T CELL-MEDIATED IMMUNITY TO *COWDRIA RUMINANTIUM* IN MICE: THE PROTECTIVE ROLE OF LYT-2⁺ T CELLS

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ABSTRACT

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The inability of athymic nude mice to make a drug-aided recovery from infection with either the Kümm or the Welgevonden stocks of *Cowdria ruminantium* and their inability to mount an immune response, suggest that immunity in heartwater is cell-mediated. The adoptive transfer of immunity with the spleen cells of mice immune to the Welgevonden stock is supportive evidence. Immune spleen cells depleted of Lyt-2⁺ T cells are unable to confer resistance to challenge to recipient mice, whereas the depletion of L₃T₄⁺ T cells had no effect on the protection conferred by immune spleen cells. This is conclusive evidence that immunity in heartwater is largely cell-mediated.

Immune serum, *C. ruminantium* and complement incubated in the presence of mouse peritoneal macrophages, inhibits the infectivity of the heartwater agent, but not in the absence of macrophages.

The decreased resistance to challenge of immune mice treated with gloxazone adds further support to the concept that in heartwater persistence of *C. ruminantium* in the host is associated with immunity.

INTRODUCTION

Cowdria ruminantium, the causal agent of heartwater, is a strict obligate, intracellular pathogen. Recent review articles on the immunity of domestic ruminants (Stewart, 1987a) and mice (Stewart, 1987b) have revealed the scanty knowledge on the mechanism of the immunity induced in this disease. Serum or large quantities of gamma globulin from immune animals, whether given at the time of infection or during the incubation period, does not influence the outcome of the disease (Alexander, 1931; Du Plessis, 1970 & 1982).

The first substantial evidence that immunity in heartwater is cellular rather than humoral, was obtained with a mouse model in which mice were infected with the Kümm stock of *C. ruminantium* (Du Plessis, 1982). The finding in this study that peritoneal macrophages from re-infected immune mice failed to elicit disease in mice injected with these cells, whereas those injected with cells from infected susceptible mice consistently died, was consistent with the concept of cellular immunity. Furthermore, the total resistance to challenge of mice simultaneously or previously injected with immune spleen cells, strongly suggested that acquired resistance to *C. ruminantium* was cell-mediated. The importance of the persistence of *C. ruminantium* in the host in the development and maintenance of protective immunity is not known.

MATERIALS AND METHODS

C. ruminantium stocks

One hundred engorged *Amblyomma hebraeum* nymphae, infected with the Kümm stock (Du Plessis, 1982), were homogenized in 40 mℓ buffered lactose peptone (BLP), centrifuged at 1 000 r.p.m. for 5 min and the supernatant deepfrozen in liquid nitrogen in suitable quantities. A sample was withdrawn and an infectivity titre of 10⁴·⁸ determined in mice as previously described (Du Plessis, 1982).

The Welgevonden stock (Du Plessis, 1985) infective inoculum was prepared from the lungs, hearts and spleens of mice *in extremis* homogenized in BLP

on a 10 % mass/volume basis and stored in liquid nitrogen in suitable quantities. A sample was withdrawn and an infectivity titre of 10⁴·³⁷ determined.

Mice

Specified pathogen free (SPF), inbred BALB/c mice, 6–8 weeks old of both sexes were used in the adoptive transfer of immunity experiments. Female, conventional, outbred Swiss white mice, 6–8 weeks old, were used in other experiments.

Athymic nude mice

In an attempt to immunize congenitally athymic nude mice against *C. ruminantium*, SPF nude mice of both sexes and 10–12 weeks old, were kept in a laminar flow cabinet at a temperature of 20–22 °C. The mice were infected with varying LD₅₀ doses of both stocks of *C. ruminantium* and treated with oxytetracycline at varying dosage levels and at various time intervals after infection (Table 1). Twenty-three mice were injected intraperitoneally (i.p.) with the Kümm and 16 with the Welgevonden stock along the intravenous (i.v.) route.

Mice that survived were either challenged with a known lethal dose of the homologous stock 4–6 weeks post-infection (p.i.), or they were sacrificed and homogenates prepared from their tissues. Their lungs, spleens and hearts were homogenized in BLP on a 20 % mass/volume basis and 0,2 mℓ inoculated into 5 susceptible white mice. The pooled tissues of 3 and 6 moribund nude mice infected with the Welgevonden and Kümm stocks, respectively, were sub-inoculated in this manner. Serum samples collected from mice showing clinical signs prior to death 30–36 days p.i. or from mice that had survived, were subjected to the indirect fluorescent antibody (IFA) test conducted as previously described (Du Plessis & Malan, 1987).

To compare the susceptibility of nude mice with that of their euthymic littermates, 5 nude (nu/nu) mice and 5 euthymic (nu/⁺) littermates were infected with 23 LD₅₀ Kümm stock doses and 5 each with 3,2 LD₅₀ Welgevonden stock doses.

T cell mediated immunity

In all 3 experiments the Welgevonden stock was used and inoculated i.v., but the method of immunizing donor mice differed in the dosage of the infective inoculum and the treatment of the mice after infection.

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Adoptive transfer of immunity

In *Exp. 1* donor mice were infected with a low dose (3,2 LD_{50}) of the Welgevonden stock. The mice showed no clinical signs, they were left untreated and their spleen cells transferred to susceptible mice on Days 10 and 20 p.i. The spleens of 6 or 7 mice were snipped in phosphate buffered saline (PBS), forced through a 1 mm diameter sieve and centrifuged for 5 min at 1 000 r.p.m. The sediment was taken up in 7 mℓ PBS and drawn into a syringe through a 25G needle several times. All operations were carried out at 4 °C. A trypan blue vital stain was used to count the cells. The spleen cells of uninfected mice were harvested and treated in a similar manner as a control group. Serum collected from the donor mice when they were killed, was subjected to the IFA test.

Without delay approximately 10^8 spleen cells in 0,3 mℓ PBS were injected i.v. into each of 5 mice, followed within an hour by 400 LD_{50} doses of *Cowdria* (Welgevonden stock). A second control group of 5 mice were inoculated with the challenge inoculum alone.

The donor mice in *Exp. 2* were infected with 400 LD_{50} doses and treated with oxytetracycline[1] on Days 9, 10, 16, 17 and 18 p.i. at a dosage rate of 30 µg/g body mass. On Days 10, 15 and 21 p.i. cells were transferred to recipient mice as described above. Two control groups were included each time and on each occasion serum for antibody assaying was collected from the donor mice.

In vitro *depletion of T cell subsets*

The donor mice in *Exp. 3* were also infected with a lethal dose of 400 LD_{50}, but they were treated with oxytetracycline[2] as early as Day 8 and again on Day 10. They were subsequently re-infected with the same inoculum 30 days after the last treatment. No additional treatment was necessary. Thirty days after re-infection, cells were prepared from the donor immune mice for transfer and depletion of T cells in a manner similar to that described in Exp. 1 and 2, except that the cell suspension was transferred to a Roux flask before counting the cells, in order to remove as many macrophages and histiocytes as possible. The non-adherent cells were used for the transfer and T cell depletion experiments.

Since complement (C') in too high a concentration is cytolytic to splenic lymphocytes, a preliminary experiment was done to determine the optimal volume of reconstituted freeze-dried rabbit serum, the source of C', to be added to the cell suspensions. Spleen cells were prepared from uninfected BALB/c mice in the manner described. Ninety-four % of the cells were viable and 1×10^8 cells were distributed in each of 6 test tubes. To each tube 0,8; 0,6; 0,4; 0,2; 0,1 and 0,05 mℓ rabbit serum was added. The tubes were incubated at 37 °C for 45 min and the viable cells counted again. Twenty-six, 42, 59, 76, 88 and 93 % of cells, respectively, were viable and it was decided to use 0,1 mℓ rabbit serum per 1×10^8 cells in future experiments.

Three aliquots of immune cells were prepared. One was treated with monoclonal antibodies (mAb) against the Lyt-2$^+$ (CD8$^+$) T cell subset (Pierrés, Goridis & Golstein, 1982) and a 2nd aliquot with mAb against the $L_3T_4^+$ (CD4$^+$) subset (Wilde, Marrack, Kappler, Dialynas & Fitch, 1983), produced by the H35.17.2 and GK 1–5 hybridomes, respectively. The supernatants of the hybridome cultures were added to the cell suspensions at a rate of 1–1,5 mℓ/8 $\times 10^6$ cells in the presence of C'. The test tubes were incubated at 37 °C for 45 min, the viable cells counted again and the volumes adjusted so that 2–4 $\times 10^7$ cells were injected i.v. per mouse in 2 groups of 7 mice. The 3rd aliquot of immune cells, incubated in the presence of C' but not exposed to mAb, were injected into a 3rd group of 7 mice. A control group of mice were injected with the spleen cells of uninfected mice incubated in the presence of C'. Twenty-four hours later all 4 groups and an additional control group not previously injected with cells, were challenged with 400 LD_{50} doses of the Welgevonden stock.

In vivo *depletion of T cells*

Twelve groups of 6 mice each were used to determine the effect of anti-$L_3T_4^+$ and anti-Lyt-2$^+$ mAb on the course of *C. ruminantium* on one hand and on the immunity of mice that had made a drug-aided revovery from the infection on the other (Table 4). Groups 1–11 were infected with 400 LD_{50} doses Welgevonden stock and treated with oxytetracyline at a dosage rate of 40 µg/g body mass on Days 9, 10 and 11 p.i. The 31 mice that survived the infection were re-infected 28 days after the last treatment.

Since persistence of *C. ruminantium* may possibly be associated with immunity (Du Plessis, 1982; Wassink, Franssen, Uilenberg & Perié, 1987), it was decided to ascertain the influence of the absence of a persistent infection on the ability of mAb to abolish the immunity *in vivo*. This was done by treating Groups 3, 4, 6 and 7 with gloxazone (dithiosemicarbazone) at a dosage rate of 60 µg/g body mass 27 and 34 days after re-infection. This is the only drug known to sterilize Kümm stock infected carrier mice (Du Plessis, 1982).

Anti-$L_3T_4^+$ and anti-Lyt-2$^+$ culture supernatants were injected i.p. either 2 days before and 5 days after infection (Groups 1 & 2) or after re-infection (Groups 5, 8, 9 & 10) and gloxazone treatment (Groups 3, 4, 6 & 7). The 21 mice in Groups 2–7 were challenged either 7 or 21 days after they had been treated with mAb. Treatment with mAb within 2 days induces a long-lasting depletion of subset T-cells (Cobbold, Jayasuriya, Nash, Prospero & Waldmann, 1984). A control immune group not treated with mAb (Group 11) and a susceptible group were challenged at the same time. Sixteen days after the challenge inoculation, blood was drawn from the orbital sinus of 2 mice of Groups 2–6, pooled, mixed with an equal volume of citrated BLP and 0,2 mℓ inoculated i.v. into 2 susceptible white mice. When the latter failed to show clinical signs their serum was collected a month later and subjected to the IFA test.

Infectivty and the *in vitro* effect of immune serum

In an earlier study (Du Plessis, 1982) to determine the effect of immune serum on the infectivity of the heartwater agent, the role possibly played by complement was not taken into consideration. In order to do so and since there are indications that bovine complement is detrimental to the infectivity of *C. ruminantium* (Du Plessis, Malan & Kowalski, 1987), a preliminary experiment was done to ascertain the amount of rabbit C' detrimental to the infectivity of the Kümm stock.

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TABLE 1 Survival rate, resistance to challenge, tissue infectivity and antibody response of oxytetracycline treated athymic nude mice infected with *C. ruminantium*

No. of mice	Infective LD_{50} dose	Treatment, µg/g body mass (days p.i.)	No. of mice that died (days p.i.)	No. of survivors resistant to challenge	Tissue infectivity	Antibody response
		Kümm stock				
9	2344	40–80 (8, 10 & 12)	7(15–30)	1/1	5/5[3]	0/3
9	234	2(10–30)[1]	8(18–36)	0/1	5/5[3]	2/3 (1:10)[5]
5	23	No treatment	0	0/3[2]	0/5[4]	0/5
		Welgevonden stock				
3	316	80(10); 20(12, 14, 16 & 18)	3(24–26)	—	5/5[3]	0/3
2	316	20(8); 2(14–31)[1]	2(25–36)	—	—	0/2
6	32	20(10); 2(11, 13, 15 & 18)	1(15)	0/3	0/5[4]	0/5
5	3,2	No treatment	0	0/3	0/5[4]	0/5

[1] Every alternate day

[2] 0/3 = not one resistant out of 3 challenged

[3] 5 out 5 mice inoculated with the pooled tissues of 3 moribund nude mice died

[4] Not one out of 5 mice inoculated with the pooled tissues of 5 survivor mice died

[5] IFA test titre of 1:10 in 2 out of 3 moribund mice

One mℓ of reconstituted freeze-dried rabbit serum and one mℓ of a 10-fold dilution thereof in bovine foetal serum (BFS) were added to one mℓ of the Kümm stock infective inoculum and to two 10-fold serial dilutions thereof in BLP. The test tubes were placed in a waterbath at 37 °C for 30 min and 0,2 mℓ per mouse inoculated i.p. into 6 groups of 5 mice.

For the *in vitro* immune serum test, it was decided to use 0,1 mℓ undiluted rabbit serum per 5 mice and a 10^{-2} dilution of the Kümm stock inoculum. Antisera to the Kümm, Ball 3, Breed and Welgevonden stocks were obtained from sheep infected with deep-frozen sheep blood infected with the above stocks as previously described (Du Plessis, Van Gas, Olivier & Bezuidenhout, 1989). The sheep were treated with oxytetracycline on the 3rd day of the febrile reaction and bled 4 weeks after infection. The sera were subjected to the IFA test and stored at −18 °C.

A volume of 0,25 mℓ of each of the 4 antisera was added to 0,65 mℓ BFS, 0,1 mℓ freeze-dried rabbit serum and 1 mℓ of a 10^{-2} dilution Kümm stock. The mixtures were incubated in a waterbath at 37 °C for 30 min and 0,4 mℓ per mouse inoculated i.p. into 4 groups of 5 mice. A control group was inoculated with the infective inoculum incubated in the presence of a negative serum, C' and BFS.

In vitro effect of immune serum in the presence of macrophages

To ascertain the *in vitro* effect of immune serum on the infectivity of the heartwater agent in the presence of macrophages, mice were infected with the Kümm stock, treated and re-infected. Eight weeks after re-infection, the peritoneal macrophages from 5 immune mice were collected in Eagle's medium with heparin (200 IU/mℓ) as previously described (Du Plessis, 1982). The cells were centrifuged at 1 500 r.p.m. for 5 min, resuspended in 4 mℓ Eagle's medium and counted. Non-immune peritoneal macrophages were collected from 5 susceptible mice in a similar manner.

A volume of 0,25 mℓ of the anti-Kümm sheep serum used in the previous experiment was added to a volume of macrophages containing 10×10^6 cells, 0,1 mℓ rabbit serum (C'), 0,75 mℓ BFS and 1 mℓ of a 10^{-2} dilution Kümm stock. In this manner combinations of immune and non-immune serum and immune and non-immune macrophages were composed (Table 7). The mixtures, including a control group consisting of infective inoculum, C' and 0,9 mℓ BFS, were incubated at 37 °C for 30 min. The cells were centrifuged at 1 500 r.p.m. for 5 min, taken up in 1 mℓ Eagle's medium and 0,2 mℓ per mouse inoculated i.p. into 5 mice per group. The infective inoculum control tube was not centrifuged and 0,4 mℓ per mouse inoculated.

Persistence of Welgevonden stock in immune mice

Ninety-five mice were inoculated i.v. with 400 LD_{50} doses of the Welgevonden stock and treated with oxytetracycline at a dosage rate of 40 µg/g body mass on Day 11. The 67 mice that survived the infection, were re-infected 40 days later. Twenty-two of the 43 mice that survived the re-infection were treated i.p. with gloxazone at a dosage rate of 60 µg/g body mass 40 and 47 days after re-infection.

TABLE 2 Susceptibility of nude mice to *Cowdria*

C. ruminantium stock	LD_{50} dose	Mice mortality score	
		Euthymic	Athymic
Kümm	23	4/5*	0/5
Welgevonden	3,2	3/5	0/5

* 4 out of 5 mice inoculated died

TABLE 3 Immunization of donor mice and adoptive transfer of spleen cells to recipient mice

Exp. No.	Donor mice					Recipient mice
	Days p.i. of transfer	LD$_{50}$ infective dose/mouse	Days p.i. treated	Reciprocals of IFA test titres	No. of cells/ mouse	Interval between injection of cells and *Cowdria*
1	10 20	4	No treatment	–ive	1×10^8	1 h
2	10 15 21	400	9, 10, 16 17 & 18	10(3/5)[1] 80(5/5) 1 280(5/5)	1×10^8	1 h
3	30 days after re-infection	400	8 & 10	1 280	$2-4 \times 10^7$	24 h

[1] 10(3/5) = 3 out of 5 mice had IFT titres of 1:10

TABLE 4 Anti-L$_3$T$_4^+$ and anti-Lyt-2$^+$ mAb *in vivo* depletion of T cells; survival of mice both after infection and challenge with *C. ruminantium*

Group No.	mAb intraperitoneally			Mouse mortalities		
	anti-L$_3$T$_4$	anti-Lyt-2	Gloxazone	After infection	Challenge, days after mAb	
					7	21
1	0,3/2/pre.i.[1] 0,3/5/p.i.[2]	–	–	1(21); [4] 5 (24)	–	–
2	–	0,3/2/pre.i. 0,3/5/p.i.	–	2(21); 2(23)	0/2	1
3	1/60/p.r.i.[3] 1/65/p.r.i.	–	+	1(20)	0/5[5]	–
4	1/50/p.r.i. 1/56/p.r.i. 1/65/p.r.i.	–	+	1(10); 1(22)	–	0/4
5	1/60/p.r.i. 1/65/p.r.i.	–	–	1(23)	0/5	–
6	–	1/60/p.r.i. 1/65/p.r.i.	+	2(25)	0/4	–
7	–	1/50/p.r.i. 1/56/p.r.i. 1/65/p.r.i.	+	2(20); 3(24)	–	0/1
8	–	1/60/p.r.i. 1/65/p.r.i.	–	1(11); 4(22) 1(23)	–	–
9	1/60/p.r.i.	–	–	1(20); 1(22)	4 survivors not shallenged, no mortalities	
10	–	1/60/p.r.i. 1/65/p.r.i.		1(11); 3(21); 1(23)	1 survivor not challenged, no mortality	
11	Infected controls not treated with mAb			1(22)	0/5	–
12	Non-immunized challenge controls				6/6	–

[1] 0,3 mℓ culture supernatant i.p. 2 days pre-infection
[2] 0,3 mℓ culture supernatant i.p. 5 days post-infection
[3] 1 mℓ culture superatant i.p. 60 days post-re-infection
[4] 1 mouse died 21 days post-infection
[5] Not 1 mouse out of 5 challenged, died

One month after the gloxazone treatment and at intervals of 3 months thereafter (Table 8), 2 gloxazone- and 2 non-gloxazone-treated immune mice were killed and their pooled hearts and lungs homogenized in BLP on a 10 % mass/volume basis. Five susceptible mice were inoculated i.v. with 0,2 mℓ of the homogenate. The sera of the 2 mice were pooled for the IFA test. At the same time 2 gloxazone-treated immune, 2 non-gloxazone-treated immune and 5 control mice were challenged i.v. with the homologous stock.

RESULTS

Athymic nude mice

The survival rate of nude mice infected with the Kümm and Welgevonden stocks and subsequently

treated, is shown in Table 1. It can be seen that irrespective of the dosage rate and the days p.i. on which treatment was given, very few mice survived. A single dose of 20 µg/g followed by low dosages of oxytetracycline (2 µg/g body mass) over a long period to mice infected with 32 Welgevonden LD_{50} doses did facilitate the survival of 5 out of 6 mice, but these mice were susceptible to challenge, they were sero-negative and the sub-inoculation of their tissues was negative. A high enough dosage of oxytetracycline given long enough enabled nude mice to survive, but death invariably intervened as soon as treatment was discontinued. In the case of both stocks, mice injected with sub-lethal doses survived unaided by treatment and without showing any clinical signs. Once again, however, these mice were susceptible to challenge, sero-negative and the sub-inoculation of their tissues negative.

Only trace amounts of antibody were detectable with the IFA test in the sera of 2 mice infected with the Kümm stock 36 days earlier. One of them died as soon as treatment was discontinued and the other survived but was fully susceptible to challenge.

It can be seem from Table 2 that in the case of both the Kümm and the Welgevonden stock athymic nude mice survived infection with numbers of organisms that were lethal to their euthymic littermates. Nude mice are therefore less susceptible to *C. ruminantium* than euthymic mice.

T cell-mediated immunity

Adoptive transfer of immunity

The transfer of spleen cells of donor mice inoculated with a low dose of the Welgevonden stock failed to render recipient mice more resistant to challenge (Fig. 1). The mortality rate of mice injected with the cells collected from donor mice infected either 10 or 20 days earlier was no different from that of mice injected with the cells of non-immunized control mice and that of mice inoculated with the challenge inoculum.
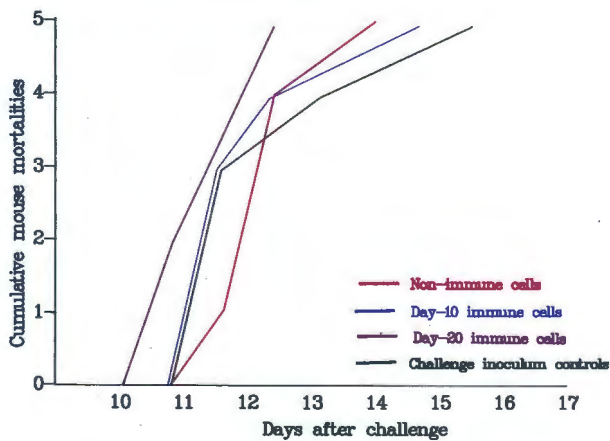

FIG. 1 Challenge mortality rate of BALB/c mice injected with spleen cells collected on Days 10 and 20 p.i. from donor mice infected with a low dose of *C. ruminantium*

The mortality rate of recipient mice injected with the cells of immune donor mice infected with a 100-fold higher infective dose and treated 5 times (Table 3), was also no different from that of the 2 control groups, irrespective of whether the donor mice were killed 10 or 15 days after infection (Fig. 2). The mortalities of the mice injected with cells collected 21 days p.i., however, occurred markedly later than those of the 2 control groups. The rise in antibody
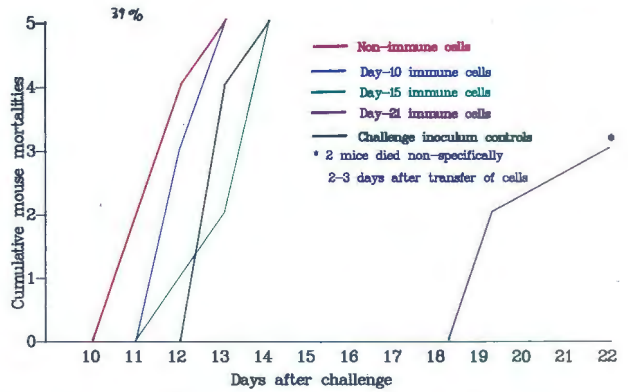

FIG. 2 Challenge mortality rate of BALB/c mice injected with spleen cells collected on Days 10, 15 and 21 p.i. from donor mice immunized with a single infective dose of 400 LD_{50}

titres over the course of 11 days (Table 3) suggests that the donor mice sacrificed 10 and 15 days p.i. had not yet mounted any significant immune response.

In the 3rd experiment the transfer of spleen cells from donor mice infected with the same Welgevonden dose, but treated a day earlier and re-infected subsequently, rendered 3 out of 7 mice resistant to challenge and retarded the mortality rate of those that eventually died (Fig. 3). Apart from re-infection of the donor mice, this experiment also differed from Exp. 2 in that the recipient mice were challenged 24 h in stead of 1 h after the cell transfer (Table 3).

In vitro depletion of T cells

The donor mice used in Exp. 3, the cells of which facilitated a successful adoptive transfer, were also used to prepare the immune spleen cells that were exposed to mAb and a control aliquot. Viable cell counts showed that, compared to the number of immune spleen cells incubated in the presence of C' but not exposed to mAb, there was a reduction of 41 % after depletion with anti-$L_3T_4^+$ mAb and 17 % after depletion with anti-Lyt-2^+ mAb. Since normal spleen cell populations consist of 43–47 % $L_3T_4^+$ and 19–23 % Lyt-2^+ T cells (Baldridge, Barry & Hinricks, 1990), it is evident that substantial numbers of specific T cells had been eliminated during the depletion process.

Immune spleen cells depleted of Lyt-2^+ T cells were unable to protect recipient mice, the mortality rate of which was no different from that of the control group given non-immune cells and that of the control mice inoculated with the challenge inoculum (Fig. 3). The ability of the spleen cells to confer
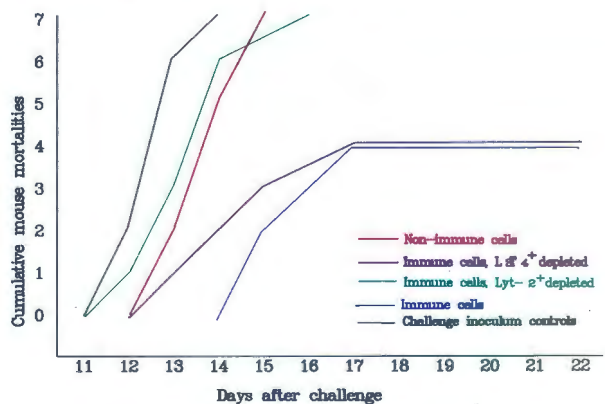

FIG. 3 Challenge mortality rate of mice injected with immune spleen cells depleted of $L_3T_4^+$ and Lyt-2^+ T cells and challenged with *C. ruminantium*

TABEL 5 The effect of complement on the infectivity of the Kümm stock of *C. ruminantium*

		Dilution of Kümm stock inoculum		
		10⁻¹	10⁻²	10⁻³
Complement	0,2	5/5[1]	3/5	0/5
mℓ/5 mice	0,1	5/5	5/5	3/5

[1] 5/5 = 5 out of 5 mice died

TABLE 6 The *in vitro* effect of immune serum and complement on the infectivity of the Kümm stock of *C. ruminantium*

Group	*C. ruminantium* stock antiserum	IFA titre of antiserum	Mouse mortality
1	Kümm	1: 20 480	5/5(13-16)[1]
2	Ball 3	1: 5 120	5/5(13-14)
3	Breed	1: 1 280	5/5(12-16)
4	Welgevonden	1: 1 280	5/5(13-14)
5	-ive control serum		5/5(14)

[1] 5/5 = 5 out of 5 mice died 13–16 days p.i.

TABLE 7 The *in vitro* effect of immune serum and complement on the infectivity of the Kümm stock in the presence of immune and non-immune peritoneal macrophages

Group	Immune serum	Non-immune serum	Immune macrophages	Non-immune macrophages	Mouse mortality
1	+		+		0/5
2	+			+	1/5[1]
3		+	+		2/5
4		+		+	5/5
5	Infective inoculum control				5/5

[1] 1 out of 5 mice died

protection was, however, not affected when they were depleted of the $L_3T_4^+$ subset and, as in the case of the immune cells not exposed to mAb, 3 out of 7 recipient mice were resistant to challenge and the mortality of the other 4 retarded.

In vivo *depletion of T cells*

Attempts to inhibit the immune response or abolish the immunity of mice by injecting them i.p. with mAb were unsuccessful (Table 4). Although all 6 mice given anti-$L_3T_4^+$ and 4 out of 6 given anti-Lyt^{2+} mAb 2 days before infection died 21–24 days p.i., possibly suggesting that they were unable to mount an immune response, 5 (Group 7), 6 (Group 8) and 5 (Group 10) out of 6 mice not treated with mAb, also succumbed at some stage 20–24 days p.i., i.e. 9–13 days after treatment on Day 11. The repeated injection of mAb to immune mice did not abrogate their immunity to challenge. Not a single mouse out of 21 challenged, showed clinical signs or died, whereas 6 out of 6 non-immunized challenged controls died.

There was also no indication of the replication of the heartwater agent introduced in the challenge inoculum, since none of the mice inoculated with blood drawn from mAb treated immune mice 16 days after challenge, succumbed and no antibody could be detected in their sera. Five immune mice (Groups 9 & 10) not treated with gloxazone and not challenged remained healthy for months after having been injected with mAb.

It is also clear from Table 4 that sterilization of the infection with gloxazone before the administration of mAb (Groups 3, 4, 6 & 7) had no influence on the outcome of the challenge.

Infectivity and *in vitro* effect of immune serum

It can be seen from Table 5 that the inhibitory effect of C' on the infectivity of the Kümm stock was determined by the concentrations of both *C. ruminantium* and the C'. A volume of 0,2 mℓ rabbit serum reduced the infectivity of the 10⁻² inoculum dilution so that only 3 out of 5 mice died, whereas a volume of 0,1 mℓ dit not. To ensure 100 % mortality in the presence of 0,1 mℓ rabbit serum, a 10⁻² inoculum dilution was chosen to determine the effect of immune serum of the Kümm stock.

Antisera with high IFA antibody titres to several stocks of *C. ruminantium* had no inhibitory effect on the infectivity of the Kümm stock (Table 6). Without a single exception, all 5 mice of all 5 groups died. Not even in the case of the homologous Kümm stock antiserum were the mortalites retarded.

The *in vitro* effect of immune serum in the presence of macrophages

The anti-Kümm immune serum that had no effect on the infectivity of *C. ruminantium* in the *in vitro* test excerted a distinct inhibitory effect not only when it was placed in contact with the heartwater agent in the presence of immune peritoneal macrophages (Group 1, Table 7), but also in the presence of non-immune macrophages (Group 2). Only 2 out of 5 mice inoculated with a non-immune serum and immune cells died (Group 3), but when this serum was incubated in the presence of non-immune macrophages, it had no inhibitory effect (Group 4), as was the case with the infective inoculum control group (Group 5).

Persistence of Welgevonden stock in immune mice

Three out of 5 mice inoculated with tissues from immune mice 2 months after immunization, died, whereas no mortalities occurred in mice inoculated with the tissues of immune mice treated with gloxazome (Table 8).

TABLE 8 Persistence of Welgevonden stock of *C. ruminantium* in mice and their resistance to homologous challenge

Interval in months after gloxazone treatment	Tissue sub-inoclation		Reciprocal of IFA test titre		Resistance to challenge		
	GTI[1]	NGTI[1]	GTI	NGTI	GTI	NGTI	Susceptible controls
1	0/5	3/5[2]	1 000	1 000	0/2	0/2	5/5
4	0/5	0/5	ND	ND	0/2	0/2	5/5
7	0/5	0/5	100	100	1/2	0/2	5/5
11	ND[3]	ND	ND	ND	1/2	0/2	5/5
13	ND	ND	10	10	2/2	2/2	5/5

[1] GTI = Gloxazone treated immune mice

NGTI = Non gloxazone treated immune mice

[2] 3/5 = 3 out of 5 mice inoculated died

[3] ND = not done

Three months later, however, no mortalities occurred amongst recipient mice inoculated with tissues from non-gloxazone-treated mice.

Until 4 months after having been treated with gloxazone, immune mice were fully resistant to homologous challenge (Table 8). At 7 and 11 months after gloxazone treatment, however, 1 out of 2 gloxazone-treated immune mice succumbed to challenge, whereas those not treated were immune. It was only at the 13 month interval that all the mice, irrespective of whether they had been treated with gloxazone, succumbed to challenge. Mice immune to the Welgevonden stock are therefore resistant to challenge for a year after immunization, but only for 5 months if sterilized from a persistent infection.

DISCUSSION

In an earlier study it has been found that immune serum, irrespective of whether it was placed in contact with the Kümm stock of C. ruminantium or injected into mice prior to or after infection, had no effect on the course of the disease (Du Plessis, 1982). Since the influence of C' was not taken into consideration in this earlier work, the in vitro test was repeated in the present study with addition of freeze-dried rabbit serum as a source of C'. Once again no inhibitory effect could be demonstrated with the sera of sheep containing high levels of antibody against several stocks of C. ruminantium, suggesting that C' has no direct lytic effect on the heartwater agent through the antibody-dependent pathway.

Peritoneal macrophages incubated in the presence of immune serum, however, clearly reduced the infectivity of the heartwater agent. This is not surprising in the case of peritoneal cells collected from immune mice, since 80,5% of mice inoculated with peritoneal macrophages from re-infected immune mice survived, whereas all those inoculated with cells from infected susceptible mice died (Du Plessis 1982). The fact that in the present study mice inoculated with non-immune macrophages incubated in the presence of an immune serum, C. ruminantium and complement were resistant to challenge, however, suggests that antibodies do play a role, possibly having an opsonic effect. Further clarification is required, because the question arises why the same immune serum did not have the same effect in the in vitro situation where, in the absence of macrophages, the heartwater agent coated with antibody was injected into the peritoneal cavity where macrophages abound. Furthermore, the only difference between the present finding and that in an earlier study when the infectivity of the Kümm stock was unchanged after having been incubated in the presence of an immune serum and non-immune macrophages (Du Plessis, 1982), was the presence of complement in the former.

Observations made on athymic nude mice on one hand and the adoptive transfer of immunity in BALB/c mice on the other, lend further support to the concept that in heartwater immunity is T cell-mediated (Du Plessis, 1982). Although thymic rudiments have been demonstrated in congenital athymic nude mice (Loor & Roelants, 1974) and a high frequency of T-lineage lymphocytes in their spleens (Holub, Rossman, Tlaskalova & Vidmarova, 1975), it is accepted that these mice are devoid of functional T lymphocytes which are required for the expression of cell-mediated immunity (Pantelouris, 1971). The inability of nude mice to recover from and mount an immune response against both the Kümm and the Welgevonden stocks, even if they are treated, is therefore supportive evidence that in heartwater the immunity is T cell-mediated.

Although C. ruminantium behaves much like several other intracellular parasites in nude mice, there are some important differences. Whereas in the case of Rickettsia akari (Kenyon & Pedersen, 1980), Coxiella burnetii (Kishimoto, Rozmiarek & Larson, 1978) and Listeria monocytogenes (Newborg & North, 1980), nude mice eventually make a drug-aided recovery and survive as carriers, C. ruminantium-infected nude mice survive as long as they are treated but invariably succumb as soon as treatment is withdrawn. High antibody titres were recorded in nude mice infected with R. akari and C. burnetii, whereas in C. ruminantium-infected mice only trace amounts of antibody were rarely detectable. The production of chronically infected nude mice was possible in the case or R. akari (Kenyon & Pedersen, 1980) and L. monocytogenes (Emmerling, Finger & Bockemühl, 1975), but not in the case of Cowdria.

The constantly fatal course of C. ruminantium infection in nude mice cannot be ascribed to a particularly high susceptibility, since in the case of both the Kümm and the Welgevonden stocks, nude mice withstood a dose that was lethal to their euthymic littermates. This is not an unprecedented phenomenon, since nude mice also have an unexpected heightened resistance to experimental infection with L. monocytogenes (Emmerling et al. 1975) and Brucella abortus (Cheers & Walker, 1975), in all probability as a result of naturally occurring activated macrophages in nude mice (Nickol & Bonventre, 1977). It is therefore much rather the lack of functional T cells that renders nude mice incapable of mounting an immune response against Cowdria.

The adoptive transfer of immunity with immune spleen cells further supports a T cell mediated immunity. The observation that almost 50 % of the recipient mice withstood challenge after having been injected with cells from re-infected immune mice, whereas there was only a retardation in the eventual mortality of the mice injected with cells from donor mice not fully recovered from a single infection 21 days earlier, needs to be explained. If one considers that the mortality rate of mice given cells harvested 10 and 15 days p.i. was no different from that of mice injected with non-immune spleen cells, it would seem that unless during the immunization process the donor mice develop the disease with ample replication of the heartwater agent and the infection running its full uninterrupted course, their spleen cells are unable to confer protection to the recipient mice. It is uncertain whether the fact that in one case the infective inoculum was inoculated within an hour after the cells and in the other 24 h thereafter, could have played a role, since the spleen cells of mice immune to L. monocytogenes confer resistance in recipient mice to a challenge inoculum given within an hour after the transfer of the cells (Berche, Gaillard & Sansonetti, 1987). The finding that the complement-mediated depletion of specific subset T cells abrogated the ability of immune spleen cells to confer protection to recipient mice, prove conclusively that immunity in heartwater is largely cell-mediated. It was found that the Lyt-2$^+$ and not the L$_3$T$_4^+$ T cell subset played a major role in the immune response, because adoptive transfer was still readily achieved with L$_3$T$_4^+$ depleted spleen cells.

Lyt-2$^+$ T cells also play a major role in the acquired cellular resistance of mice infected with *L. monocytogenes,* while the L$_3$T$_4^+$ T cells are responsible for the delayed-type hypersensitivity (Berche, Decreusefond, Theodorou & Stiffel, 1989; Baldridge *et al.,* 1990). Furthermore, mice infected with *Trypanosoma cruzi* developed higher parasitaemias and increased or earlier mortality when depleted of Lyt-2$^+$ T cells before infection (Tarleton, 1990). In mice infected with *Mycobacterium bovis,* however, L$_3$T$_4^+$ depletion led to a dramatic increase in the number of viable bacteria, while Lyt-2$^+$ depletion had no significant effect (Pedrazzini, Hug & Louis, 1987).

Two major funtions have been attributed to Lyt-2$^+$ T cells, suppression and cytotoxicity. In addition, this subset of T cells is the source of lymphokines, although they are probably less important in this role than L$_3$T$_4^+$ T cells (McGuire, Yang & Rothenberg, 1988). While cytotoxicity against *C. ruminantium*-infected cells has as yet not been demonstrated in heartwater immunity (Stewart, 1987b), it is known that immune macrophages play an important role in this respect (Du Plessis, 1982). Should macrophage-activating lymphokines be involved in heartwater immunity, Lyt-2$^+$ T cells may well be the source of the lyphokines.

Electron microscopy has shown that at no stage during the developmental cycle in macrophages do limiting membranes separate *C. ruminantium* inclusions from the cytoplasm of the host cell (Du Plessis, 1975). The absence of *Cowdria*-containing endosomes is therefore reconcilable with the cytosolic pathway of antigen processing, in which antigens and micro-organisms in the cytoplasm of antigen-presenting cells are processed outside endosomes with the participation of the endoplasmic reticulum and the Golgi complex. This process favours a T cell-mediated immune response and in particular the activation of Lyt-2$^+$ T cells (Yewdell & Bennink, 1990) and is therefore consistent with the major role attributed to these cells in this study.

It is not clear why the *in vivo* depletion of Lyt-2$^+$ cells failed to affect either the course of the infection or the resistance of immune mice to challenge. There are differences between the successful *in vitro* and the unsuccessful *in vivo* situations and there are also several unknown facts. A crucial unknown factor is the lifespan of specifically activated, immune macrophages. If it exceeds 3 weeks, it is conceivable why immune mice depleted of T cells were still resistant to challenge at this interval. In a previous adoptive transfer experiment mice were still resistant to challenge 2 months after the i.p. transfer of spleen cells from mice immune to the Kümm stock (Du Plessis, 1982). Although the depletion of T cells may have been satisfactory in the present study, it may be that the residual capacity of the macrophages to have withstood the challenge was still adequate. The crux of the matter may well be that in the *in vitro* setup naive, unconditioned macrophages, the site of the primary, uninhibited growth of *Cowdria,* are temporarily alerted and activated to avert a single challenge, favourably timed for the defense of the recipient mice. The *in vivo* setup is different: by eliminating specific T cells, an attempt is made to prevent the activation and conditioning of a new generation of macrophages on the uncertain assumption that existing immune macrophages no longer protect.

Adoptive transfer of immunity was achieved much more readily by introducing both the spleen cells of mice immune to the Kümm stock and the challenge inoculum into the peritoneal cavity (Du Plessis, 1982). Contrary to the i.v. route used in the present study, the contact between the donor T cells and a large number of macrophages in a confined cavity was perhaps conducive to the conditioning of a large population of cells. Likewise, the injection of the challenge inoculum along the i.p. route exposed the full dose to a large number of activated, hostile macrophages in a confined space, whereas along the i.v. route the organisms are distributed much more widely.

The question of whether immunity in heartwater is associated with persistence of *C. ruminantium* has often been raised (Du Plessis, 1982; Stewart, 1987a; Wassink *et al.,* 1987). In the present study it was found that the Welgevonden stock was demonstrable in the tissues of mice sub-inoculated into susceptible mice 2 months after re-infection of the donor mice, but not 4 months thereafter. This agrees with what was found with the Kwanyanga stock (Wassink *et al.,* 1987), but differs from the observation in mice infected with the Kümm stock that tissues collected from immune mice up to a year after infection were still infective (Du Plessis, 1982).

It does not seem that these widely varying periods of persistence are related to the differences in pathogenicity to mice of the different *C. ruminantium* stocks. On one hand the Kwanyanga, Kümm and Welgevonden stocks are highly pathogenic to mice (Du Plessis *et al.,* 1989), but on the other the sub-inoculation of tissues from mice infected with the Senegal stock, which is infective but not pathogenic to mice, caused fatal heartwater in a goat a year later (Wassink, Jongejan, Gruys & Uilenberg, 1990).

Perhaps the method of subinoculating tissues of immune animals is not sensitive enough to detect a persistent infection. Whereas the heartwater agent could be demonstrated for only 35 (Alexander, 1931) and 50 days (Ilemobade, 1978) after infection in the blood of sheep sub-inoculated into susceptible sheep and goats, respectively, *Amblyomma hebraeum* adult ticks that had fed as uninfected nymphae on sheep and cattle 223 and 246 days after infection, respectively, transmitted *C. ruminantium* to susceptible sheep (Andrew & Norval, 1989). There remains little doubt therefore that mice can be carriers for as long as a year and domestic ruminants for as long as 8 months.

There is conflicting evidence as to whether the duration of immunity in heartwater is dependent on the persistence of *C. ruminantium.* Against this view on one hand was the finding in the present study that persistence could be demonstrated for only 2 months p.i., while immunity lasted for 11 months. Wassink *et al.* (1987) also found that whereas mice remained immune for at least 4 months, the heartwater agent could not be demonstrated in immune mice for the same period of time. On the other hand, mice were no longer immune 7 months after having been treated with gloxazone, whereas mice not treated were still immune 11 months after infection and only became susceptible 13 months p.i. Furthermore, gloxazone-treated immune sheep lost their immunity much sooner than untreated immune controls (Du Plessis, 1981).

The discrepancy between the persistence of infection and the duration of immunity can probably be attributed to the combined effect of a residual

immunity after the disappearance of *C. ruminantium* from the host endothelial cells and the lack of sensitivity of the sub-inoculation technique. If this is true, findings in this study suggest that immunity in heartwater is dependent on the persistence of *C. ruminantium*.

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