T with 3 stocks of C. ruminantium, Ball 3 and Mara stocks (2 cattle each) and Kwanyanga (one cow).

Group B2. There were 10 sera in this group collected from cattle (B196. B199. B200 to B206, B210 and B211) in a heartwater endemic area of South Africa. Group B3. In the third group were thirteen sera from Kenyan cattle. Nine of the sera were obtained from 3 cattle (S295, S296. and S304) immunized with IEBs of the Gardel stock of *C. ruminantium* then challenged with virulent homologus organism. They were collected on day 0, 36. pi and day 31 post challenge (pc) respectively. The remaining 4 sera were obtained from two control cattle (S234 and S264) collected on day 0 and day 11 pc.

Mouse sera : Sera from 4 I/T out-bred TO white mice and another 5 mice immunized with IEBs were collected at day 34 pi were examined for IgG_1 and IgG_2 antibody responses to C. ruminantium. Additionally pooled sequential mouse sera (day 0. 14, 21 and 34 pi) obtained from mice which had been immunized with a recombinant 58kDa Hsp were also examined for IgG_1 and IgG_2 antibodies to a recombinant 35kDa subclone protein of C. ruminantium by ELISA.

Monoclonal antibodies : The monoclonal antibodies (MoAb) used to determine sheep and goat IgG_1 and IgG_2 responses were rat anti-sheep IgG_1 (IRS) and IgG_2 (2RS). Their optimal working dilutions was 1/20 in phosphate buffer saline containing 0.02%Tween 20 to which was added 4% normal rabbit serum in (PBST/4%NRS). For the mice tests. rat-anti-mouse IgG_1 and IgG_2 monoclonal antibodies (Serotech, UK) were used. For the bovine tests, sheep anti-bovine IgG_1 HRP conjugated polyclonal serum was obtained from Bethyl Laboratories (USA) used at a dilution of 1/50,000 to detect IgG_1. Mouse anti-bovine IgG_2 obtained from Sigma (Sigma UK) was used at a dilution of 1/10,000 to detect bovine IgG_2. All the working dilutions for the bovine ELISA were carried out in phosphate buffered saline containing 0.02%Tween 20 to which was added 4% normal goat serum in (PBST/4%NGS)).

Detection of IgG_1 and IgG_2 isotype responses of goats, sheep and mice and cattle infected with live or immunized with *inactivated C. ruminantium* antigens by

indirect ELISA

The method used to determine isotype responses was a modification of that described by Soldan et al., (1993). Optimal working dilutions of each monoclonal antibody (MoAb) and antigens were determined by checkerboard titration.

Antigens : The antigen used in ELISA tests to detect IgG_1 and IgG_2 responses of goats, sheep and mice were from the Welgevonden stock of *C. ruminantium* prepared as described by Soldan et al., (1993). The optimal dilution of this antigen used to coat the ELISA plates was 1/1000 in coating buffer (0.05M carbonate/bicarbonate buffer pH 9.6). For the bovine IgG_1 and IgG_2 tests the Gardel stock of *C. ruminantium* was used at a dilution of 1/4,000 in coating buffer for the Kenyan bovine sera while the Welgevonden stock was used at a dilution of 1/6,000 for the South African bovine sera.

Buffers : The blocking buffer for the goat, sheep and mouse tests was PBS containing 0.02%Tween 20 to which was added 4% normal rabbit serum in (PBST/4%NRS). While the blocking buffer for the bovine tests was PBST to which 4% normal goat serum was added. The washing buffer for all the tests was PBST containing 0.9% sodium chloride.

The ELISA tests carried as follows: Ninety six (96) well flat-bottomed microtiter ELISA plates (Immulon 1, Dynatech Laboratories) were coated with 50 µl of respective ELISA antigen in coating buffer The plates were covered with cling film and incubated at 4°C overnight. Then the contents were discarded and the plates were washed three times (3 min/wash) in 0.9% sodium chloride containing 0.05% (v/v) Tween 20 (0.9% NaCl/PBST). The plates were blocked with 100 µl per well of PBST/4% NRS for all sheep, goat and mice tests. For the bovine tests, the plates were blocked with 100 µl per well PBST/4% NGS. All the tests were incubated for 1 hour at room temperature and the buffer was discarded. Then, fifty microlitters (50 µl) of goat, sheep, and mice sera diluted to 1/50 in blocking buffer (PBST/4%NRS) were added to respective wells. The bovine sera were diluted to 1/800 in blocking buffer (PBST/4%NGS) then 50 µl were added to all the wells and the wells were topped up 100 µl with respective blocking buffer.

Detection of goat and sheep IgG_1 and IgG_2 responses was achieved by adding 50 µl of rat anti-sheep IgG_1 (1RS) and IgG_2 (2RS) monoclonal antibody (diluted to 1/20) to respective wells prior to incubation at 37°C for 1 h. While for the mice tests, rat anti-mouse IgG_1 and IgG_2 monoclonal antibodies (Serotech, UK) were used. After incubation the plates were washed 3 times as above. This was followed by the addition of 50 µl of goat anti-rat IgG whole molecule horse radish peroxidase (Sigma) conjugate (diluted 1/1,000) to all wells and incubated at 37°C for 1 h. Detection of bovine IgG_1 was achieved by using a 1/50,000 dilution of sheep anti-bovine IgG_1 horse radish peroxidase (HRP) conjugated ploYclonal antibodies (Bethyl Labs UK) and detection of bovine IgG_2 was achieved by adding goat-anti-bovine IgG whole molecule HRP conjugate (Sigma) diluted to 1/2,000.

The plates were washed 3 times and 50 µl of the peroxidase substrate tetramethyl benzidine (TMB, Kirkegaard and Perry laboratories) was added to all wells. The reaction was stopped after 15 minutes by addition of 50 µl of 0.2M sulfuric acid. The absorbency (Optical density/ODs) at 450 nm was read using a plate reader (Multiscan Plus, Version 2.03, Labsystems). The mean of 2 tests was calculated, then the OD of the negative control was subtracted from this value to remove background absorbency. The results were expressed as the mean OD values of 2 tests

Detection of IgG_1 and IgG_2 isotype responses of immunized goats to *C. ruminantium* by Western blotting

Elementary bodies of the Welgevonden stock of *C.ruminantium* were used as antigens. SDS-PAGE and Western blotting was carried out as described by Lally et al (1995) with the following modifications to enable specific isotype responses to be detected:

Optimal working dilutions of each monoclonal antibodies (MoAb) used in Western blot analysis were established to be 1/10 for 2RS and 1/20 for 1RS. Following blocking of the membranes and incubation with primary sera, the test blots were reacted with monoclonal antibodies to sheep IgG_1 and IgG_2. They were then incubated for 60 minutes and color was developed in the usual way. The Molecular masses of the bands were estimated by a standard curve then the blots were photographed for permanent record.

Analysis of statistics

The student's t test was used to analyze for differences between IgG_1 and IgG_2 antibody responses in sera from IEB immunized goats which survived challenge with virulent homologous organism.

RESULTS

IgG_1 and IgG_2 responses of goats immunized by I/T or with IEBs measured by ELISA

Goats immunized with IEBs developed a predominant IgG_1 response before challenge (Figures 1 d34). There were significant differences between the ELISA OD values of IgG_1 and IgG_2 isotypes following IEB immunization ($p<0.05$). The IgG_1: IgG2 ratios for 4 goats in-groups 1 and 2 were 5:1 for (G476), 2:1 (G614, G668) and 1.5:1 (G601).

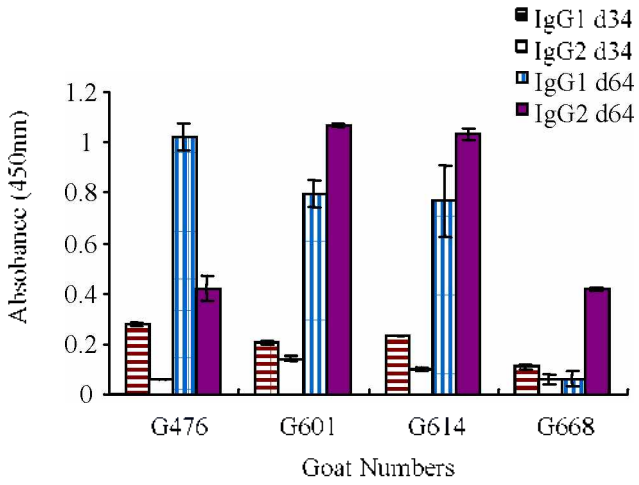

Figure 1. The post immunization (p.i) IgG_1 and IgG_2 responses of 4 goats immunized with IEB of C. ruminantium detected by ELISA. Post immunization and post-challenge sera were collected day 34 p.i and day 28 pc (= Day 64 pi) from each animal. Goats G476 & G601 belong to Gp C1 and G614 & G668 belong to Gp C). The bars show the mean OD of 2 tests.

Table 1. The mean ELISA absorbance values of *Cowdria* specific IgG_1 and IgG_2 of antibodies and IgG_1: IgG2 ratios in sera from South African cattle immunized by infection and treatment method (Gp.B1)

Mean absorbance at 450nm of isotypes			
Immunized cow/stock	IgG_1	IgG_2	IgG_1:IgG_2 ratios
B9/Ball 3	0.580	0.068	8:1
B155/Ball 3	0.237	0.200	1:1
B10/Kwanyanga	0.747	0.146	5:1
B25/Mara	0.324	0.018	18:1
B61/Mara	0.302	0.134	2:1

Comparison of IgG_1 and IgG_2 responses show that there are no significant differences p=0.006

Absorbance values at 450nm given are the means of two tests

After live virulent challenge of the IEBs immunized goats, the level of IgG_2 isotype as measured by OD value in ELISA, rose to between 7 and 10 times their pre-challenge levels and IgG_1 ODs in post challenge sera increased by a factor of 3 to 6 times. Analysis for differences between IgG_2 responses pre-challenge and post-challenge indicate that the levels of IgG_2 had increased after challenge ($p<0.05$). The rise in IgG_2 had the result that the differences in IgG_1 and IgG_2 levels were no longer significantly different (p=0.06). After challenge the ratio between IgG_1 and IgG_2 responses had changed to between 2:1 to 1:1.6.

IgG_1 and IgG_2 antibodies in post immunization and post-challenge sera from IEBs immunized goats recognize specific antigens of the elementary body in Western blots

To confirm that IgG_1 and IgG_2 responses following IEB immunization were to *Cowdria* antigens and not to endothelial cell antigens, which contaminate IEB preparations, Western blotting was performed. Sera collected at day 28 from an I/T goat (G74) reacted strongly with EB antigens of molecular masses of 21 kDa, 24 kDa, 28kDa and 32kDa. A few antigens of higher molecular masses reacted faintly. These reactions were however only with IgG_1 (Figure 2, lanes 2 and 4) but not IgG_2. The IgG_1 and IgG_2 responses of sera collected on day 34 pi from an IEB goat (G668, group C2) were the same as those of the I/T goat C4 (Figure 2 lanes 6 and 7). The very significant

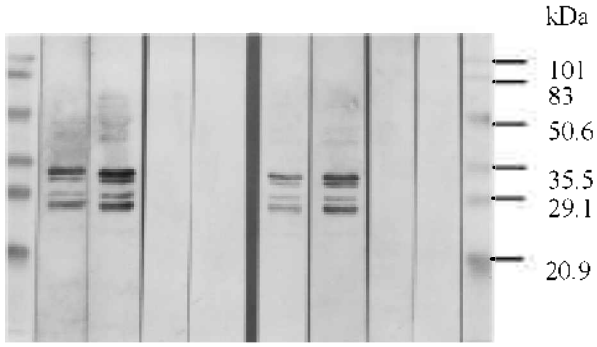

Figure 2. Evidence of specific IgG_1 response against Cowdria antigens following by Infection/Treatment(I/T) or immunization with Inactivated elementary bodies (IEB_S) of goats.

Lenes 1 and 10 SDS low range molecular weight protein standards; Western blots were reacted with sera lenes 2 amd 3, IgG_1 positive day 28 sera from I/T goat (G74), lenes 4 and 5 day 28 I/T serum negative for IgG_2, lanes 6 and 7 pre-challenge day 34 sera from IEB goat (G668) tested for IgG_1; lane 8 and 9 pre-challenge sera from G668 tested IgG_2. Note the apparent absence of IgG_2 in both I/T and pre-challenge IEB sera.

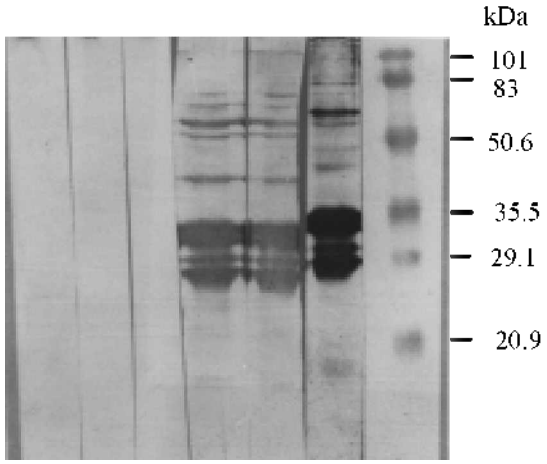

Figure 3. Evidence of *Cowdria* specific IgG_2 responses following challenge of IEBs immunized goats with virulent homologous of *C. ruminantium*.

Lanes 1 to 3, negative control serum (G106); lanes 4 to 6 serum collected day 28 post challenge of IEBs immunized goat (G476); lanes 7 SDS protein standards.

Table 2. The mean ELISA absorbance values of *Cowdria* specific IgG_1 and IgG_2 antibodies and ratios of IgG_1: IgG_2 in sera collected from naturally challenged cattle in South Africa (Gp B2).

Mean ELISA absorbance values at 450nm of each isotype			
Sample No.	IgG_1	IgG_2	IgG_1:IgG_2 ratios
B196	0.122	0.338	3:1
B199	1.746	0.529	3:1
B201	1.627	0.426	4:1
B202	0.829	0.057	14:1
B203	0.765	0.126	6:1
B204	0.449	0.008	56:1
B205	0.945	0.076	12:1
B206	0.193	0.062	3:1
B211	0.327	0.031	10:1

Comparison of IgG_1 and IgG_2 responses show that there are no significant differences p=0.008

Absorbance values given are the means of two tests.

IgG_2 response after challenge of goat G476 of IEB immunized goats was confirmed by the presence of reactions to *Cowdria* antigens (Figure 3, lanes 4, to 6).

IgG_1 and IgG_2 responses of sheep immunized by infection and treatment and those inoculated with recombinant 58kDa antigen determined by ELISA

Infection of sheep with live virulent organisms in blood resulted in a predominant IgG_1 response and an apparent absence IgG_2 response. The IgG_1 and IgG_2 responses of sheep (group S2) immunized with recombinant 58 kDa Hsp were also characterized by a predominant IgG_1 response with a ratio of at least 2:1 and no detection of IgG_2 in 3 out of 6 animals.

IgG_1 and IgG_2 responses of cattle following live infection by experiment (I/T) or by field exposure

The *Cowdria* specific IgG_1 and IgG_2 responses to experimental infection of 7 cattle (group B1) with 3 different stocks of *C. ruminantium* were characterized by a dominant IgG_1 although IgG_2 was detectable above background. The IgG_1: IgG2 ratio had a median of 5:1 (range 1:1 to 8:1, Table 1). The IgG_1: IgG2 ratios of field sera from South Africa (group B2) were characterized by IgG_1: IgG2 ratios greater than 1 (Table 2) with a range from 3:1 to 56:1 (mean 5:1). Except for one animal which had a ratio of 1:2. These ratios were similar to those obtained following experimental infection (Tables 1 and 2).

IgG_1 and IgG_2 responses of cattle following immunization with IEBs

Immunization of cattle with IEBs of *C. ruminantium* led to production of both similar amounts of IgG_1 and IgG_2 (Table 3, Figure 4). The level of IgG_2 was much higher after immunization with IEBs than that obtained in cases of the experimental infection and field sera (Figure 4, Table 3). The IgG_1: IgG2 ratios in sera of three immunized cattle

Table 3. The mean ELISA absorbance values of *Cowdria* specific IgG1 and IgG2 antibodies and ratios between the isotype responses in sera from Kenyan cattle collected day 36 post immunization and day 31 post challenge from cattle immunized with IEBs (Gp.B 3) of *C. ruminantium* and challenge with live Gardel stock

Mean ELISA absorbance values at 450nm of each isotype				
Animal No.	Days serum was collected	IgG_1	IgG_2	ratios of IgG_1: gG2.
S295	-1	0.044	0.005	NA
	36 pi.	1.319	0.881	2:1
	31 pc.	1.554	1.149	1:1
S296	-1	0.022	0.004	NA
	36 pi.	0.889	0.714	1:1
	31 pc.	1.347	1.304	1:1
S304	-1	0.013	0.006	NA
	36 pi.	1.116	0.995	1:1
	31 pc.	1.347	1.260	1:1
Control infections				
S234	0	0.0014	0.016	
	11	0.035	0.016	
S264	0	0.036	0.039	
	11	0.014	0.022	
Positive control		0.609	0.044	
Negative ontrol		0.038	0.015.	

Comparison of IgG_1 and IgG_2 responses show that there is no significant difference p=0.031

pc. = post challenge. Absorbance values given are the means of two tests.

pi. = Post inoculation, NA = not applicable.

(S295, S296, S304) were the same before challenge and after challenge although there was an increase in OD values of both isotypes after challenge. In contrast to goats the increase in OD value of the two IgG isotypes was of a similar magnitude (Figure 4), and did not show greater increase in IgG_2. The mean OD values of sera obtained before immunization of the immunized group and those from two control cattle (S234, S264) remained low and their IgG_1: IgG_2 ratios were same or IgG_1 ratio was higher than 1.

IgG_1 and IgG_2 responses of mice to *C. ruminantium* after immunization with I/T, with IEBs or recombinant antigens

Immunization of mice with live *C. ruminantium* was observed to result in a dominant IgG_2 isotype response, in contrast to that obtained in live infection of ruminants. Sera obtained on day 34 pi. From 4 mice infected with live EBs had higher IgG_2 OD values in comparison to sera from mice inoculated with IEBs and collected at day 34 after immunization (Figure 5). The IgG_1: IgG_2 ratios of the sera from I/T mice was 1:2 and that of sera obtained after immunization with inactivated EB was 2:1. The antibody responses of mice immunized with recombinant 58 kDa Hsp were characterized by predominantly of IgG_1 isotype as determined using the 35 kDa antigen (Figure 6).

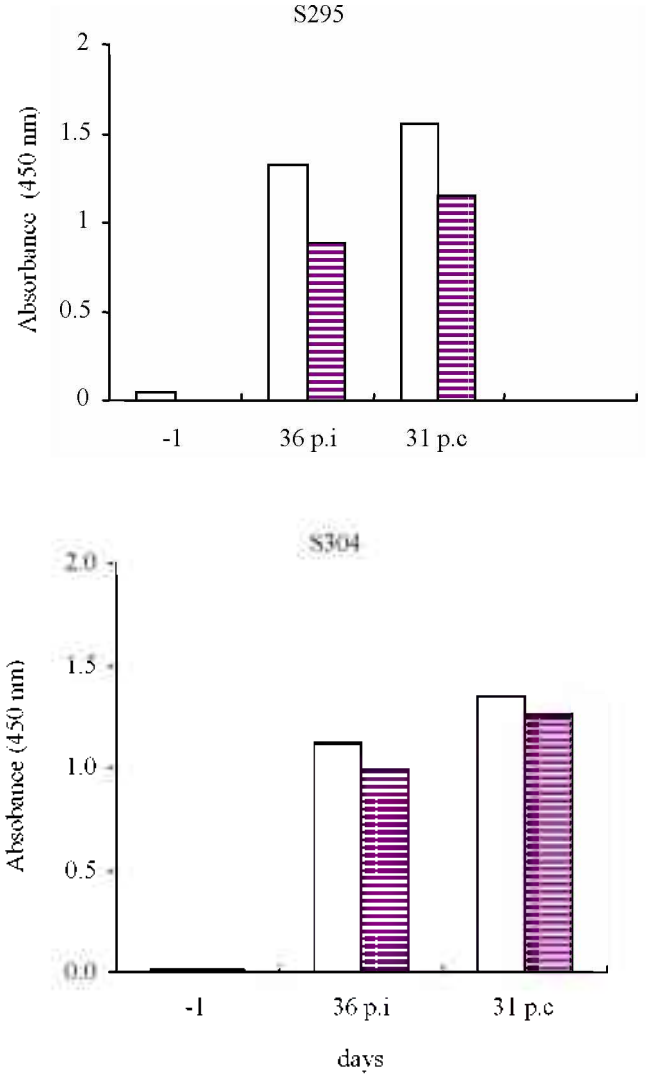

Figure 4. IgG_1 and IgG_2 responses of pre-challenge (day 36 pi) and post-challenge (day 31 pc) sera of two Kenyan cattle (S295, S296) immunized with IEBS (gp B3) of C. ruminantium (Gardel). IgG_1 responses (□) and IgG_2 responses (▤).
Bars represent the mean ELISA OD 450 of two tests of sera collected 36 days

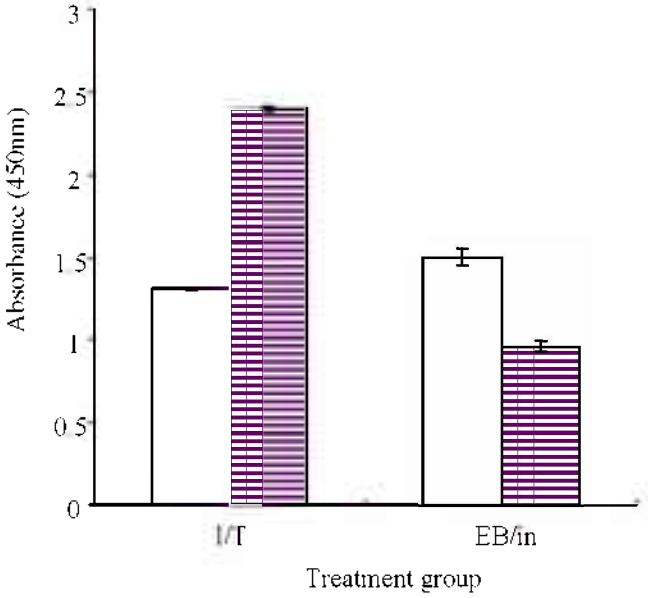

Figure 5. The IgG_1 and IgG_2 responses of mice immunized by I/T or with IEB of C. ruminantium. Sera were collected 34 days after infection or immunization, pooled and tested for IgG_1 and IgG_2. IgG_1 responses (□) and IgG2 responses (▤).
Results are express as the mean OD 450 nm of 2 tests.
Error bars show standard error of the mean.

DISCUSSION

Immunization of goats with inactivated elementary bodies led to the development of stronger IgG_1 than IgG_2 responses pre-challenge. After challenge the ELISA OD values of both isotypes were 7 and 10 times those of pre-challenge levels. The increase in IgG_2 titers after challenge was such that in two of four goats which survived challenge, the IgG_2 OD values were higher than those of IgG_1, indicating that this isotype was being preferentially produced. The shift in antibody isotype from IgG_1 to IgG_2 or an increase in IgG_2 after challenge indicated that a Th1 type response was stimulated by the challenge infection. In a Th1 type response $CD4^+$ lymphocytes secrete the cytokines IFN-γ, IL-2 and TNF-α, promoting cell-mediated immunity and IgG_2 production. These findings are in agreement with the evidence that stimulation of bovine B cells sorted for IgM with IFN-γ induces secretion of IgG_2 *in vitro* (Estes et al., 1995). In contrast immunization of cattle with IEB stimulated production of both IgG_1 and IgG_2, and challenge of the immunized cattle led to a further increase in the titters of both isotypes. The differences in IgG isotype responses in goats and cattle to *C. ruminantium* may be associated with the greater resistance of cattle to heartwater (Uilenberg, 1983; Camus et al., 1996).

Sera from two I/T goats (Group 4, G69, G74) infected with the Welgevonden stock and treated to prevent death, had higher levels of IgG_1 responses. The reason for these exceptions is not clear. However it suggests that *C. ruminantium* may be inducing production of IL-4 or IL-10 at the beginning of infection which drives the immune responses to a Th2 type antibody responses rather than a Th1 type IgG_2 immune response.

Cattle immunized by I/T (group B1) and field sera (group B2) had dominant IgG_1 responses. It is possible that live infection and immunization by I/T does not lead to a classical Th1 response but to an IL-4/IL-10 driven response since bovine IL-4 upregulates production of IgG_1, IgM and IgE in the presence of a variety of costimulators

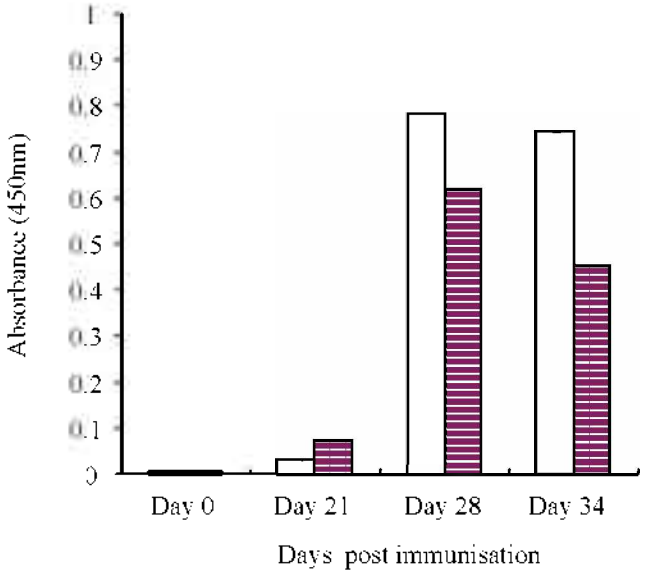

Figure 6. The IgG_1 and IgG_2 response of sequential sera from mice (10) immunized with recombinant 58-kDa Hspantigen of C. ruminantium at days 21, 28 and 34 after immunization. IgG_1 responses (□) and IgG2 responses (▤) were detected by ELISA. Bars represent the mean of two tests and the error bars show the standard error of the mean.

(Estes et al., 1995). Immunization of cattle with IEBs induced a strong IgG_2 response in addition to IgG_1, which suggests that a mixed Th1 type and Th2 type response was induced by inactivated organisms in contrast to live, or natural (tick mediated) infection. The results suggest live *Cowdria* shift responses towards Th2. The fact that natural infection and I/T may lead to an IL-4 driven Th2 type responses indicates that *C. ruminantium* may circumvent the host immune system by directing it towards a Th2 type response. However, I/T leads to solid immunity, which indicates that, other effector mechanisms such as cell-mediated immune responses mediated by cytotoxic T cell, IFN- and NK cells are probably involved in protective immunity. Other workers have found that cytotoxic T cell populations are induced by *Cowdria* infection but only at a suprisingly late stage (Ben Said, Personal Communication). The IgG_1 response of mice immunized with inactivated EBs was characterized by higher titters of IgG_1 whereas that of I/T mice had higher IgG_2 titters. These findings are in agreement with those of Du Plessis et al. (1991) who showed that following I/T immunity is cell mediated and that $CD8^+(Lyt2^+)$ T cells were responsible for protection as indicated by adoptive transfer of immune cells to un-immunized mice. The situation in ruminants appears different in that in natural infections and I/T leads to a Th2 type response characterized by a dominant IgG_1 response.

The role of antibodies in immunity to heartwater is not clear. Experimental transfer of serum or gamma globulins by *in vivo* or *in vitro* neutralization tests has given variable results (Du Plessis, 1993) found no correlation between antibody titters and immunity to heartwater in calves. Furthermore Martinez et al. (1993) observed that sera from survivors of a challenge experiment after immunization did not neutralize *C. ruminantium* infection of endothelial cell cultures *in vitro*. However the same authors observed that serum from immune mice and cattle inhibited adhesion and entry of endothelial cell cultures by *C. ruminantium*. In other experiments, Byrom et al., (1993) used mouse serum with or without complement or purified antibodies administered simultaneously into mice with *C. ruminantium* during incubation or clinical reaction. They observed each treatment did confer immunity or alter the course of *C. ruminantium* infection in mice. In contrast addition of complement to immune serum which was subsequently mixed with infectious *C. ruminantium* (Kumm) inhibited their infectivity (Du Plessis, 1993). The Kumm stock however appears to have a tropism for macrophages and this may be responsible for the different results.

Live immunization by infection and treatment leads to development of very low IgG_2 antibodies in the ruminant whereas it leads to development of a high concentration in the mouse. These results indicates that the two species respond differently to live infections of *C. ruminantium* and therefore the use of mice to study immune responses to *C. ruminantium* does not seem to be appropriate.

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