

Experimental *Schistosoma bovis* Infections in Goats

Studies on the Host-Parasite Relationship with Special Reference to
Immunoregulatory Effects and Immunopathology

Kaisa Sörén

*Faculty of Veterinary Medicine and Animal Science
Department of Biomedical Sciences and Veterinary Public Health
Uppsala*

Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2009

Acta Universitatis Agriculturae Sueciae

2009:17

Cover: A West African Dwarf goat (Photo: Lena Sörén Plantin). Transverse section of a *Schistosoma bovis* worm pair in an intestinal vein (Photo: Kaisa Sörén). Picture created by Leif Sörén.

ISSN 1652-6880

ISBN 978-91-86195-64-9

© 2009 Kaisa Sörén, Uppsala

Tryck: SLU Service/Repro, Uppsala 2009

Experimental *Schistosoma bovis* Infections in Goats. Studies on the Host-Parasite Relationship with Special Reference to Immunoregulatory Effects and Immunopathology.

Abstract

The ruminant trematode *Schistosoma bovis* occurs endemically mainly in Africa and the Middle East, where a considerable negative impact of infection on production has been appraised. The pathological effects in schistosomosis are due to immune responses of the host to the eggs of the parasite, leading to intestinal and hepatic granulomatous disease and hepatic fibrosis. Ruminant schistosomosis is most often chronic under natural transmission conditions, and development of an acquired resistance has been proven in cattle and goats. Several aspects of chronic *S. bovis* infection such as duration of resistance to challenge infection, and effects of anthelmintic treatment on immunoregulatory events and regression of infection with increasing time after treatment, have not been explored previously. Although perioval granulomas are key factors in disease caused by schistosome infections, knowledge on the immunopathology of granulomatous inflammation in ruminant *S. bovis* infection is very limited.

The overall aim of this thesis was to gain further knowledge on the host-parasite relationship in ruminant schistosomosis by use of the *S. bovis*/West African Dwarf goat model. Parasitological data from experimental infections revealed that resistance to both primary and challenge infection persisted in long-standing infection, with suppression of the female worm fecundity as the key effector mechanism. Furthermore, resistance to challenge was not abolished by anthelmintic elimination of the primary infection. The fact that host immune responses, as reflected by humoral anti-worm antibodies and histopathological immune cell reactions, persisted both in very late primary infection and after treatment, may explain the sustained anti-fecundity effect observed. Quantification of cytokine gene expressions suggested a role for the Th1 type cytokines IFN- γ , IL-2 and TNF- α in the inflammation of the intestine at 13 weeks of infection, and indicated that IFN- γ may be produced by cells in the *S. bovis* egg granuloma.

Keywords: *Schistosoma bovis*, goats, granuloma, chronic infections, praziquantel, resistance, serology, immunopathology, cytokines

Author's address: Kaisa Sören, Department of Biomedical Sciences and Veterinary Public Health, SLU, Box 7028, 750 07 Uppsala, Sweden
E-mail: Kaisa.Soren@bvf.slu.se

To My Girls

Contents

List of Publications	7
Abbreviations	8
1 Introduction	9
1.1 General background	9
1.2 <i>Schistosoma bovis</i>	10
1.3 Life cycle	11
1.4 Pathogenesis and clinical signs	13
1.5 Pathology	14
1.5.1 Gross pathology	14
1.5.2 Histopathology	15
1.6 Immune responses	17
1.6.1 Resistance	17
1.6.2 Humoral responses	17
1.6.3 Immunopathology	18
1.7 Diagnosis	20
1.8 Treatment and control	21
1.8.1 Treatment	21
1.8.2 Vaccines	22
1.8.3 Water hygiene	23
1.9 The <i>S. bovis</i> /goat model	24
2 Aims of the thesis	27
3 Material and methods	29
3.1 Animals and experimental designs	29
3.1.1 Experiment I (Papers I-II)	29
3.1.2 Experiment II (Paper III)	29
3.2 Parasites and experimental infections (Papers I-III)	30
3.3 Treatment (Papers I and II)	30
3.4 Clinical pathology (Paper I)	31
3.5 Serology (Paper II)	31
3.6 Fecal egg counts (Papers I and III)	31
3.7 Perfusion for worm recovery (Paper I)	31
3.8 Tissue egg counts (Paper I)	32
3.9 Gross liver pathology (Paper I)	32
3.10 Necropsy and histopathological laboratory procedures (Papers II-III)	33

3.11	Histopathological examination (Papers II and III)	33
3.12	Immunohistochemistry (Paper III)	34
3.13	Quantitative real-time reverse transcriptase polymerase chain reaction (Paper III)	35
3.14	Statistical methods (Papers I-III)	36
4	Results and general discussion	39
4.1	Experiment I (Papers I-II)	39
4.1.1	Long-standing infection	39
4.1.2	Treatment effects	42
4.1.3	Resistance to challenge infection	43
4.2	Experiment II (Paper III)	44
4.2.1	Fecal egg excretion and histopathology	44
4.2.2	Immunohistology	45
4.2.3	Cytokine responses	46
5	General conclusions	51
6	Suggestions for future research	53
7	References	55
8	Acknowledgements	67

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Monrad, J., Sörén, K., Johansen, M. V., Lindberg, R. and Ørnbjerg, N. (2006). Treatment efficacy and regulatory host responses in chronic experimental *Schistosoma bovis* infections in goats. *Parasitology*, 133, 151-158.
- II Sörén, K., Monrad, J., Johansen, M. V. and Lindberg, R. (2008). Persistent immune responses in late infection and after treatment in experimental *Schistosoma bovis* infections in goats. *Research in Veterinary Science*, article in press, doi:10.1016/j.rvsc.2008.10.006.
- III Sörén, K., Monrad, J., Tallkvist, J., Hurst, M. and Lindberg, R. (2009). Experimental *Schistosoma bovis* infection in goats: A study of cytokine gene expression and immune cell phenotypes in the small intestine and mesenteric lymph nodes (manuscript).

Papers I-II are reproduced with the permission of the publishers.

Abbreviations

Ab	Antibody
ANOVA	Analysis of variance
CD	Cluster of differentiation
C _t	Threshold cycle
ELISA	Enzyme-linked immunosorbent assay
epgf	Eggs per gram feces
FFPE	Formalin-fixed paraffin-embedded
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
MHC	Major histocompatibility complex
mRNA	Messenger RNA
pi	Post infection
qRT-PCR	Quantitative real-time reverse transcriptase polymerase chain reaction
RNA	Ribonucleic acid
Sb28GST	<i>Schistosoma bovis</i> 28-kilodalton glutathione S-transferase
Th	T helper
TNF	Tumor necrosis factor
WC	Workshop cluster
ZSF	Zinc salts fixative

1 Introduction

1.1 General background

Schistosomiasis¹, also called Bilharziasis after Theodor Bilharz who first described the disease in 1851, is a snail-borne parasitic disease of large public- and animal health importance in endemic countries. The disease is restricted to subtropical and tropical countries and is estimated to affect the health of around 200 million people in countries in Africa, Asia, the Middle East and South America (Chitsulo et al., 2000). Humans and animals get infected when coming into contact with snail infested waters, where the cercarial stage of the parasite is allowed to penetrate the skin (Malek, 1980).

Schistosomes are dioecious trematode worms of the family Schistosomatidae. The name schistosoma originates from the Greek words for split (schistos) and body (soma), referring to the ventral longitudinal cleft, the gynecophoric canal, of the male worm in which the female worm is carried during copulation and movement. A characteristic feature of schistosomes is that the female worm is longer and more slender than the male worm. The life cycle of schistosomes is indirect, sexual reproduction taking place in the definitive mammalian host and asexual reproduction in the intermediate fresh-water snail host. Adult schistosomes live in pairs in the mesenteric veins of the mammalian host, where the female worms deposit their eggs. (Malek, 1980). Pathological effects in schistosomiasis are

¹ In this thesis, schistosome infection in humans is referred to as 'schistosomiasis', according to recommendations by the World Health Organization. For infection in animals, the term 'schistosomosis' is used, according to recommendations by the World Association for the Advancement of Veterinary Parasitology (Kassai et al., 1988).

mainly due to immune responses to the eggs, leading to perioval granuloma formation and hepatic fibrosis (Taylor, 1987).

There are several species of the genus *Schistosoma*, affecting different mammalian hosts. *Schistosoma mansoni*, *Schistosoma haematobium* (located to the urogenital veins instead of mesenteric veins) and *Schistosoma japonicum* are the major species infecting humans, *S. japonicum* also infecting several animal species, thus being a zoonotic schistosome (Cheever and Neafie, 2000). The most important ruminant schistosomes are *Schistosoma bovis* and *Schistosoma mattheei* (Taylor, 1987).

1.2 *Schistosoma bovis*

The first description of *S. bovis* was made by Sonsino (1876), who described the parasite in a bull from Egypt. It has been suggested that African *S. bovis* originated in the Mediterranean area after 668 A.D. and that it was widely spread by the migration of domestic animals with nomads into northeast Africa, and continued gradually to the south via the eastern coast of Africa (Faulkner and Epstein, 1957; Dinnik and Dinnik, 1965). Today, *S. bovis* is present in large parts of Africa, mainly in the eastern countries from Sudan southward to the northern part of Zambia, but it can also be found in the northern, western and central parts of Africa. Outside Africa, *S. bovis* has been found in several Mediterranean countries and the Middle East (Pitchford, 1977).

Together with *S. haematobium* and *S. mattheei*, *S. bovis* belongs to the species complex of 'terminal-spined' schistosomes, which means that they all have eggs that possess a terminal spine, in contrast to e.g. *S. mansoni* eggs that have a lateral spine (Malek, 1980).

Naturally acquired *S. bovis* infections have been described in cattle, sheep, goats, camels, dromedars, horses, donkeys, pigs, several wild bovines and also various rats and mice (Pitchford, 1977). From the animal husbandry point of view, schistosomosis bovis in cattle, sheep and goats play the most important role.

Ruminant *S. bovis* infection is most often chronic under natural transmission conditions (Taylor, 1987). Although death can occur in heavily infected animals, the most common outcome of the disease is weight loss and reduced growth (Dargie, 1980; Saad et al., 1980; McCauley et al.,

1983). However, it has been shown that there are species differences in the severity of disease, and goats become more severely affected clinically than both cattle and sheep (Massoud, 1973; Saad et al., 1984 a). A negative impact of infection on production has been appraised, particularly in cattle (McCauley et al., 1984; Makundi et al., 1998), leading to major economic losses in infected herds. The impact of infection on production is probably still underestimated, but more attention is bound to be directed towards this disease as small ruminants are important for smallholder farmers in African countries, and improved livestock husbandry by these farmers is a strategy for reaching the United Nations Millennium Development Goal to end poverty and hunger (www.un.org/millenniumgoals).

1.3 Life cycle

A schematic drawing of the *S. bovis* life cycle is shown in Fig. 1. The following account is based on descriptions by Malek (1980) of the schistosome life cycle and on detailed studies by Lengy (1962 a, b) on all stages of the *S. bovis* life cycle.

Adult schistosomes live in pairs in the portal and mesenteric veins of the definitive host. The male transports the female against the current of the portal blood stream into the mesenteric venules. The female partially or totally leaves the gynecophoric canal of the male to lay her eggs in the finest branches of the venules. The uterus of the female worm contains from 3 to 65 eggs (Lengy, 1962 b) and a paired female produces hundreds of eggs per day. Some of the eggs will make a passage from the intravascular lumen through the intestinal mucosa to the intestinal lumen, from where they are passed out with feces. Other eggs are washed away by the bloodstream to the portal circulation and are later found in various organs of the body, mainly the liver. The eggs pass from the vessels through the mucosa to the intestinal lumen assisted by lytic factors secreted from the eggs and by forceful piercing of the tissues by the pointed end of the egg (Semuguruka, 1992). Deposited eggs are immature, but after 6-8 days of maturation the egg contains a fully developed miracidium (Lengy, 1962 a), which hatches when the egg has passed out with feces and come into contact with water.

The miracidium swims actively in search of its intermediate host, a fresh water snail of the genus *Bulinus* (*B. truncatus*, *B. africanus*, *B. forskalii*) (Christensen et al., 1983). It penetrates several species of snails and other organisms, but only in the *Bulinus* snail asexual reproduction will take place.

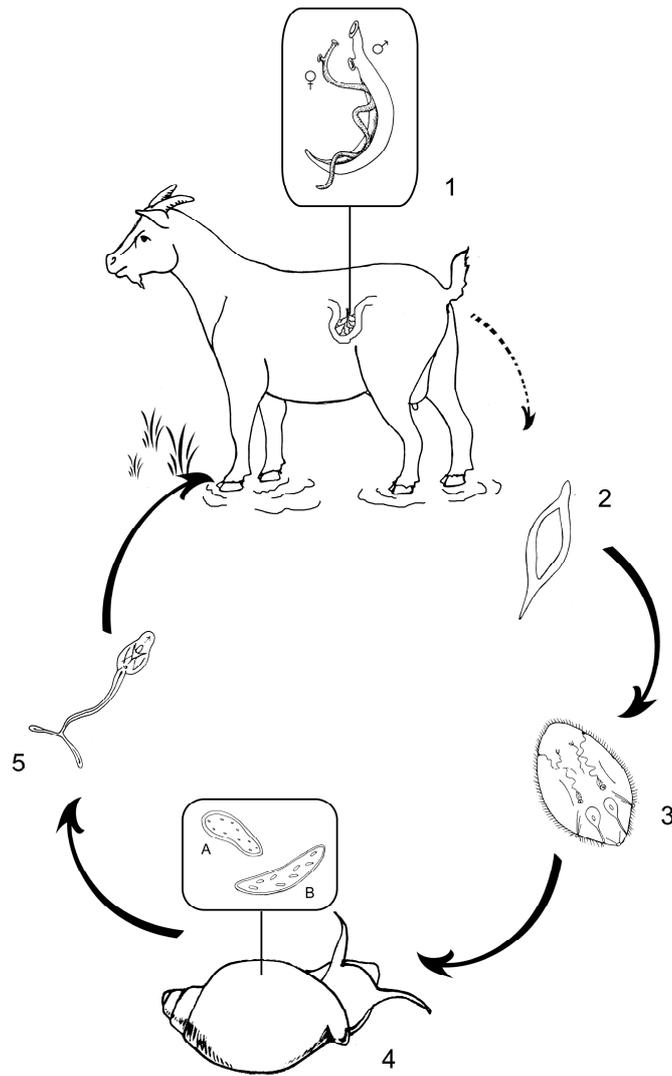


Figure 1. The *Schistosoma bovis* life cycle. (1) Adult worm pair in the mesenteric veins of the definitive host. (2) Egg. (3) Miracidium. (4) Intermediate host with: a) Primary and b) Secondary sporocyst. (5) Cercaria. (Drawing: Karolina Larsson)

After entering the snail, the miracidium transforms into a primary sporocyst, which in turn produces a number of secondary sporocysts. These migrate to the digestive gland and gonad of the snail, where they produce large numbers of fork-tailed cercariae, which will escape from the snail into fresh water. Under optimal temperature conditions (at about 25°C), the time elapsed from the penetration by the miracidium to the liberation of the cercaria is approximately four weeks.

The cercaria penetrates the skin of the definitive host, after which it is transformed to a schistosomulum. In addition to penetration of skin, an oral route of infection has been proven in goats (Kassuku et al, 1985). The schistosomulum travels by the venous system or the lymphatics to the right heart and lungs and then most probably continues through the systemic circulation to the liver, where it matures into an adult worm. Adult male and female worms form pairs, and migrate to the mesenteric veins, where they may live for years. The time from cercarial penetration of the definitive host to the onset of egg laying by female worms, the prepatent period, is around six weeks.

1.4 Pathogenesis and clinical signs

The development of pathological lesions in schistosomosis is the result of a complex interaction between the host and the parasite. Although different developmental stages of the parasite induce lesions in the skin and other tissues during their migration through the body of the host, the most pathogenetic factor is the egg (Taylor, 1987). Eggs that do not succeed in penetrating the intestinal mucosa, or eggs that are carried to other organs by the portal blood flow, remain trapped in the tissues, where secretions from the miracidia provoke strong local inflammatory responses (von Lichtenberg, 1964; Dunne et al., 1981). In schistosomosis bovis, the most severe pathology is located to the intestines, where perioval granulomatous inflammatory lesions are formed around deposited eggs. The same type of granulomas can be found in the liver, where egg antigens also induce increased fibrosis of the portal tracts. Antibody-dependent killing of different developmental stages of the parasite by eosinophils is essential in host defence mechanisms in schistosomosis (Butterworth et al., 1979; Hagan et al., 1985; Capron and Capron, 1986), and as a result, tissue eosinophilia is a common feature.

In caprine schistosomosis bovis, fecal egg excretion commences 4-8 weeks post infection (p.i.), reaches its maximum by week 8-10 p.i., and declines to minimum values 16-24 weeks p.i. (Saad et al., 1984 a; Kassuku, 1986; Monrad et al. 1991, 1995; Johansen et al., 1997). A decrease in the egg excretion with time is seen in both cattle and goats, and has been suggested to be an effect of worm death, egg retention in the tissues and reduced worm fecundity (Saad et al., 1980). However, evidence indicates that the decline in egg excretion results from lowered worm fecundity rather than from attrition of worm populations (Bushara et al., 1980; Johansen et al., 1997).

At the beginning of egg excretion, infected animals develop hemorrhagic diarrhea, anemia, hypoalbuminemia, hyperglobulinemia and eosinophilia. The severity of the disease is related to the fecal egg count: the higher the fecal egg count, the more severe is the disease (Dargie, 1980; Saad et al. 1980, 1984 b; Kassuku 1986, Monrad et al. 1991; Labbo et al., 2007). The intestinal hemorrhaging and diarrhea result from damage caused by the eggs when migrating through the gut wall (Dargie, 1980). Balemba et al. (2000) suggested destruction and inflammation of the intestinal nervous tissue as a possible contributing factor to diarrhea. Due to the intestinal disturbances, the animals develop inappetence, maldigestion and malabsorption of nutrients, leading to weight loss or failure to thrive (Dargie, 1980).

1.5 Pathology

Several studies in cattle, sheep and goats infected with *S. bovis* have provided knowledge on pathological lesions due to infection (Hussein, 1971; Massoud, 1973; Hussein et al., 1975, 1976; Saad et al., 1984 a; Lindberg et al., 1993, 1995, 1997; Johansen et al., 1996 a; Ferreras-Estrada et al., 1998). Pathology in schistosomosis bovis is mainly restricted to the intestines, liver and corresponding lymph nodes.

1.5.1 Gross pathology

Sometimes, a considerable loss of mesenteric and omental body-fat as well as ascitic fluid, hydrothorax and hydropericardium are observed (Massoud, 1973; Hussein et al., 1975). Generally, the intestines show a catarrhal inflammation with a hyperemic mucosa, focal petechial hemorrhages and sometimes a mucous exudate. Minute nodules, corresponding to granulomas, may appear in the intestinal wall, and the submucosal and subserosal veins can be engorged and tortuous. The intestinal lesions are

most prominent in the small intestines, the cecum and the proximal part of colon (Hussein, 1971; Johansen et al., 1996 a). The liver is often enlarged, dark and firm, and sometimes shows fibrous streaks and numerous greyish-white foci beneath the capsule and deep in the substance of the organ. The portal tracts may be thickened and surrounded by stellate-shaped, whitish areas of fibrous tissue. The portal and mesenteric lymph nodes are enlarged, edematous and nodular with petechial hemorrhages. The spleen is congested with a thickened capsule and show prominent follicles. In the lung, focal patches of bronchopneumonia and congestion have been observed (Hussein et al., 1976), as well as adult schistosomes in pulmonary vessels (Ferrerias-Estrada et al., 1998).

1.5.2 Histopathology

Histopathology in the intestines is characterized by the presence of numerous eggs and granulomas formed around eggs in the intestinal wall (Fig. 2), mainly in the lamina propria of the mucosa and in the submucosa. Eggs without a surrounding inflammatory cellular reaction can often be found in rows (Hussein, 1971). The perioval granulomas consist of macrophages, epithelioid cells, scattered lymphocytes and eosinophils and sometimes giant cells, and may be surrounded by a peripheral rim of fibrosis (Lindberg et al., 1993; Johansen et al., 1996 a). Splendore-Hoepli reactions (von Lichtenberg et al., 1966) and an intense eosinophilia may be present in granulomas, most commonly in early patency (Lindberg et al., 1995, 1997). Depending on the developmental stage of the granuloma, eggs in granulomas can be either intact or degenerated, or seen only as empty egg shells. Beyond inflammatory foci, the mucosa may show epithelial desquamation, lymphoid hyperplasia, tissue eosinophilia and plasmacytosis, and increased numbers of globule leukocytes (Hussein, 1971; Hussein et al., 1975, 1976; Saad et al., 1984 a; Lindberg et al., 1993, 1995; Johansen et al., 1996 a). Hypertrophy of the intestinal blood vessels occurs (Hussein, 1971; Hussein et al., 1975, 1976), as well as perivascular cellular infiltrates of lymphocytes and plasma cells around submucosal veins (Lindberg et al., 1995, 1997).

Granulomatous lesions are also present in the liver, both in portal areas and in the parenchyma. Portal tracts show inflammatory cell infiltration and mildly to moderately increased fibrous connective tissue proliferation (Hussein, 1971; Hussein et al., 1975, 1976; Saad et al., 1984 a; Lindberg et al., 1993, 1995, 1997; Ferrerias-Estrada et al., 1998).

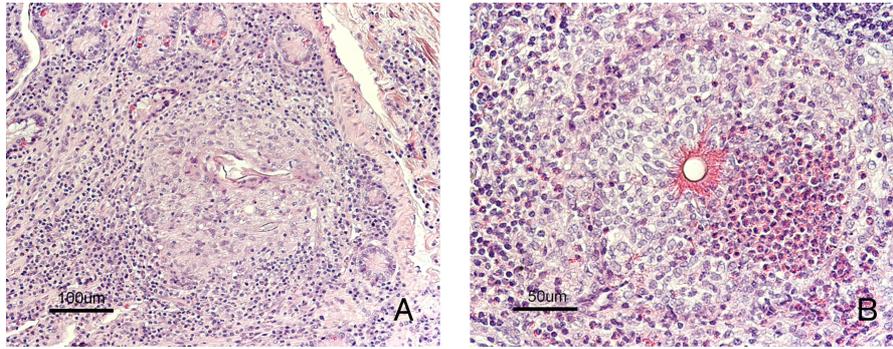


Figure 2. Different appearances of *Schistosoma bovis* egg granulomas in the intestine: (A) granuloma consisting mainly of macrophages and epithelioid cells, with scattered lymphocytes and remnants of egg-shells. (B) Granuloma showing an egg-shell surrounded by Splendore-Hoeppli material, and with a focus of eosinophils. (Photo: Kaisa Sörén)

Intravascular granulomas and lymphoid nodules may be seen, the latter sometimes showing remnants of disintegrated worms in their centre (Hussein 1971; Hussein et al., 1975). Schistosomal pigment is usually present within swollen, proliferative Kupffer cells but may also be found in inflamed portal tracts and near granulomas (Hussein, 1971; Hussein et al., 1975, 1976; Saad et al., 1984 a). The bile duct epithelium is infiltrated by globule leukocytes (Lindberg et al., 1993; Ferreras-Estrada et al., 1998).

Portal and mesenteric lymph nodes have been described to show reticuloendothelial hyperplasia, infiltration of eosinophils and plasma cells in the medullary sinuses, 'starry-sky' appearance of lymphoid follicles and local accumulations of schistosomal pigment and hemosiderin. Eggs and granulomas are frequent findings. (Hussein, 1971; Hussein et al., 1975, 1976; Saad et al., 1984 a; Ferreras-Estrada et al., 1998).

Findings in the spleen may include trabecular thickening, hemosiderosis, and hyperplasia of the germinal centres of the splenic follicles (Hussein, 1971; Hussein et al., 1975, 1976; Saad et al., 1984 a) as well as increased plasma cell infiltration of the red pulp (Lindberg et al., 1993).

In the lungs, alveolar epithelial hyperplasia may be present (Lindberg et al., 1993), as well as peribronchiolar lymphocytic infiltration (Saad et al., 1984 a) and focal eosinophilic infiltration of the interstitium (Hussein, 1971). Pulmonary vessels are thickened due to medial hypertrophy and hyalinization, sometimes leading to complete obliteration of the vessel

lumen. Granulomatous lesions may be found, (Hussein, 1971; Hussein et al., 1975, 1976; Saad et al., 1984 a) and embolized worms can be present (Hussein et al., 1976; Saad et al., 1984 a).

Granulomas and eggs may also be found in the interstitium and parenchyma of the pancreas, accompanied by mild perivascular lymphocytic infiltration. The pancreatic architecture is often well preserved, but focal necrosis and atrophy may occur. Adult schistosomes may be present in pancreatic veins. (Hussein, 1971; Hussein et al., 1975, 1976; Saad et al., 1984 a).

1.6 Immune responses

1.6.1 Resistance

In several studies on schistosomiasis bovis in cattle and goats, development of acquired resistance has been proven (Majid et al., 1980; Bushara et al., 1980, 1983 a, b, c; Saad et al., 1980, 1984 a; Kassuku et al., 1986; Monrad et al., 1991, 1995; Johansen et al., 1997). The resistance is expressed as a reduction of the egg excretion with time and has been shown to remain after challenge exposure (Bushara et al., 1983 b; Monrad et al., 1991, 1995; Johansen et al., 1997). The regulating mechanism - presumably immunologically mediated - has proved to be an anti-fecundity effect with reduced egg production and excretion, but with a persistent worm population (Johansen et al., 1997). Regulation of egg excretion through fecundity suppression is a feature shared particularly by infections with *S. bovis* and the closely related human species *S. haematobium*, and this 'anti-fecundity resistance' should be distinguished from resistance to worm establishment. In a serum transfer study, Bushara et al. (1994) showed that serum received from immune donors caused reduced egg counts in *S. bovis*-infected calves, a result suggesting a role for humoral factors in the anti-fecundity resistance phenomenon.

1.6.2 Humoral responses

Compared to the great number of studies addressing human schistosomiasis, very little work has been done on the humoral responses to infection in ruminant schistosomiasis bovis.

Oleaga and Ramajo (2004) observed a continuous increase in serum IgG levels for 51 weeks in response to worm antigens in experimentally infected

sheep, and showed that the serum antibody levels were proportional to the number of worms recovered. In contrast, in another experimental study of ovine schistosomosis bovis, the circulating anti-worm antibody levels decreased continuously after a peak at 9 weeks p.i. until the end of the 15 week study period (Rodriguez-Osorio et al., 1999). In the latter study no attempt to correlate antibody levels to worm burden was made.

In caprine schistosomosis bovis, both anti-worm and anti-egg IgG and IgM levels in serum have been shown to increase significantly after experimental infection. In untreated animals antibody levels continued to rise throughout the 16 week study period. After treatment at week 13 p.i., antibody levels started decreasing, but only after an initial peak in levels for anti-worm antibodies and anti-egg IgM. Neither the specific antibodies against worm antigens nor egg antigens showed correlation to fecal egg counts (Johansen et al., 1996 b).

1.6.3 Immunopathology

In schistosomosis, pathology is mainly a result of host immune responses to the eggs of the parasite. Adult worms are not directly pathogenic, but yet strongly immunogenic (Dunne et al., 1988). However, they manage to evade host immune responses by incorporating host-derived macromolecules into their tegumental membrane and by losing expression of their own antigens (Smithers and Terry, 1969; Pearce et al., 1986). The larval schistosomula on the other hand are vulnerable to host responses and may be killed by antibody-dependent cellular cytotoxicity (ADCC), which, in human schistosomiasis, has been shown to be mediated mainly by eosinophils and IgE (Butterworth, 1993).

Antigens secreted from the miracidia within the egg induce an inflammatory response leading to granuloma formation (von Lichtenberg, 1964; Dunne et al., 1981). The main function of the granuloma is to protect the surrounding tissue from the potentially toxic substances secreted from the egg and then eventually to destroy the egg, but at the same time granuloma formation is harmful as it leads to focal organ damage (von Lichtenberg, 1964; Warren, 1973). The granuloma is mainly formed by macrophages, epithelioid cells and multinucleated giant cells that are gathered around the egg, but other inflammatory cells such as lymphocytes, eosinophils, fibroblasts and sometimes neutrophils and mast cells are also involved in granuloma formation (Weinstock, 1992). Granuloma development undergoes stages of initiation, maturation and involution, and

eventually healing when the egg has been destroyed, leaving a fibrous scar (Warren and Domingo, 1970; Weinstock, 1992).

Studies on the regulation of the formation and development of granulomas have mostly been conducted in murine models of human *S. mansoni* infection. Granuloma formation has been shown to be a T cell mediated delayed type hypersensitivity reaction (Warren et al., 1967), dependent on major histocompatibility complex (MHC) class II-restricted $\alpha\beta$ receptor CD4⁺ T lymphocytes (Mathew and Boros, 1986; Iacomini et al., 1995; Hernández et al., 1997). In contrast to the well studied murine schistosomiasis *mansoni*, there are only occasional reports on the immunopathology of schistosomiasis in ruminants, and those were limited to immune cell phenotyping of the inflammatory response in the intestine (Lindberg et al., 1999; Ferreras et al., 2000). In the single study of intestinal granulomas in *S. bovis*-infected goats, macrophages and epithelioid cells in granulomas were shown to express MHC class II consistently. Whereas CD2⁺ (CD4⁺ and CD8⁺) $\alpha\beta$ receptor T cells indeed were present in granulomas, they were outnumbered by $\gamma\delta$ T cells (Lindberg et al., 1999). In contrast to humans and mice, $\gamma\delta$ T cells constitute a large proportion of the T cell system in ruminants (Mackay and Hein, 1991). Gamma/delta T cells differ from $\alpha\beta$ T cells in that they do not require antigens to be presented by MHC molecules (Schild et al., 1994).

Based on studies in mice, CD4⁺ cells (T helper cells) are designated to one of two subsets depending on which cytokines they produce. T helper (Th) 1 cells are characterized by expression of IL-2 and IFN- γ , whereas Th2 cells express IL-4 and IL-5 (Mosmann and Coffin, 1989). In murine schistosomiasis, the host immune response shifts from an initial moderate Th1 to a robust Th2 - dominated response with the onset of egg deposition in tissues (Pearce and MacDonald, 2002). Although diverging results on the contribution of different cytokines to granuloma formation have been obtained from different experimental models of murine schistosomiasis *mansoni* (Stavitsky, 2004), it has been shown that exacerbated granulomatous inflammation driven by Th1 responses is controlled by a well established Th2 response associated with well-circumscribed granulomas consisting of alternatively activated macrophages, lymphocytes and eosinophils (Anthony et al., 2007). Cytokine responses in ruminant schistosomiasis *bovis* are as yet unstudied.

Another result of host immune responses to the eggs of the parasite is the formation of connective tissue in portal tracts of the liver, referred to as portal fibrosis (Wilson et al., 2007). Fibrogenesis is a dynamic process including interactions between fibroblasts, macrophages and T cells (Grimaud, 1987). Whereas immature fibrous tissue is rapidly degradable, mature collagen is more resistant to degradation due to stabilization by cross-linking molecules (Andrade, 1994). However, liver fibrosis has been shown to be reversible after chemotherapy in *S. japonicum* and *S. mansoni* infections in humans, mice and rabbits (Dunn et al., 1994; Olds et al., 1996; Cheever et al., 2002; Andrade, 2004). In chronic human schistosomiasis, hepatic fibrosis is the major cause of morbidity as it is associated with portal hypertension that can lead to the development of porto-systemic shunts and bleeding in the upper gastrointestinal tract. In comparison, hepatic fibrosis in caprine schistosomosis *bovis* is most often only mildly to moderately expressed (Lindberg et al., 1993, 1995, 1997).

1.7 Diagnosis

Diagnosis of infection is essential in epidemiological surveys as well as in prevention and control of schistosomosis in domestic livestock. Ruminant schistosomosis can not be diagnosed merely by clinical signs, since these are unspecific and often resemble the signs of other diseases, such as paratuberculosis, fasciolosis and trypanosomosis (Kassuku, 1985). Instead, detection of eggs in feces is a common method for diagnosis. Since fecal egg counts in chronic schistosomosis *bovis* are usually very low, the sensitivity of the diagnostic method is critical for correct diagnosis. In experimental laboratory studies, methods with high sensitivity such as the Pitchford and Bell filtration techniques (Bell, 1963; Pitchford and Visser, 1975) are used for fecal egg counting. However, since these methods require time, resources and expertise, they are poorly suited for routine diagnostics in the field. In a comparative study by Olaechea et al. (1990) it was shown that a modified syringe filtration technique originally described by Bradley (1965) and the Teesdale smear technique (Teesdale and Amin, 1976) were acceptable alternatives for diagnosis under field conditions.

Immunodiagnostic methods have not yet been developed for routine diagnostics in ruminant schistosomosis. Although specific serum antibodies towards worms and eggs have been detected in experimental work with *S. bovis*-infected sheep and goats (Johansen et al., 1996 b; Rodriguez-Osorio et al., 1999; Oleaga and Ramajo, 2004), a poor correlation between antibody

levels and worm burdens or fecal egg excretion was shown in goats (Johansen et al., 1996 b). In contrast, the level of the schistosome circulating cathodic antigen (CCA) in serum has been shown to significantly correlate to worm burdens and fecal egg counts during peak egg excretion, making it a possible candidate for diagnostic purposes (Johansen et al., 1996 b). A new area for intensive research is schistosome glycans and glycoproteins, which are regarded as good immunodiagnostic targets and potential targets for vaccine development (Nyame et al., 2003, 2004). In a recent study by Ramajo-Hernández et al. (2007), the glycans and the proteins which they are expressed on in *S. bovis* were identified, thus presenting a new opening in diagnostics.

1.8 Treatment and control

1.8.1 Treatment

Praziquantel, a drug effective against a range of both human and veterinary trematode and cestode infections (Andrews et al., 1983), is today the anthelmintic of choice for treatment of human schistosomiasis, and it has also proven effective against ruminant *S. bovis* (Bushara et al., 1982; Johansen et al., 1996 c; Cioli and Pica-Mattocia, 2003). When praziquantel was introduced in the 1970s it opened new possibilities for the control of schistosomiasis, as it proved to be not just effective but also safe (King and Mahmoud, 1989). However, although the cost of a single dose has decreased during the past decades, it is still high enough to limit the use of praziquantel in the treatment of both humans and animals in developing countries (King and Mahmoud, 1989; Chitsulo et al., 2000).

In cattle and goats, praziquantel treatment of *S. bovis* infection has been shown to be highly effective and lead to a near complete reduction (99.2-100%) in worm loads (Bushara et al., 1982; Johansen et al., 1996 c). A higher praziquantel dose (60 mg/kg) is required in goats compared to cattle (20 mg/kg) for successful treatment. The exact mode of action of praziquantel is still unknown, but it is believed that it alters the Ca^{2+} -homeostasis in the worm in a way that ultimately leads to tegumental disruption and induction of paralytic muscle contraction (Becker et al., 1980; Melhorn et al., 1981; Andrews et al., 1983; Xiao et al., 1984). As the worms are killed, they are flushed to the portal veins and the liver. In *S. bovis*-infected goats studied up to four weeks after treatment, acute inflammation in the liver followed by induration, necrosis and calcification

was seen, together with cellular reactions in relation to dead worms (Johansen et al., 1996 a).

There has been some concern about whether praziquantel treatment could lead to a loss of acquired resistance. In a study by Bushara et al. (1983 c), it was shown that naturally acquired resistance to *S. bovis* was not affected at reinfection seven weeks after treatment of cattle, but adequately controlled studies on the long-term effects of treatment on immunoregulatory events in ruminant schistosome infections have so far been lacking. In *S. japonicum* infected mice, acquired resistance was lost thirteen weeks after treatment (Moloney et al., 1987). In human *S. haematobium* and *S. mansoni* infection on the other hand, there is circumstantial evidence that treatment might even confer augmented protection to reinfection (Medhat et al., 1998; Karanja et al., 2002).

Although praziquantel is effective in removing the parasite burden, it offers no protection against reinfection and repeated treatment is therefore necessary for successful control. As for other anthelmintics, there is a major risk of development of drug resistance. A praziquantel resistant *S. mansoni* strain has already been found in experimentally infected mice (Fallon and Doenhoff, 1994), and in Egypt, failure of treatment of human schistosomiasis mansoni led to the conclusion that a resistant strain might be present (Ismail et al., 1994).

1.8.2 Vaccines

Due to the risk of development of drug resistance, there is a need to continue the search for vaccine candidates for the control of ruminant schistosomosis. There are two possible strategies for vaccine development with the aim to reduce morbidity. The first would be to reduce worm burden and the other to reduce female worm fecundity and egg viability. The work on developing a vaccine for bovine schistosomosis has been going on for decades, and good results with respect to reduction in worm and egg burdens were achieved already in the 1970s and 1980s using homologous live vaccines with attenuated cercariae or schistosomulae (Taylor, 1987). However, these vaccines were not suited for large scale field application due to difficulties in producing and storing enough cercariae, and the work was re-directed to developing antigen vaccines.

Today, two different antigens with vaccine potential to ruminant *S. bovis* have been identified, the *S. bovis* 28-kilodalton glutathione S-transferase

(Sb28GST), and keyhole limpet hemocyanin (KLH), which is a commercially available high molecular-weight glycoprotein shown to share a protective carbohydrate epitope with the protective *S. mansoni* schistosomular surface glycoprotein GP38. Both native Sb28GST and KLH have been shown to reduce fecal and tissue egg counts in vaccinated calves, without however having any effect on the worm burden (Bushara et al., 1993). In contrast, vaccination of goats and sheep with recombinant Sb28GST was shown to result in reduced worm loads, but no reduction in worm fecundity (Boulanger et al., 1994, 1999). The vaccination protocol and animal model thus seem to have an impact on vaccination results. A recombinant *S. haematobium* 28GST vaccine candidate for humans is now in the process of clinical trials (Capron et al., 2005), but no further steps have been taken towards producing a commercially available antigen vaccine for ruminant schistosomosis.

As both Sb28GST and KLH only elicit partial protection from the effects of infection, the search for new proteins involved in host-parasite interplay continues. Recently, the *S. bovis* 14-3-3 protein was characterized (Uribe et al., 2007 a), and the injection of recombinant Sb 14-3-3 protein together with immunomodulators was shown to give significant protection to *S. bovis* in mice (Uribe et al., 2007 b). In a study by Pérez-Sánchez et al. (2006), sixteen new excreted/secreted and tegumental *S. bovis* proteins were identified, opening new possibilities for vaccine design. Another area gaining interest is the identification of schistosomal pathogen associated molecular patterns (PAMPs). PAMPs are microbial molecules recognized by the innate and acquired immune systems, primarily through Toll-like receptors (TLRs) (Janeway and Medzhitov, 2002). A schistosomal PAMP termed lysophosphatidylserine has been shown to affect immune polarization through activation of TLR 2 (van der Kleij et al., 2002). Further knowledge of the PAMPs and their interaction with the host could potentially provide a basis for new strategies in vaccine development.

1.8.3 Water hygiene

Ultimately, improved water hygiene giving access to safe water would probably be the most effective way to prevent transmission of schistosomes. One way to accomplish this would be to eliminate the snail vector by the use of molluscicides, but so far this has failed. Although not toxic for humans, domestic animals and crops, synthetic molluscicides can be toxic to fish and are therefore not considered environmental friendly (Lardans and Dissous, 1998). Unfortunately, suitable snail habitats have increased as the

requirements of increasing populations and development in several endemic countries have led to large scale water impoundment for electricity and irrigation (Kay, 1990; Hunter et al., 1993).

1.9 The *S. bovis*/goat model

In order to study schistosomosis bovis in a ruminant host, the use of a *S. bovis*/West African Dwarf goat model has been introduced. As a natural ruminant host of the parasite, the West African Dwarf goat (Fig. 3) is a costefficient and practical model for ruminant schistosomosis. Work by Kassuku et al. (1986), Monrad et al. (1991, 1995) and Johansen et al. (1997) has given information on the clinical pathology and development of acquired resistance in primary and challenge infections in caprine schistosomosis bovis. Studies on pathology and phenotypic characteristics of cells of the inflammatory response in *S. bovis*-infected goats have been performed by Lindberg et al. (1993, 1995, 1997, 1999). Johansen et al. (1996 a, b, c.) has elucidated the effects of treatment on clinico-pathological parameters, pathology and serology in infected goats for a time period of up to four weeks post treatment. All these studies have concerned infections of maximally about eight months' duration.



Figure 3. A West African Dwarf goat. (Photo: Lena Sörén Plantin)

The *S. bovis*/goat model is thus well established for studies of ruminant schistosomosis bovis. Apart from giving information on the effects of

infection on a natural host species, the *S. bovis*/goat model is attractive from the comparative medicine viewpoint, since *S. bovis* is considered an immunological analogue of the human pathogen *S. haematobium* (Agnew et al., 1989). Finally, the *S. bovis*/goat model also provides an opportunity to gain increased knowledge on basic aspects of ruminant immunology.

2 Aims of the thesis

The overall aim of this thesis was to gain further knowledge on the host-parasite relationship in ruminant schistosomiasis bovis by use of the *S. bovis*/West African Dwarf goat model.

The specific aims of this thesis were:

- To study immunoregulatory responses and treatment effects in chronic, up to one year old, infection.
- To study host immune responses and their possible relationship to fecundity suppression in female worms – the anti-fecundity phenomenon.
- To further elucidate the immunological features of the inflammatory response in ruminant schistosomiasis bovis by studying immune cell characteristics and cytokine gene expression in target organs.

3 Material and methods

This thesis is based on the results from two different experiments, approved by the Danish Animal Ethical Committee, and performed at the Danish Centre for Experimental Parasitology, University of Copenhagen, Denmark. Details on the material and methods used are given in the papers I-III in the appendix. Below, material and methods are described in a summarized form.

3.1 Animals and experimental designs

In both experiments, the animals used were young West African Dwarf goats bred and raised at the Copenhagen Zoo.

3.1.1 Experiment I (Papers I-II)

In the first experiment, forty-seven, three to five months old, castrated goats were allocated into eight groups of five to six animals each, and treated as shown in Table 1. The goats were kept indoors randomly mixed in three pens independently of infection and treatment status. For the necropsies, the goats were killed by pentobarbital intravenously. In paper I, all groups except the group necropsied at week 36 and treated with anthelmintics (group PT36) were included. In paper II, all groups were included.

3.1.2 Experiment II (Paper III)

In the second experiment twelve goats, six to seven months of age, were used. Eleven of them were castrated males and one was a female, and they were all sired by the same buck. The goats were allocated to four groups and kept indoors in a pen. Two of the groups, consisting of four goats each, were infected and the goats were necropsied after 7 and 13 weeks of infection, respectively. Two groups with two uninfected goats each served

as controls. For the necropsies, the goats were killed with a captive bolt and bled.

Table 1. Experimental design showing timing of experimental exposures, praziquantel treatment and necropsy

Group	No. of goats	Primary infection (week 0)	Praziquantel treatment (week 13)	Challenge infection (week 36)	Necropsy	
					week 36	week 52
P36 ^a	6	+			+	
P52	6	+				+
PT36 ^b	5	+	+		+	
PT52 ^c	6	+	+			+
PTCh	6	+	+	+		+
PCh	6	+		+		+
Ch ^d	6			+		+
F	6	-	-	-	-	+

^a One goat was excluded week 25 due to intestinal intussusception.

^b This group was not included in paper I.

^c This group is named PT in paper I and PT52 in paper II.

^d One goat was euthanized week 51 due to central nervous system disturbances; this goat was necropsied according to the protocol.

3.2 Parasites and experimental infections (Papers I-III)

For the infections, a bovine isolate of a Tanzanian strain of *S. bovis*, maintained in *Bulinus* snails and passaged in hamsters at the Danish Bilharziasis Laboratory, were used. Prior to infection all goats were drenched with albendazole. The goats were infected by the percutaneous route using the leg immersion technique as described by van Wyk et al. (1975). The infection dose was 800 cercariae per goat for both primary and challenge infections.

3.3 Treatment (Papers I and II)

Praziquantel was used for anthelmintic treatment. Praziquantel powder in a dose of 60 mg kg⁻¹ was dissolved in propylene glycol and applied orally using a syringe.

3.4 Clinical pathology (Paper I)

In order to monitor the clinical impact of infections, the goats were weighed and blood samples for hemoglobin measurement were taken every two weeks throughout the experiment. Hemoglobin concentrations were determined as described by Monrad et al. (1991).

3.5 Serology (Paper II)

Serum samples were collected at twelve times throughout the one-year study period, and serum levels of antigen-specific antibody levels were measured by an indirect enzyme-linked immunosorbent assay (ELISA). Detection of IgG and IgM antibodies against *S. bovis* adult worm antigens was performed for the primary infected and challenged groups, praziquantel-treated (PTCh) or untreated (PCh), and for the non-infected control group (F). Detection of IgG antibodies against *S. bovis* egg antigens was performed for all groups except groups P36 and PT36. Worm antigen was produced from adult worms collected at the vascular perfusion of killed infected goats. For the production of egg antigen, eggs were obtained from the livers of the same goats. Microtitration plates (Maxisorp, Nunc) were coated with either worm or egg antigen. After blocking, goat sera in different dilutions dependent on the antigen and antibody were added to the wells and incubated. Horse radish peroxidase-conjugated rabbit anti-goat antibodies (Bethyl Laboratories Inc., USA) were applied, and after incubation, the plates were developed with OPD substrate (Sigma-Aldrich, Sweden) and read in an ELISA reader (Labsystems Multiskan MS) at 492 nm. From the results, group means for OD values were calculated.

3.6 Fecal egg counts (Papers I and III)

In paper I, sampling of feces was carried out every two weeks throughout the experiment. In paper III, sampling of feces was performed at 6, 8 and 12 weeks of infection, as well as at the days of necropsies at week 7 and 13. Fecal egg counting, expressed as eggs per gram feces (epgf) was performed using a modified Bell technique as described by Johansen et al. (1997).

3.7 Perfusion for worm recovery (Paper I)

Perfusions for worm recovery were done in order to establish the worm loads of the infected goats, and were performed as previously described by Johansen et al. (1996 c). In brief, before the goats were killed, 0.25 mg kg⁻¹

heparin was administered intravenously in order to prevent blood clotting during the subsequent perfusion of the portal and mesenteric vascular systems for collection of worms. After opening the abdominal cavity, the aorta was clamped proximal to the renal arteries and a second pair of forceps was used to close the caudal vena cava. A tube was inserted through an incision in the aorta and connected to a peristaltic pump which pumped perfusion solution from a glass flask into the goat. The portal vein was cut open and a mixture of blood and perfusion fluid was sucked from the vein, using a tube connected to a vacuum pump. The perfused suspension was poured through a sieve and the worms were collected. Following perfusion the intestines were examined for residual worms. The number of worms (males, females and immature worms) per goat was determined.

3.8 Tissue egg counts (Paper I)

For determination of the number of eggs in the tissues, representative tissue samples were taken from the liver and predetermined sites of the duodenum, jejunum, ileum, cecum and colon after the perfusions. The samples were frozen and egg counts per gram tissue were later determined using a KOH digestion technique described by Bjørneboe and Frandsen (1979). The liver and the different intestinal sections were weighed in order to determine organ-specific total tissue egg counts.

3.9 Gross liver pathology (Paper I)

At necropsy, livers were examined macroscopically and photographed in all groups except P36. Hepatic fibrosis was assessed semi-quantitatively. Two types of fibrous changes, i.e., fibrosis affecting the liver diffusely (generalized fibrosis) and multi-focal fibrous scarring, were evaluated separately. Generalized fibrosis was assessed both visually and by the degree of firmness of the liver. A second observer, blinded to the identity and group affiliation of the goats, assessed the degree of fibrous scarring on colour photographs of the parietal and visceral aspects of the entire organ. Both types of lesions were graded as 0 = no fibrosis, 1 = mild fibrosis (mild generalized fibrosis or presence of 1-2 fibrous scars), 2 = moderate fibrosis (moderate generalized fibrosis or presence of multiple fibrous scars), and 3 = marked fibrosis (severe generalized fibrosis or marked fibrous scarring). Mean scores for generalized fibrosis and fibrous multi-focal scarring were determined for each group.

3.10 Necropsy and histopathological laboratory procedures (Papers II-III)

Paper II:

At necropsy, intestinal specimens were taken from predetermined sites of the proximal, middle and distal jejunum, the distal ileum, and the cecum and colon (centrospiral and distal parts). A mesenteric lymph node and one specimen each from the central parts of the liver and spleen were collected, respectively. In groups P36 and PT36, sampling did not include lymph node, ileum, distal colon or spleen. The specimens were fixed in 10% neutral buffered formalin, and those from the intestines and liver were further trimmed into two or more pieces. Small intestinal specimens included the whole circumference of the gut, and 1 x 2 cm large pieces were cut from the liver specimens. Paraffin sections were cut 4 µm thick and stained with hematoxylin and eosin. The slides were given coded labels, the investigator thus being blinded to the identity of all animals, inclusive of non-infected controls.

Paper III:

At necropsy, intestinal specimens from a predetermined site of the proximal and distal jejunum, respectively, and a jejunal mesenteric lymph node, were collected from each goat. Specimens were fixed in two different solutions, zinc salts fixative (ZSF) (Beckstead, 1994; González et al., 2001) for subsequent use in histology and immunohistochemistry (IHC), and 10% neutral buffered formalin for subsequent use in histology, IHC and quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR). After a fixation time of 48 hours (ZSF) or 8-12 days (formalin), specimens were further trimmed, processed using routine methods and paraffin embedded. For histological assessment, 4 µm thick paraffin sections were cut from both the ZSF-fixed and formalin-fixed material and stained with hematoxylin and eosin.

3.11 Histopathological examination (Papers II and III)

Paper II:

After initial scrutiny of morphological changes, certain parameters were chosen for quantitative assessment or subjective grading. For details see paper II. For the evaluation of the results, mean values were calculated for the pieces examined from each intestinal specimen and the pieces examined from each liver, spleen and lymph node specimen, respectively. In each goat, values recorded for jejunal and ileal specimens were pooled as

representing small intestine and those for colonic and cecal specimens as large intestine. For every histological characteristic assessed in any organ, group means were calculated based on the results from each individual.

Paper III:

Sections from both the ZSF-fixed material and formalin-fixed material were used for a descriptive histopathological assessment. In order to perform studies on the correlation between cytokine gene expressions measured by qRT-PCR (see below) and the numbers of granulomas in the tissues, the total number of perioval granulomas in the formalin-fixed material from the proximal jejunum and mesenteric lymph node, respectively, of each goat, was counted.

3.12 Immunohistochemistry (Paper III)

Phenotypic characterization of the cells of the inflammatory response in the small intestine and mesenteric lymph nodes was performed with IHC, using the streptavidin-biotin complex/horse radish peroxidase method (Dako, Glostrup, Denmark). ZSF-fixed material was chosen for IHC since this fixative has proven to have several advantages. Morphology is better preserved than in frozen material, and at the same time antigen retrieval steps that are needed in formalin-fixed material can be avoided, thus allowing the demonstration of cell surface epitopes considered to be processing-sensitive, e.g., CD4 and CD8 (Beckstead, 1994; González et al., 2001). Table 2 describes the primary antibodies used. All primary antibodies were applied to 4 μ m thick serial sections from the ZSF-fixed tissues, except CD79 α for which formalin-fixed tissues were used. For the latter, an antigen retrieval step was included, according to the manufacturer's instructions. Biotinylated goat-anti mouse IgG (Chemicon Int., CA, USA) and goat-anti-rabbit IgG (Vector Lab. Inc., CA, USA), respectively, were used as secondary antibodies. The sections were developed with the SigmaFastTM 3,3'-diaminobenzidine (DAB) tablet set (Sigma-Aldrich, Sweden). On control sections, primary antibodies were replaced by indifferent mouse IgG or rabbit immunoglobulins. The number of positively stained cells in each section was assessed semiquantitatively to describe the phenotypic characteristics of cells in the jejunum and mesenteric lymph nodes.

Table 2. Primary antibodies (Ab) used in immunohistochemistry, differentiation molecules against which they were raised (Specificity), cell types marked and the dilution employed

Ab	Specificity	Cells marked	Dilution
H42A	Ruminant MHC class II ^a	Antigen presenting cells	1:4000
HM57	Human CD79 α ^b	B lymphocytes	1:200
17D1	Ovine CD4 ^a	T helper cells	1:100
CACT80C	Bovine CD8 ^a	T cytotoxic cells	1:100
19.19	Ovine WC1 (T19) ^c	$\gamma\delta$ T cells	1:100
Rabbit anti-human T cell	Human CD3 ϵ ^b	T cells	1:200

^a Supplied by VMRD, Inc., Pullman, WA, USA

^b Supplied by Dako, Glostrup, Denmark

^c Supplied by AbD SEROTEC, Oxford, U.K.

3.13 Quantitative real-time reverse transcriptase polymerase chain reaction (Paper III)

Gene expression of the cytokines IL-4, IFN- γ , IL-2 and TNF- α in formalin-fixed paraffin-embedded (FFPE) material from the proximal jejunum and lymph node, respectively, was measured by qRT-PCR on a Rotorgene, RG 3000 (Corbett Research, Mortlake, Australia), using the QuantiTectTM SYBR[®]Green RT-PCR kit (Qiagen) according to the manufacturer's instructions (for details, see paper III). The primers used, which were specific for caprine mRNA, are described in Table 3. Gene expressions were normalized to total RNA instead of housekeeping genes since finding suitable housekeeping genes for the comparison of normal tissues to those with an altered cell composition due to granulomatous inflammation is difficult. Relative quantification of normalized gene expressions was performed by comparing the mean threshold cycles (C_t) of three technical triplicates between uninfected and infected goats.

Table 3. Primer sequences for quantitative real time RT-PCR for caprine IL-4, IFN- γ , IL-2 and TNF- α

Primer	Sequence	Product size (bp)
IL-4 forward	5' GTACCAGCCACTTCGTCCAT 3'	75
IL-4 reverse	3' TGAGGATGTTTCAGCGTTTTG 5'	
IFN- γ forward	5' TTCAAATTCGGTGGATGAT 3'	84
IFN- γ reverse	3' ATTTTGGCGACAGGTCATTC 5'	
IL-2 forward	5' GTGAAGTCATTGCTGCTGGA 3'	76
IL-2 reverse	3' TCCTGGAGAGCTTGAGGTTC 5'	
TNF- α forward	5' CATCTACCAGGGAGGGTCT 3'	72
TNF- α reverse	3' TATTCGGCTGGTTGATCTC 5'	

3.14 Statistical methods (Papers I-III)

In all assessments, $P < 0.05$ was considered statistically significant.

Paper I:

SAS version 8(2) (SAS Institute 1999) was used for statistical analysis. Tissue egg counts, total worm burdens and body weight gains were compared by one-way analysis of variance (ANOVA), with subsequent paired comparisons of group means. The female-male worm ratios were assessed by logistic analysis, comparing the proportions of female:total adult worms (PROC GENMOD/SAS). Longitudinal data on epgf and hemoglobin values were analyzed by repeated measures analysis of variance (PROC MIXED/SAS).

Paper II:

In the analysis of serological data linear models were used by means of the PROC MIXED/SAS, version 9 for Windows (Littell et al., 1996). The response variables were analyzed using a model including treatments and

time. Since repeated measures were used, a covariance structure to account for the dependence was included, where no mathematical pattern was imposed on the covariance matrix. After a significant interaction between treatment and time, pair-wise comparisons of the treatments were made for selected time-points. Histopathological data from each goat were compared by ANOVA (SAS 9.1), with subsequent paired comparisons of group means.

Paper III:

For cytokine gene expression, differences between groups were analyzed with the non-parametric Kruskal-Wallis method by the use of Minitab, version 15. Cytokine gene expressions in the proximal jejunum and mesenteric lymph node were correlated to the numbers of granulomas in sections from the proximal jejunum and the mesenteric lymph node, using the Spearman correlation test (PROC CORR/SAS 9.1).

4 Results and general discussion

4.1 Experiment I (Papers I-II)

The first experiment was designed in order to give information for the first time on immunoregulatory responses and treatment effects in chronic, up to one year old, *S. bovis* infections in goats. This kind of information is of particular importance since ruminant *S. bovis* infection is most often chronic under natural transmission conditions (Taylor, 1987), and because reinfections after treatment are to be expected under field conditions. Several aspects of long-standing *S. bovis* infection such as duration of resistance to challenge infection, effects of anthelmintic treatment on immunoregulatory events as well as regression of infection and of treatment-induced hepatic damage with increasing time after treatment, have not been explored previously. Serum antibody responses to worm and egg antigens in *S. bovis*-infected goats have only been studied in up to sixteen week old primary infection, and up to three weeks post treatment (Johansen et al., 1996 b), and previous histopathological studies of caprine schistosomiasis *bovis* have concerned infections of maximally about eight months' duration (Lindberg et al., 1993, 1995, 1997; Johansen et al, 1996 a).

4.1.1 Long-standing infection

Previous studies have shown a strong regulatory response in goats with a primary *S. bovis* infection, expressed as a reduction in egg excretion with time after a peak level 8 to 12 weeks after exposure (Monrad et al., 1991, Johansen et al., 1997), whilst no attrition of the adult worm population was seen (Johansen et al., 1997). This was confirmed in the present study which furthermore showed that egg excretion was persistently suppressed for up to 52 weeks, remaining constantly low (< 20 epgf), and that the adult worm

burden was comparable at 36 and 52 weeks. These observations, together with relatively low and similar tissue egg counts in goats infected for 36 and 52 weeks, indicate that acquired resistance expressed through an anti-fecundity effect persists also in a very late and chronic stage of infection. However, in contrast to earlier studies where clinico-pathological effects of infection gradually diminished in parallel with decreasing egg excretion (Monrad et al., 1991, 1995), anemia and poor weight gains persisted throughout the one-year infection time, indicating that no protection seemed to develop against clinico-pathological consequences of infection.

The goats mounted significant circulatory antibody responses to both worm and egg antigens after primary infection, in agreement with a previous short-term study by Johansen et al. (1996 b). Both anti-worm IgG and IgM were furthermore shown to remain at high levels throughout the one year course of primary infection, likely mirroring the substantial persisting worm loads. Circulating anti-egg IgG levels also remained at a high level throughout the 52 week primary infection, decreasing only slowly. This occurred despite that egg excretion was long-term continuously minimized and only low burdens of tissue bound eggs were found. Significant cross-reactivity with worm antigens to account for the persisting antibody levels was unlikely since the rise of anti-egg IgG occurred later than that of anti-worm antibodies. Instead, this result indicates that even low numbers of deposited eggs during chronic infection may be sufficient to maintain consistently high antibody levels.

In long-term primary infections (at 36 and 52 weeks p.i.), granulomatous reactivity in the intestines and liver explored by light microscopy was considerably diminished as compared with that in shorter primary infection (16 weeks), likely reflecting lowered worm fecundity over time. Granuloma appearances did not change markedly with the age of infection, and acute-stage granulomas characteristic for early patency, with Splendore-Hoeppli reactions and prominent eosinophilia (Lindberg et al., 1995, 1997) were sparse, which can be explained by the fact that the goats were examined as late as 16-52 weeks of infection. A surprising finding was that the proportion of intestinal granulomas with mature intact eggs was significantly higher in the group with the oldest infection (P52) than in any other infection group. This was not observed in previous studies on caprine *S. bovis* infection of shorter duration (Lindberg et al., 1993, 1997). The finding might indicate diminished egg destruction in the granuloma as the infection

grows older, possibly due to altered immune reactivity within the granuloma.

In *S. bovis*-infected goats, infiltrates of mononuclear inflammatory cells may occur around intestinal submucosal small veins and venules, most prominently in early patency. Though reactions against schistosome antigens seem likely, the antigen-specific reactivity of the infiltrates is unknown (Lindberg et al., 1999). In the present study, similar perivascular cell infiltrates consisting of lymphocytes, plasma cells and few eosinophils was a common finding. Interestingly, the perivascular inflammation was as prominent at 52 weeks of infection, when egg excretion had been minimized for over four months, as in infection of shorter duration. Furthermore, infiltrates of eosinophils in the hepatic portal triads were as abundant in the P52 group as in the others. These apparently longstanding intestinal and hepatic inflammatory reactions were never observed in goats investigated after treatment, emphasizing the possibility that they may target schistosome antigens.

Liver fibrosis is a typical feature of most schistosome infections, and is related to the immune responses to the eggs (Wilson et al., 2007). To the best of the author's knowledge, long-term studies on the resolution of liver fibrosis during infection and after chemotherapy in *S. bovis*-infected ruminants have not been published previously. In the present study, two different methods to assess the degree of fibrosis affecting the liver diffusely were used. At necropsy, 'generalized fibrosis' was assessed both visually and by the degree of firmness of the liver. Later, the amount of fibrous connective tissue of the portal tracts was examined by light microscopy. Gross examination indicated that the livers of goats in all infected groups were mildly to moderately fibrotic. By light microscopy, portal hepatic fibrosis, although not pronounced, was still apparent in the untreated goats at 36 weeks p. i., ten weeks after fecal egg output had declined to low levels. However, by week 52, portal connective tissue was significantly reduced and almost reached the level of the control group. The difference in results gained by the use of macroscopic and microscopic examination, respectively, might in part be due to effects on the gross appearance of livers of the perfusions for worm recovery that were performed post mortem in all infected goats.

4.1.2 Treatment effects

Praziquantel, an anthelmintic that has earlier proven effective against ruminant *S. bovis* (Bushara et al., 1982; Johansen et al., 1996 c), was used for treatment of the goats. The treatment efficacy was as high as 97.7%, resulting in an abrupt decline in egg excretion (< 10 epgf) one week after treatment, and to a dramatic reduction in the total tissue egg count of both the intestines and liver. The long-term beneficial effects of treatment were proved by that treated goats responded with a prompt improvement in weight gains, and with increasing hemoglobin values, as compared with untreated goats.

After chemotherapy, circulating anti-worm IgG and IgM decreased continuously, except for an IgG peak immediately after treatment. The latter phenomenon is consistent with previous results in goats with *S. bovis* and in mice with *S. mansoni*, where it has been proposed to reflect an immune response to antigens suddenly released from killed worms (Tawfik et al., 1986; Johansen et al., 1996 b). Anti-worm antibodies as well as anti-egg IgG levels were significantly reduced by week 20, i.e. seven weeks after treatment. In contrast to anti-worm IgM and anti-egg IgG levels, which both declined to control group values by week 26, anti-worm IgG levels remained significantly elevated compared to controls until the time of challenge infection ($P < 0.01-0.05$ vs controls).

The histopathological evaluation of treated goats revealed that only occasional perioval granulomas were present in the intestines and liver 23 and 39 weeks after treatment. Interestingly enough, plasma cell infiltration in the spleen was significantly higher in treated goats than in controls as late as 39 weeks after treatment (Group PT52 vs F: $P < 0.01$), suggesting remaining systemic immune responses several months after the infection had been eliminated. Fibrous scarring of livers was prominent in treated goats, but no negative effects of liver lesions were reflected in weight gains of these goats. Histopathological examination of a fibrous scar from a liver of a treated goat revealed two sites with a granulomatous reaction around necrotic and mineralized material with adjacent schistosome pigment, consistent with material from dead worms. Inflammatory responses, including fibrotic lesions, to dead worms ending up in the liver after treatment, have previously been shown within four weeks after treatment (Johansen et al., 1996 a). Apparently, cellular inflammatory reactions may persist as late as nine months post treatment. The finding is of interest based on hypotheses that protective immune responses after therapy in human schistosomiasis

may result from exposure to antigens released from dead worms in response to treatment (Woolhouse and Hagan, 1999; Mutapi, 2001). In contrast to fibrous scars, generalized portal hepatic fibrosis induced by infection was absent 23 weeks after chemotherapy. This was expected based on results from *S. japonicum* and *S. mansoni* infections of humans, mice and rabbits, where liver fibrosis has been shown to be reversible after chemotherapy (Dunn et al., 1994; Olds et al., 1996; Cheever et al., 2002; Andrade et al., 2004).

4.1.3 Resistance to challenge infection

It has earlier been shown that resistance to challenge infection expressed through an anti-fecundity effect is marked in goats when the time interval between primary and challenge infection is up to 16 weeks (Johansen et al., 1997). The present study demonstrated the same effect, also when challenge was delayed until the primary infection was 36 weeks old. The challenge infection established fully, but the egg deposition was markedly low, reflected by low fecal egg excretion and an only limited increase in the total tissue egg counts. These events were reflected by a relatively small, yet significant, further elevation of anti-egg IgG in serum. Weight gains and hemoglobin values were both unaffected by the challenge infection. It thus appears that in goats, the degree of chronicity of *S. bovis* infection is not an important factor for resistance to challenge infection. Furthermore, an almost complete anthelmintic elimination of the primary infection in week 13 did not diminish the ability of the goats to develop marked resistance to challenge infection given 23 weeks post treatment. The resistance after treatment was apparently again expressed through an anti-fecundity effect. However, the results actually indicated that a partial anti-worm effect may have contributed, since the challenge worm burden following a treated primary infection (group PTCh) was significantly reduced a. in comparison with that of the challenge control group (Ch), and b. compared with the difference between the worm burdens of the primary-infected and challenged group (PCh) and the primary control group (P36), i.e. the estimated net challenge worm burden of group PCh. Several immuno-epidemiological studies indicate that chemotherapy influences resistance also in human schistosomiasis, conferring augmented protection to reinfection after chemotherapy, associated with altered immune responses (Hagan et al., 1991; Roberts et al., 1993; Medhat et al., 1998; Karanja et al., 2002).

The sustained worm fecundity depression at challenge in both untreated and treated goats demonstrated in the present study may be a result of

immune responses that were maintained both at a very late stage of primary infection and after treatment. Apart from persisting histopathological immune cell reactions that could be shown both in late infection and after treatment, anti-worm IgG levels in serum also remained elevated in treated goats until the time of challenge infection. It has been shown in *S. bovis*-infected calves that immunization against *S. bovis* glutathione S-transferase worm antigen may significantly depress worm fecundity. Furthermore, egg counts were reduced after serum transfer from immune donors (Bushara et al., 1993, 1994). Hence, antibody production against worm antigens may be involved in immune mediated fecundity suppression. Thus, in the present study, persistent levels of serum anti-worm antibody at challenge might have reflected on-going anti-fecundity immune responses. If so, this should provide one explanation for the persistence of the fecundity depression phenomenon in challenge infection even several months post treatment.

4.2 Experiment II (Paper III)

Although granulomas formed around deposited eggs are key factors in disease caused by schistosome infections, only occasional studies of the immunology of the granulomatous response in ruminant schistosomiasis have been carried out. Those studies are limited to immunohistological characterization of immune cells in the inflammatory response in the intestine of *S. bovis*-infected goats or sheep, and indicate the importance of MHC class II-restricted immune events and of $\gamma\delta$ T cells in granuloma pathogenesis (Lindberg et al., 1999; Ferreras et al., 2000). No studies have explored the roles of different cytokines in granulomatous inflammation of schistosomiasis in any ruminant host. Such information should be essential for increasing the basic understanding of immunological scenarios in the infections, and increased knowledge in this field may be useful for development of treatment strategies and vaccines. This calls for further studies of the immune response. In this second experiment, goats were infected with *S. bovis* and the gene expression of selected cytokines in the small intestine and its lymph nodes was studied together with an immunohistological delineation of immune responses and histopathological observations.

4.2.1 Fecal egg excretion and histopathology

With the aim to elucidate possible variations in cytokine responses between various phases of infection, we chose to study the immune reactivity at the time-point that would coincide with very early patency, i.e. at the onset of

egg deposition, and at the time-point shortly after the peak in egg excretion when granulomatous reactivity should be marked (Lindberg et al., 1995; Johansen et al., 1997). The fecal egg excretion curves and histopathology indicated that the infection lengths of 7 and 13 weeks, respectively, were accurate in this respect. Egg excretion started between week 6 and 7 p.i., and peaked between week 8 and 12 p.i.. On histopathology, perioval granulomas and free eggs without any obvious adjacent inflammatory reaction were present in the intestine and mesenteric lymph node of both the 7 and 13 week infection groups. However, granuloma numbers were much higher at 13 weeks, particularly in the lymph node (Tables 4 and 5).

4.2.2 Immunohistology

Immunohistochemical characterization of cell phenotypes of the inflammatory response was primarily performed in order to provide background data of relevance for the cytokine analysis. Perioval granulomas in the intestine displayed MHC class II⁺ macrophages and epithelioid cells, mainly peripherally located CD3⁺ (mixed CD4⁺, CD8⁺ and WC1⁺) lymphocytes, and rare B (CD79⁺) cells. Overall, lymphocyte populations were predominated by CD4⁺ cells instead of WC1⁺ cells, but otherwise the findings were accordant with previous results in *S. bovis*-infected goats (Lindberg et al., 1999). The present study also confirmed earlier observations that eggs in the mucosa were surrounded by thin 'collars' of MHC class II⁺ cells of the macrophage type (Lindberg et al., 1999). In the present study, accumulations of CD4⁺ T cells around the eggs were also shown. The MHC class II⁺ and CD4⁺ cell clusters should indicate immune reactivity to the eggs, but whether the response may signal an initial step of an ovicidal granulomatous reaction, or possibly may serve to protect eggs in transit for excretion, is not known and deserves further study. Gamma/delta T cells are a remarkably large component of the T cell population in ruminants, and are considered important in the mucosal immune defenses of the gut (Mackay and Hein, 1991; Nanno et al., 2007). Gamma/delta T cells in cattle may express MHC class II, present antigen to CD4⁺ T cells and produce cytokines such as IL-2, IFN- γ and TNF- α (Hanby-Florida et al., 1996; Collins et al., 1996, 1998; Fikri et al., 2001; Sopp and Howard, 2001). In the present study, we noticed a substantial increase of WC1⁺ $\gamma\delta$ T cells in the lamina propria, beyond granulomas, of infected goats, suggesting a proliferation of this cell type. Though not previously recorded in schistosomiasis, proliferation/activation of WC1⁺ $\gamma\delta$ T cells of the gut has been shown in a variety of gastrointestinal infections of sheep or cattle, e.g., *Ostertagia ostertagi*, *Haemonchus contortus*, *Cryptosporidium parvum* and

Salmonella typhimurium (Almeria et al., 1997; Abrahamsen, 1998; Balic et al. 2000; Hedges et al., 2007).

In the lymph nodes, granulomas were similar to intestinal granulomas with respect to immune cell types found, differing only in that CD8+ cells were not demonstrable.

4.2.3 Cytokine responses

The immunology of granulomas in schistosomiasis has been studied mainly in mice infected with the human schistosome *S. mansoni*, where it is a complex process involving an array of cytokines produced by several cell types, including CD4+ Th cells. Th2 type cytokines, especially IL-4, are considered to play a primary role in granuloma development (Wynn and Cheever, 1995). Although IL-5 does not seem to be directly involved in granuloma formation (Rosa Brunet et al., 1999), it is required for the differentiation of eosinophils, which are major sources of IL-4 in granulomas (Rumbley et al., 1999). The Th1 type cytokines TNF- α , IL-2 and IFN- γ likely contribute to the granulomatous response, IL-2 by promoting the Th2 response (Amiri et al., 1992; Joseph and Boros, 1993; Wynn and Cheever, 1995; Rezende et al., 1997).

In goats, only few studies on Th1/Th2 polarization of cytokine responses to infectious agents are on record. Esteves et al. (2004) showed a biased Th1 response in goats after immunization against *Ehrlichia ruminantium*, and polarized Th1 and Th2 responses, associated with different disease status, were reported in caprine arthritis-encephalitis virus infection (Cheevers et al., 1997). In bovines, most T cell clones differ from those in mice in that they co-express Th1 and Th2 type cytokines, but biased type 1 and type 2 cytokine responses to specific antigens exist (Brown et al., 1998). Cattle mount biased Th1 or Th2 type immune responses to other types of granulomatous disease than schistosomiasis, such as paratuberculosis (Coussens, 2001) and tuberculosis (Welsh et al., 2005), and it may therefore be assumed that polarized cytokine responses in different stages of infection might occur also in ruminant *S. bovis* infection.

To the author's knowledge, there are no previous records of cytokine expression in the intestines in goats, and information on cytokine expression in caprine mesenteric lymph nodes is restricted to one *in vitro* study of stimulated lymph node cells in neonatal goats (Tourais-Esteves et al., 2008). In the present qRT-PCR study, it was decided to use FFPE tissues. The

choice of FFPE material over frozen material, which is more commonly used, was based on the superior morphological quality and larger size of specimens in formalin-fixed than in frozen tissue sections, facilitating studies correlating histopathology and cytokine gene expression. The potential of using FFPE material was also of interest since this kind of material would due to better morphology be preferred if combining qRT-PCR with other methods, such as laser capture microdissection, in future studies. A drawback, however, is that nucleic acids isolated from FFPE material are highly fragmented, so that primers producing a short enough PCR product to enable strong detection must be selected. An amplicon size of about 80 bp, earlier shown suitable for the analysis of RNA isolated from FFPE material (Lehmann and Kreipe, 2001), was used as a guideline.

For this first study on cytokine gene expression in *S. bovis*-infected goats, only relevant cytokines with available complete caprine mRNA sequences were included, thus restricting the choice to IL-4, IFN- γ , IL-2 and TNF- α . mRNA expression of all those cytokines were detected in both the jejunum and mesenteric lymph node in all goats. The results are presented in Tables 4 and 5, respectively, expressed as 2^{-C_t} (C_t = threshold cycle value). In both tissues, the level of expression differed markedly between the different cytokines. In both infected goats and control goats, TNF- α was the most expressed cytokine in the intestine and IL-4 was the most expressed in the lymph node, whereas IFN- γ was the least expressed in both tissues. Only few studies on the relative levels of gene expression of different cytokines in the intestines and mesenteric lymph nodes of other ruminants exist, but higher gene expression of TNF- α than IFN- γ has been shown in ileal tissue of uninfected control sheep in a study on natural paratuberculosis (Smeed et al., 2007).

The present study did not reveal any significant differences in cytokine gene expressions between uninfected and infected goats at the group level, but still indicated increased expression of Th1 cytokines in the intestine in three out of four goats at 13 weeks of infection (Table 4). The expression of both IFN- γ (goats no. 5 and 7) and IL-2 mRNA (goats no. 5, 7, and 8) was substantially increased, whereas the increase in expression of TNF- α (goat no. 8) was less pronounced. The result, though not supported by statistical significance, should suggest a role for these Th1 type cytokines in the intestinal inflammation at this stage of infection.

Table 4. Mean number of granulomas per histological section of the proximal jejunum, and gene expression of cytokines investigated in consecutive sections from the same tissue block in *Schistosoma bovis*-infected goats and uninfected control goats. C_t = threshold cycle in quantitative real-time RT-PCR

Group/Goat no.	No. of granulomas	Gene expression of cytokines ($2^{-C_t} \times 10^{-9}$)			
		IL-4	IFN- γ	IL-2	TNF- α
<i>Infected 7 weeks</i>					
1	3	44	0.36	6.97	104
2	0.7	49	0.22	5.71	109
3	1.7	37	0.40	6.91	87
4	3.7	43	0.30	5.96	92
<i>Infected 13 weeks</i>					
5	5	29	1.42	9.66	175
6	10.5	39	0.22	4.73	91
7	10.5	31	2.11	8.91	97
8	0.5	54	0.24	9.62	206
<i>Uninfected controls</i>					
9	0	33	0.16	4.69	167
10	0	81	0.02	0.49	10
11	0	41	0.19	6.08	149
12	0	29	0.58	4.67	110

Furthermore, expression of IFN- γ tended to correlate ($\rho = 0.56$; $P = 0.05$) with granuloma density, providing support for production of IFN- γ in the granulomas, but such correlation was not shown for the other Th1 type cytokines. An increased Th1 type response in the lamina propria beyond granulomatous lesions may have contributed to the increased cytokine expression in infected goats. Since WC1+ $\gamma\delta$ T cells may produce Th1 cytokines, the obvious expansion of the lamina propria $\gamma\delta$ T cell population might be of relevance in this context. In contrast, we could not demonstrate any difference in the gene expression of IL-4 in the intestine between uninfected and infected goats, independent of the duration of infection, indicating that this Th2 type cytokine may not play any part in the immune response to *S. bovis* infection in the intestine at the time-points studied.

Table 5. Number of granulomas per histological section of the mesenteric lymph node, and gene expression of cytokines investigated in consecutive sections of the same tissue block in *Schistosoma bovis*-infected goats and uninfected control goats. C_t = threshold cycle in quantitative real-time RT-PCR

Group/Goat no.	No. of granulomas	Gene expression of cytokines ($2^{-C_t} \times 10^{-9}$)			
		IL-4	IFN- γ	IL-2	TNF- α
<i>Infected 7 weeks</i>					
1	5	38	0.15	3.06	27
2	9	54	0.27	5.63	25
3	0	30	0.18	2.88	15
4	0	26	0.15	3.65	25
<i>Infected 13 weeks</i>					
5	35	43	0.17	3.56	33
6	6	32	0.03	2.07	18
7	16	28	0.09	1.91	23
8	14	43	0.12	4.92	39
<i>Uninfected controls</i>					
9	0	14	0.01	0.73	13
10	0	41	0.11	4.71	24
11	0	35	0.15	2.74	28
12	0	62	0.31	9.94	58

Studies on murine schistosomosis mansoni have revealed increased mRNA expression of both IFN- γ and IL-4 in mesenteric lymph nodes in the acute stage of infection, indicating that mesenteric lymph nodes are involved in the immune response to this infection (Henderson et al., 1991, 1992). In comparison, we could not demonstrate any obvious differences between control goats and infected goats in the mRNA expression of any of the cytokines explored in the mesenteric lymph nodes. The histology and immunohistology of the lymph nodes in infected goats did not indicate any hyperplastic changes of the lymphoid tissue, but the result is still unexpected since perioval lymph node granulomas were common in most goats, and the granulomas essentially conformed to those of the intestines both morphologically and with regard to cellular phenotypes investigated.

The results of this experiment have demonstrated the usefulness of qRT-PCR performed on FFPE material for measurement of gene expression of

cytokines in goat tissues. However, in order to get more reliable data for the comparison of cytokine gene expression in *S. bovis*-infected *vs* uninfected goats, future studies including larger numbers of animals would be required.

5 General conclusions

- Acquired resistance to both primary and challenge infection in *S. bovis* infections in goats persisted in long-standing infections, with anti-fecundity as the key effector mechanism. Furthermore, resistance to challenge was not abolished by anthelmintic elimination of the primary *S. bovis* infection, even when the time span between treatment of the goats and challenge infection was several months.
- Host immune responses, as reflected by humoral anti-worm antibodies and histopathological immune cell reactions, persisted both at very late primary *S. bovis* infection and after treatment. This may provide an explanation for the sustained anti-fecundity effect observed at challenge infection in the goats.
- Quantification of cytokine gene expressions suggested a role for the Th1 type cytokines IFN- γ , IL-2 and TNF- α in the inflammation of the intestine at thirteen weeks of *S. bovis* infection in goats, and indicated that IFN- γ may be expressed by cells in the *S. bovis* egg granuloma.

6 Suggestions for future research

Below, some suggestions for future research in relation to the present work are given.

- The sustained anti-fecundity resistance after treatment demonstrated in this thesis was observed after a single, moderate dose of cercariae. In order to better mimic natural transmission conditions, characterized by continuous low-level uptake of cercariae from water, it would be of interest to study effects of treatment on immunoregulatory responses after repeated low-level exposure to cercariae.
- T regulatory cells (Tregs) that are CD4+Foxp3+ have been shown to be present in significant amounts in granulomas in the liver and corresponding lymph nodes in *S. mansoni*-infected mice. In the present study, CD4+ T cells were demonstrated both in granulomas and around free eggs in the lamina propria, and it would be interesting to perform studies in order to investigate whether some of these cells could be Tregs.
- The fact that increased IL-4 gene expression was not demonstrated in the intestine and mesenteric lymph nodes of infected goats at the time-points studied, does not necessarily exclude Th2 type cytokine activity. It would therefore be of interest to expand the study on cytokine responses by studying other Th2 type cytokines.
- In order to investigate whether the demonstrated increases in cytokine gene expressions were antigen specific, it would be of interest to combine the method used in this study with measuring cytokine

responses after antigen specific stimulation of mesenteric lymph node cells or spleen cells *in vitro*.

- When studying cytokine responses to infection, it would be important to verify increased cytokine mRNA expression in target organs by protein detection *in situ*. Given that appropriate antibodies were available this might be accomplished by the use of immunohistochemistry.

7 References

- Abrahamsen, M. S., 1998. Bovine T cell responses to *Cryptosporidium parvum* infection. *Int. J. Parasitol.*, 28, 1083-1088.
- Agnew, A. M., Murare, H. M., Lucas, S. B. and Doenhoff, M. J., 1989. *Schistosoma bovis* as an immunological analogue of *S. haematobium*. *Parasite Immunol.*, 11, 329-340.
- Almeria, S., Canals, A., Zarlenga, D. S. and Gasbarre, L. C., 1997. Quantification of cytokine gene expression in lamina propria lymphocytes of cattle following infection with *Ostertagia ostertagi*. *J. Parasitol.*, 83, 1051-1055.
- Amiri, P., Locksley, M., Parslow, T. G., Sadick, M., Rector, E., Ritter, D. and McKerrow, J. H., 1992. Tumor necrosis factor α restores granulomas and induces parasite egg-laying in schistosome-infected SCID mice. *Nature*, 356, 604-607.
- Andrade, Z. A., 1994. Extracellular matrix degradation in parasitic diseases. *Braz. J. Med. Biol. Res.*, 27, 2273-2281.
- Andrade, Z. A., 2004. Schistosomal hepatopathy. *Mem. Inst. Oswaldo Cruz*, 99, 51-57.
- Andrews, P., Thomas, H., Pohlke, R. and Seubert, J., 1983. Praziquantel. *Med. Res. Rev.*, 3, 147-200.
- Anthony, R. M., Rutitzky, L. I., Urban Jr, J. F., Stadecker, M. J. and Gause, W. C., 2007. Protective immune mechanisms in helminth infection. *Nat. Rev. Immunol.*, 7, 975-987.
- Balemba, O. B., Mbassa, G. K., Assey, R. J., Kahwa, C. K. B., Makundi, A. E., Hay-Schmidt, A., Dantzer, V. and Semuguruka, W. D., 2000. Lesions of the enteric nervous system and the possible role of mast cells in the pathogenic mechanisms of migration of schistosome eggs in the small intestine of cattle during *Schistosoma bovis* infection. *Vet. Parasitol.*, 90, 57-71.
- Balic, A., Bowles, V. M. and Meeusen, E. N. T., 2000. Cellular profiles in the abomasal mucosa and lymph node during primary infection with *Haemonchus contortus* in sheep. *Vet. Immunol. Immunopathol.*, 75, 109-120.
- Becker, B., Melhorn, H., Andrews, P., Thomas, H. and Eckert, J., 1980. Light and electron microscopic studies of the effect of praziquantel on *Schistosoma mansoni*, *Dicrocoelium dendriticum* and *Fasciola hepatica* (Trematoda) *in vitro*. *Z. Parasitenkd.*, 63, 113-128.
- Beckstead, J. H., 1994. A simple technique for preservation of fixation-sensitive antigens in paraffin embedded tissues. *J. Histochem. Cytochem.*, 42, 1127-1134.

- Bell, D. R., 1963. A new method for counting *Schistosoma mansoni* eggs in feces. With special reference to therapeutic trials. *Bull. W. H. O.*, 29, 525-530.
- Bjørneboe, A. and Frandsen, F., 1979. A comparison of the characteristics of two strains of *Schistosoma intercalatum* Fisher, 1934 in mice. *J. Helminthol.*, 53, 195-203.
- Boulanger, D., Schneider, D., Chippaux, J.-P., Sellin, B. and Capron, A., 1999. *Schistosoma bovis*: vaccine effects of a recombinant homologous glutathione S-transferase in sheep. *Int. J. Parasitol.*, 29, 415-418.
- Boulanger, D., Trottein, F., Mauny, F., Bremond, P., Couret, D., Pierce, R. J., Kadri, S., Godin, C., Sellin, E., Lecocq, J. P., Sellin, B. and Capron, A., 1994. Vaccination of goats against the trematode *Schistosoma bovis* with a recombinant homologous schistosoma-derived glutathione S-transferase. *Parasite Immunol.*, 16, 399-406.
- Bradley, D. J., 1965. The measurement of bilharziasis prevalence and schistosomal egg output. Aims and techniques, with an account of a field method. *Bull. W. H. O.*, 33, 503-508.
- Brown, W. C., Rice-Ficht, A. C. and Estes, D. M., 1998. Bovine type 1 and type 2 responses. *Vet. Immunol. Immunopathol.*, 63, 45-55.
- Bushara, H. O., Bashir, M. E. N., Malik, K. H. E., Mukhtar, M. M., Trottein, F., Capron, A. and Taylor, M. G., 1993. Suppression of *Schistosoma bovis* egg production in cattle by vaccination with either glutathione S-transferase or keyhole limpet haemocyanin. *Parasite Immunol.*, 15, 383-390.
- Bushara, H. O., Hussein, M. F., Majid, M. A., Musa, B. E. H. and Taylor, M. G., 1983 a. Observations on cattle schistosomiasis in the Sudan, a study in comparative medicine. IV. Preliminary observations on the mechanisms of naturally acquired resistance. *Am. J. Trop. Med. Hyg.*, 32, 1065-1070.
- Bushara, H. O., Gameel, A. A., Majid, B. Y. A., Khitma, I., Haroun, E. M., Karib, E. A., Hussein, M. F. and Taylor, M. G., 1983 b. Observations on cattle schistosomiasis in the Sudan, a study in comparative medicine. VI. Demonstration of resistance to *Schistosoma bovis* challenge after a single exposure to normal cercariae or to transplanted adult worms. *Am. J. Trop. Med. Hyg.*, 32, 1375-1380.
- Bushara, H. O., Majid, B. Y. A., Majid, A. A., Khitma, I., Gameel, A. A., Karib, E. A., Hussein, M. F. and Taylor, M. G., 1983 c. Observations on cattle schistosomiasis in the Sudan, a study in comparative medicine. V. The effect of praziquantel therapy on naturally acquired resistance to *Schistosoma bovis*. *Am. J. Trop. Med. Hyg.*, 32, 1370-1374.
- Bushara, H. O., Hussein, M. F., Majid, M. A. and Taylor, M. G., 1982. Effects of praziquantel and metrifonate on *Schistosoma bovis* infections in Sudanese cattle. *Res. Vet. Sci.*, 33, 125-126.
- Bushara, H. O., Majid, M. A., Saad, A. M., Hussein, M. F., Taylor, M. G., Dargie, J. D., Marshall, T. F. and Nelson, G. S., 1980. Observations on cattle schistosomiasis in the Sudan, a study in comparative medicine. II. Experimental demonstration of naturally acquired resistance to *Schistosoma bovis*. *Am. J. Trop. Med. Hyg.*, 29, 442-451.
- Bushara, H. O., Omer, O. H., Malik, K. H. and Taylor, M. G., 1994. The effect of multiple transfer of immune serum on maturing *Schistosoma bovis* infections in calves. *Parasitol. Res.*, 80, 198-202.

- Butterworth, A. E., 1993. Immunology of schistosomiasis. In: Jordan, P., Webbe, G., Sturrock, R. F. (eds): *Human schistosomiasis*. CAB International, Wallingford, Oxon, UK, pp. 331-366.
- Butterworth, A. E., Vadas, M. A., Wassom, D. L., Dessein, A., Hogan, M., Sherry, B., Gleich, G. J. and David, J. R., 1979. Interactions between human eosinophils and schistosomula of *Schistosoma mansoni*. II. The mechanisms of irreversible eosinophil adherence. *J. Exp. Med.*, 150, 1456-1471.
- Capron, M. and Capron, A., 1986. Rats, Mice and Men – Models for immune effector mechanisms against schistosomiasis. *Parasitol. Today*, 2, 69-75.
- Capron, A., Riveau, G., Capron, M. and Trottein, F., 2005. Schistosomes: the road from host-parasite interactions to vaccines in clinical trials. *Trends Parasitol.*, 21, 143-149.
- Cheever, A. W., Lenzi, J. A., Lenzi, H. L. and Andrade, Z. A., 2002. Experimental models of *Schistosoma mansoni* infection. *Mem. Inst. Oswaldo Cruz*, 97, 917-940.
- Cheever, A. W. and Neafie, R. C., 2000. Schistosomiasis. In: Meyers, W. M. (ed): *Pathology of infectious diseases, vol. I: Helminthiases*. Armed Forces Institute of Pathology, US., pp 23-47.
- Cheevers, W. P., Beyer, J. C. and Knowles, D. P., 1997. Type 1 and type 2 cytokine gene expression by viral gp 135 surface protein-activated T lymphocytes in caprine arthritis-encephalitis lentivirus infection. *J. Virol.*, 71, 6259-6263.
- Chitsulo, L., Engels, D., Montresor, A. and Savioli, L., 2000. The global status of schistosomiasis and its control. *Acta Trop.*, 77, 41-51.
- Christensen, N. O., Mutani, A. and Frandsen, F., 1983. A review of the biology and transmission ecology of African bovine species of the genus *Schistosoma*. *Z. Parasitenkd.*, 69, 551-570.
- Cioli, D. and Pica-Mattoccia, L., 2003. Praziquantel. *Parasitol. Res.*, 90, 53-59.
- Collins, R. A., Sopp, P., Gelder, K. I., Morrison, W. I. and Howard, C. J., 1996. Bovine gamma/delta TcR+ T lymphocytes are stimulated to proliferate by autologous *Theileria annulata*-infected cells in the presence of interleukin-2. *Scand. J. Immunol.*, 44, 444-452.
- Collins, R. A., Werling, D., Duggan, S. E., Bland, A. P., Parsons, K. R. and Howard, C. J., 1998. $\gamma\delta$ T cells present antigen to CD4⁺ $\alpha\beta$ T cells. *J. Leukoc. Biol.*, 63, 707-714.
- Coussens, P. M., 2001. *Mycobacterium paratuberculosis* and the bovine immune system. *Anim. Health Res. Rev.*, 2, 141-161.
- Dargie, J. D., 1980. The pathogenesis of *Schistosoma bovis* infection in Sudanese cattle. *Trans. R. Soc. Trop. Med. Hyg.*, 74, 560-562.
- Dinnik, J. A. and Dinnik, N. N., 1965. The schistosomes of domestic ruminants in Eastern Africa. *Bull. Epizoot. Dis. Afr.*, 13, 341-359.
- Dunn, M. A., Cheever, A. W., Paglia, L. M., Kelly, E. P., Duvall, R. H., Andrade, Z. A. and Goldner, F. H., 1994. Reversal of advanced liver fibrosis in rabbits with schistosomiasis japonica. *Am. J. Trop. Med. Hyg.*, 50, 499-505.
- Dunne, D. W., Grabowska, A. M., Fulford, A. J., Butterworth, A. E., Sturrock, R. F., Koech, D. and Ouma, J. H., 1988. Human antibody responses to *Schistosoma mansoni*: the influence of epitopes shared between different life-cycle stages on the response to the schistosomulum. *Eur. J. Immunol.*, 18, 123-131.

- Dunne, D. W., Lucas, S., Bickle, Q., Pearson, S., Madgwick, L., Bain, J. and Doenhoff, M. J., 1981. Identification and partial purification of an antigen (omega 1) from *Schistosoma mansoni* eggs which is putatively hepatotoxic in T-cell deprived mice. *Trans. R. Soc. Trop. Med. Hyg.*, 75, 54-71.
- Esteves, I., Vachiéry, N., Martinez, D. and Totté, P., 2004. Analysis of *Ehrlichia ruminantium*-specific T1/T2 responses during vaccination with a protective killed vaccine and challenge of goats. *Parasite Immunol.*, 26, 95-103.
- Fallon, P. G. and Doenhoff, M. J., 1994. Drug-resistant schistosomiasis: resistance to praziquantel and oxamniquine induced in *Schistosoma mansoni* in mice is drug specific. *Am. J. Trop. Med. Hyg.*, 51, 83-88.
- Faulkner, D. E. and Epstein, H., 1957. *Publ. Colon. Adv. Comm. Agric. Anim. Hlth. For.*, No. 5, London, H. M. S. O.
- Ferreras, M. C., Garcia-Iglesias, M. J., Manga-Gonzalez, M. Y., Perez-Martinez, C., Mizinska, Y., Ramajo, V., Gonzalez-Lanza, M. C., Escudero, A. and Garcia-Marin, J. F., 2000. Histopathological and immunohistochemical study of lambs experimentally infected with *Fasciola hepatica* and *Schistosoma bovis*. *J. Vet. Med.*, 47, 763-773.
- Ferreras-Estrada, M. C., Garcia-Iglesias, M. J., Perez-Martinez, C., Manga-Gonzalez, M. Y., Ramajo-Martin, V., Escudero-Diez, A. and Garcia-Marin, J. F., 1998. A pathological study of experimental long-standing *Schistosoma bovis* infection in sheep. *J. Comp. Pathol.*, 119, 479-484.
- Fikri, Y., Denis, O., Pastoret, P.-P. and Nyabenda, J., 2001. Purified bovine WC1+ $\gamma\delta$ T lymphocytes are activated by staphylococcal enterotoxins and toxic shock syndrome toxin-1 superantigens: proliferation response, TCR V γ profile and cytokines expression. *Immunol. Lett.*, 77, 87-95.
- González, L., Anderson, I., Deane, D., Summers, C. and Buxton, D., 2001. Detection of immune system cells in paraffin wax-embedded ovine tissues. *J. Comp. Pathol.*, 125, 41-47.
- Grimaud, J. A., 1987. Cell-matrix interactions in schistosomal portal fibrosis: a dynamic event. *Mem. Inst. Oswaldo Cruz*, 82, Suppl. IV, 55-65.
- Hagan, P., Blumenthal, U. J., Dunn, D., Simpson, A. J. G. and Wilkins, A., 1991. Human IgE, IgG4 and resistance to reinfection with *Schistosoma haematobium*. *Nature*, 349, 243-245.
- Hagan, P., Moore, P. J., Adjuikiewicz, A. B., Greenwood, B. M. and Wilkins, H. A., 1985. In-vitro antibody-dependent killing of schistosomula of *Schistosoma haematobium* by human eosinophils. *Parasite Immunol.*, 7, 617-624.
- Hanby-Flarida, M. D., Okragly, A. J. and Baldwin, C. L., 1996. Autologous mixed leucocyte reaction and the polyclonal activation of bovine gamma/delta T cells. *Res. Vet. Sci.*, 61, 65-71.
- Hedges, J. F., Buckner, D. L., Rask, K. M., Kerns, H. M. M., Jackiw, L. O., Trunkle, T. C., Pascual, D. W. and Jutila, M. A., 2007. Mucosal lymphatic-derived $\gamma\delta$ T cells respond early to experimental *Salmonella* enterocolitis by increasing expression of IL-2R α . *Cell Immunol.*, 246, 8-16.
- Henderson, G. S., Conary, J. T., Summar, M., McCurley, T. L. and Colley, D. G., 1991. *In vivo* molecular analysis of lymphokines involved in the murine immune response during

- Schistosoma mansoni* infection. I. IL-4 mRNA, not IL-2 mRNA, is abundant in the granulomatous livers, mesenteric lymph nodes, and spleens of infected mice. *J. Immunol.*, 147, 992-997.
- Henderson, G. S., Lu, X., McCurley, T. L. and Colley, D. G., 1992. *In vivo* molecular analysis of lymphokines involved in the murine immune response during *Schistosoma mansoni* infection. II. Quantification of IL-4 mRNA, IFN- γ mRNA, and IL-2 mRNA levels in the granulomatous livers, mesenteric lymph nodes, and spleens during the course of modulation. *J. Immunol.*, 148, 2261-2269.
- Hernández, H. J., Wang, Y., Tzellas, N. and Stadecker, M. J., 1997. Expression of class II, but not class I, major histocompatibility complex molecules is required for granuloma formation in infection with *Schistosoma mansoni*. *Eur. J. Immunol.*, 27, 1170-1176.
- Hunter, J. M., Rey, L., Chu, K. Y., Adekolu-John, E. O. and Mott, K. E., 1993. Parasitic diseases in water resources development: the need for intersectoral negotiation. *World Health Organization*, Geneva.
- Hussein, M. F., 1971. The pathology of experimental schistosomiasis in calves. *Res. Vet. Sci.*, 12, 246-252.
- Hussein, M. F., Bushara, H. O. and Ali, K. E., 1976. The pathology of experimental *Schistosoma bovis* infection in sheep. *J. Helminthol.*, 50, 235-241.
- Hussein, M. F., Tartour, G., Imbabi, S. E. and Ali, K. E., 1975. The pathology of naturally-occurring bovine schistosomiasis in the Sudan. *Ann. Trop. Med. Parasitol.*, 69, 217-225.
- Iacomini, J., Ricklan, D. E. and Stadecker, M. J., 1995. T cells expressing the gamma delta T cell receptor are not required for egg granuloma formation in schistosomiasis. *Eur. J. Immunol.*, 25, 884-888.
- Ismail, M., Attia, M., Metweally, A. A., Farghaly, A. M., Bruce, J., Bennett, J., el-Badaway, A. A. and Hussein, M. H., 1994. Assessment of praziquantel therapy in treatment of *Schistosoma mansoni* infection. *J. Egypt. Soc. Parasitol.*, 24, 231-238.
- Janeway, C. A. and Medzhitov, R., 2002. Innate immune recognition. *Annu. Rev. Immunol.*, 20, 197-216.
- Johansen, M. V., Monrad, J., Christensen, N. O. and Lindberg, R., 1996 a. Experimental *Schistosoma bovis* infection in goats: pathological consequences of praziquantel treatment. *J. Comp. Pathol.*, 115, 1-11.
- Johansen, M. V., Fillié, Y., Monrad, J., Christensen, N. O. and Deelder, A., 1996 b. Experimental *Schistosoma bovis* infection in goats. Circulating antigen and antibody responses to egg and adult worm antigens during infection and following treatment with praziquantel. *Parasitology*, 113, 367-375.
- Johansen, M.V., Monrad, J. and Christensen, N. O., 1996 c. Effects of praziquantel on experimental *Schistosoma bovis* infection in goats. *Vet. Parasitol.*, 62, 83-91.
- Johansen, M. V., Monrad, J., Christensen, N. O. and Lindberg, R., 1997. The impact of primary *Schistosoma bovis* infection on a subsequent challenge infection in goats. *J. Parasitol.*, 83, 242-246.
- Joseph, A. L. and Boros, D. L., 1993. Tumor necrosis factor plays a role in *Schistosoma mansoni* egg-induced granulomatous inflammation. *J. Immunol.*, 151, 5461-5471.
- Karanja, D. M. S., Hightower, A. W., Colley, D. G., Mwinzi, P. N. M., Galil, K., Andove, J. and Secor, W. E., 2002. Resistance to reinfection with *Schistosoma mansoni* in

- occupationally exposed adults and effect of HIV-1 co-infection on susceptibility to schistosomiasis: a longitudinal study. *Lancet*, 360, 592-596.
- Kassai, T., Cordero del Campillo, M., Euzeby, J., Gaafar, S., Hiepe, Th. And Himonas, C. A., 1988. Standardized nomenclature of animal parasitic diseases (SNOAPAD). *Vet. Parasitol.*, 29, 299-326.
- Kassuku, A. A., 1985. African ruminant schistosomiasis. Studies on the epizootiology and clinical pathology of *Schistosoma bovis* infections. *Ph.D. Thesis*, Royal Veterinary and Agricultural University, Copenhagen, Denmark.
- Kassuku, A. A., Christensen, N. O., Nansen, P. and Monrad, J., 1986. Clinical pathology of *Schistosoma bovis* infection in goats. *Res. Vet. Sci.*, 40, 44-47.
- Kassuku, A. A., Nansen, P. and Christensen, N. O., 1985. A comparison of the efficiency of the percutaneous and per-oral routes of infection in caprine *Schistosoma bovis* infections. *J. Helminthol.*, 59, 23-28.
- Kay, B., 1990. Vector-borne disease implications and their control. *Waterlines*, 9, 3-6.
- King, C. H. and Mahmoud, A. F., 1989. Drugs five years later: Praziquantel. *Ann. Intern. Med.*, 110, 290-296.
- Labbo, R., Boulanger, D., Brémond, P. and Chippaux, J. P., 2007. Infestation expérimentale de caprins par *Schistosoma bovis* et *S. curassoni*: effets pathogènes comparés. *Parasite*, 14, 77-82.
- Lardans, V. and Dissous, C., 1998. Snail control strategies for reduction of schistosomiasis transmission. *Parasitol. Today*, 14, 413-417.
- Lehmann, U. and Kreipe, H., 2001. Real-time PCR analysis of DNA and RNA extracted from formalin-fixed and paraffin-embedded biopsies. *Methods*, 25, 409-418.
- Lengy, J., 1962 a. Studies on *Schistosoma bovis* (Sonsino, 1876) in Israel. I. Larval stages from egg to cercaria. *Bull. Res. Counc. Israel*, 10E, 1-36.
- Lengy, J., 1962 b. Studies on *Schistosoma bovis* (Sonsino, 1876) in Israel. II. The intramammalian phase of the life cycle. *Bull. Res. Counc. Israel*, 10E, 73-96.
- Lindberg, R., Johansen, M. V., Monrad, J., Christensen, N. O. and Nansen, P., 1997. Experimental *Schistosoma bovis* infection in goats: the inflammatory response in the small intestine and liver in various phases of infection and reinfection. *J. Parasitol.*, 83, 454-459.
- Lindberg, R., Johansen, M. V., Nilsson, C. and Nansen, P., 1999. An immunohistological study of phenotypic characteristics of cells of the inflammatory response in the intestine of *Schistosoma bovis*-infected goats. *Parasitology*, 118, 91-99.
- Lindberg, R., Monrad, J., Johansen, M. V., Christensen, N. O. and Nansen, P., 1993. Histopathology of experimental *Schistosoma bovis* infection in goats. *Acta Vet. Scand.*, 34, 183-191.
- Lindberg, R., Monrad, J., Johansen, M. V., Christensen, N. O. and Nansen, P., 1995. Tissue response of goats to single or repeated low-level doses and to a massive challenge dose of *Schistosoma bovis*. *Res. Vet. Sci.*, 58, 56-60.
- Littell, R. C., Milliken, G. A., Stroup, W. W. and Wolfinger, R. D., 1996. SAS[®] System for Mixed Models, Cary, NC: *SAS Institute Inc.*, 633 pp.
- Mackay, C. R. and Hein, W. R., 1991. Prominence of gamma delta T cells in the ruminant immune system. *Immunol. Today*, 12, 30-34.

- Majid, A. A., Marshall, T. F., Hussein, M. F., Bushara, H. O., Taylor, M. G., Nelson, G. S. and Dargie, J. D., 1980. Observations on cattle schistosomiasis in the Sudan, a study in comparative medicine. I. Epizootiological observations on *Schistosoma bovis* in the White Nile Province. *Am. J. Trop. Med. Hyg.*, 29, 435-441.
- Makundi, A. E., Kassuku, A. A., Maselle, R. M., Boa, M. E., 1998. Distribution, prevalence and intensity of *Schistosoma bovis* infection in cattle in Iringa district, Tanzania. *Vet. Parasitol.*, 75, 59-69.
- Malek, E. A., 1980. *Snail-transmitted parasitic diseases, vol I*. CRC Press Inc., Boca Raton, Florida, 222 pp.
- Massoud, J., 1973. Parasitological and pathological observations on *Schistosoma bovis* Sonsino, 1876, in calves, sheep and goats in Iran. *J. Helminthol.*, XLVII, 155-164.
- Mathew, R. C. and Boros, D. L., 1986. Anti-L3T4 antibody treatment suppresses granuloma formation and abrogates antigen induced interleukin-2 production in *Schistosoma mansoni* infection. *Infect. Immun.*, 54, 820-826.
- McCauley, E. H., Majid, A. A. and Tayeb, A., 1984. Economic evaluation of the production impact of bovine schistosomiasis and vaccination in the Sudan. *Prev. Vet. Med.*, 2, 735-754.
- McCauley, E. H., Majid, A. A., Tayeb, A. and Bushara, H. O., 1983. Clinical diagnosis of schistosomiasis in Sudanese cattle. *Trop. Anim. Health Prod.*, 15, 129-136.
- Medhat, A., Shehata, M., Bucci, K., Mohamed, S., Dief, A. D. E., Badary, S., Galal, H., Nafeh, M. and King, C. L., 1998. Increased interleukin-4 and interleukin-5 production in response to *Schistosoma haematobium* adult worm antigens correlates with lack of reinfection after treatment. *J. Infect. Dis.*, 178, 512-519.
- Melhorn, H., Becker, B., Andrews, P., Thomas, H. and Frenkel, J. K., 1981. *In vivo* and *in vitro* experiments on the effects of praziquantel on *Schistosoma mansoni*. A light and electron microscopic study. *Arzneimittelforschung/Drug Res.*, 31, 544-554.
- Moloney, N. A., Hinchcliffe, P. and Webbe, G., 1987. Loss of resistance to reinfection with *Schistosoma japonicum* in mice after treatment with praziquantel. *Trans. R. Soc. Trop. Med. Hyg.*, 81, 247-254.
- Monrad, J., Christensen, N. O. and Nansen, P., 1991. Acquired resistance in goats following a single primary *Schistosoma bovis* infection. *Acta Trop.*, 48, 69-77.
- Monrad, J., Christensen, N. O., Nansen, P., Johansen, M. V. and Lindberg, R., 1995. Acquired resistance against *Schistosoma bovis* after single or repeated low-level primary infections in goats. *Res. Vet. Sci.*, 58, 42-45.
- Mosmann, T. R. and Coffman, R. L., 1989. TH1 and Th2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.*, 7, 145-173.
- Mutapi, F., 2001. Heterogeneities in anti-schistosome humoral responses following chemotherapy. *Trends Parasitol.*, 17, 518-524.
- Nanno, M., Shiohara, T., Yamamoto, H., Kawakami, K. and Ishikawa, H., 2007. $\gamma\delta$ T cells: firefighters or fire boosters in the front lines of inflammatory responses. *Immunol. Rev.*, 215, 103-113.

- Nyame, A. K., Kwar, Z. S. and Cummings, R. D., 2004. Antigenic glycans in parasitic infections: implications for vaccines and diagnostics. *Arch. Biochem. Biophys.*, 426, 182-200.
- Nyame, A. K., Lewis, F. A., Doughty, B. L., Correa-Oliveira, R. and Cummings, R. D., 2003. Immunity to schistosomiasis: glycans are potential antigenic targets for immune intervention. *Exp. Parasitol.*, 104, 1-13.
- Olaechea, F. V., Christensen, N. O. and Henriksen, S. A., 1990. A comparison of the filtration, concentration, and thick smear techniques in the diagnosis of *Schistosoma bovis* infection in cattle and goats. *Acta Trop.*, 47, 217-221.
- Olds, G. R., Olveda, R., Wu, G., Wiest, P., McGarvey, S., Aligui, G., Zhang, S., Ramirez, B., Daniel, B., Peters, P., Romulo, R., Fevidal, P., Tiu, W., Yuan, J., Domingo, E. and Blas, B., 1996. Immunity and morbidity in schistosomiasis japonicum infection. *Am. J. Trop. Med. Hyg.*, 55, 121-126.
- Oleaga, A. and Ramajo, V., 2004. Efficiency of the oral, intramuscular and subcutaneous routes for the experimental infection of hamster and sheep with *Schistosoma bovis*. *Vet. Parasitol.*, 124, 43-53.
- Pearce, E. J., Basch, P. F. and Sher, A., 1986. Evidence that the reduced surface antigenicity of developing *Schistosoma mansoni* schistosomula is due to antigen shedding rather than host molecule acquisition. *Parasite Immunol.*, 8, 79-94.
- Pearce, E. J. and MacDonald, A. S., 2002. The immunobiology of schistosomiasis. *Nat. Rev. Immunol.*, 2, 499-511.
- Pérez-Sánchez, R., Ramajo-Hernández, A., Ramajo-Martin, V. and Oleaga, A., 2006. Proteomic analysis of the tegument and excretory-secretory products of adult *Schistosoma bovis* worms. *Proteomics*, 6, 226-236.
- Pitchford, R. J., 1977. A check list of definitive hosts exhibiting evidence of the genus *Schistosoma* Weinland, 1858 acquired naturally in Africa and the Middle East. *J. Helminthol.*, 51, 229-252.
- Pitchford, R. J. and Visser, P. S., 1975. A simple and rapid technique for quantitative estimation of helminth eggs in human and animal excreta with special reference to *Schistosoma* sp. *Trans. R. Soc. Trop. Med. Hyg.*, 69, 318-322.
- Ramajo-Hernández, A., Oleaga, A., Ramajo-Martin, V. and Pérez-Sánchez, R., 2007. Carbohydrate profiling and protein identification of the tegumental and excreted/secreted glycoproteins of adult *Schistosoma bovis* worms. *Vet. Parasitol.*, 144, 45-60.
- Rezende, S. A., Oliveira, V. R., Silva, A. M., Alves, J. B., Goes, A. M. and Reis, L. F., 1997. Mice lacking the gamma interferon receptor have an impaired granulomatous reaction to *Schistosoma mansoni* infection. *Infect. Immun.*, 65, 3457-3461.
- Roberts, M., Butterworth, A. E., Kimani, G., Kamau, T., Fulford, A. J. C., Dunne, D. W., Ouma, J. H. and Sturrock, R. F., 1993. Immunity after treatment of human schistosomiasis: association between cellular responses and resistance to reinfection. *Infect. Immun.*, 61, 4984-4993.
- Rodriguez-Osorio, M., Gomez-Garcia, V., Rojas, J. and Ramajo-Martin, V., 1999. Humoral immune response and antigenemia in sheep experimentally infected with *Schistosoma bovis*. Cross-reactivity with *Fasciola hepatica* antigens. *J. Parasitol.*, 85, 585-587.

- Rosa Brunet, L., Sabin, E. A., Cheever, A. W., Kopf, M. A. and Pearce, E. J., 1999. Interleukin 5 (IL-5) is not required for expression of a Th2 response or host resistance mechanisms during murine schistosomiasis mansoni but does play a role in development of IL-4-producing non-T, non-B cells. *Infect. Immun.*, 67, 3014-3018.
- Rumbley, C. A., Sugaya, H., Zekavat, S. A., El Refaei, M., Perrin, P. J. and Phillips, S. M., 1999. Activated eosinophils are the major source of Th2-associated cytokines in the schistosome egg granuloma. *J. Immunol.*, 162, 1003-1009.
- Saad, A. M., Hussein, M. F., Dargie, J. D. and Taylor, M. G., 1984 a. The pathogenesis of experimental *Schistosoma bovis* infections in Sudanese sheep and goats. *J. Comp. Pathol.*, 94, 371-385.
- Saad, A. M., Hussein, M. F., Bushara, H. O., Dargie, J. D. and Taylor, M. G., 1984 b. Erythokinetics and albumin metabolism in primary experimental *Schistosoma bovis* infections in zebu cattle. *J. Comp. Pathol.*, 94, 249-262.
- Saad, A. M., Hussein, M. F., Dargie, J. D., Taylor, M. G. and Nelson, G. S., 1980. *Schistosoma bovis* in calves: the development and clinical pathology of primary infections. *Res. Vet. Sci.*, 28, 105-111.
- SAS Institute Inc., 1999. SAS Version 8(2). Cary, NC: SAS Institute Inc.
- SAS Institute Inc. 2004. SAS/STAT 9.1 User's Guide. Cary, NC: SAS Institute Inc.
- Schild, H., Mavaddat, N., Litzenberger, C., Ehrlich, E. W., Davis, M. M., Bluestone, J. A., Matis, L., Draper, R. K. and Chien, Y. H., 1994. The nature of major histocompatibility complex recognition by gamma delta T cells. *Cell*, 76, 29-37.
- Semuguruka, W. D., 1992. Intestinal lesions associated with transmucosal migration of eggs in calves and hamsters infected with *Schistosoma bovis*. A light and electron microscopic study. *PhD thesis*, Dept. of Pharmacology and Pathobiology, Royal Veterinary and Agricultural University, Copenhagen, Denmark.
- Smede, J. A., Watkins, C. A., Rhind, S. M. and Hopkins, J., 2007. Differential cytokine gene expression profiles in the three pathological forms of sheep paratuberculosis. *BMC Vet. Res.*, 3, doi: 10.1186/1746-6148-3-18.
- Smithers, S. R. and Terry, R. J., 1969. Immunity in schistosomiasis. VI. Immunological aspects. *Ann. N.Y. Acad. Sci.*, 160, 826-840.
- Sonsino, P., 1876. Intorno ad un nuova parassito del bue (*Bilharzia bovis*). *Re. Accad. Sc. Naples.*, 15, 84-87.
- Sopp, P. and Howard, C. J., 2001. IFN γ and IL-4 production by CD4, CD8 and WC1 $\gamma\delta$ TCR⁺ T cells from cattle lymph nodes and blood. *Vet. Immunol. Immunopathol.*, 81, 85-96.
- Stavitsky, A. B., 2004. Regulation of granulomatous inflammation in experimental models of schistosomiasis. *Infect. Immun.*, 72, 1-12.
- Tawfik, A. F., Carter, C. E., Colley, D. G., 1986. Effects of anti-schistosomal chemotherapy on immune responses, protection and immunity. I. Changes in cellular and humoral responses. *Am. J. Trop. Med. Hyg.*, 35, 100-109.
- Taylor, M. G., 1987. (Ed. Soulsby, E. J. L.) *Immune responses in parasitic infections, vol II: trematodes and cestodes*. CRC Press, Boca Raton, Florida, 49-90 pp.

- Teesdale, C. M. and Amin, M. A., 1976. Comparison of the Bell technique, a modified Kato thick smear technique, and a digestion method for the field diagnosis of schistosomiasis mansoni. *J. Helminthol.*, 50, 17-20.
- Tourais-Esteves, I., Bernardet, N., Lacroix-Lamandé, S., Ferret-Bernard, S. and Laurent, F., 2008. Neonatal goats display a stronger TH1-type cytokine response to TLR ligands than adults. *Dev. Comp. Immunol.*, 32, 1231-1241.
- Uribe, N., Muro, A., Vieira, C., Lopez-Aban, J., del Olmo, E., Suárez, L., Martínez-Fernández, A. R. and Siles-Lucas, M., 2007 a. Genetic and immunological characterization of the 14-3-3 ζ molecule from *Schistosoma bovis*. *J. Parasitol.*, 93, 964-969.
- Uribe, N., Siles-Lucas, M., López-Abán, J., Esteban, A., Suarez, L., Martínez-Fernández, A. R., del Olmo, E. and Muro, A., 2007 b. The Sb 14-3-3 ζ recombinant protein protects against *Schistosoma bovis* in BALB/c mice. *Vaccine*, 25, 4533-4539.
- van der Kleij, D., Latz, E., Brouwers, J. F. H. M., Kruize, Y. C. M., Schmitz, M., Kurt-Jones, E. A., Espevik, T., de Jong, E. C., Kapsenberg, M. L., Golenbock, D. T., Tielens, A. G. M., and Yazdanbakhsh, M., 2002. A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization. *J. Biol. Chem.*, 277, 48122-48129.
- van Wyk, J. A., Heitmann, A. L. P. and van Rensburg, L. J., 1975. Studies on schistosomiasis. 7. A comparison of various methods for the infestation of sheep with *Schistosoma mattheei*. *Onderstepoort J. Vet. Res.*, 42, 71-74.
- von Lichtenberg, F., 1964. Studies on granuloma formation. III. Antigen sequestration and destruction in the schistosome pseudotubercle. *Am. J. Pathol.*, 45, 75-94.
- von Lichtenberg, F., Smith, J. H. and Cheever, A. W., 1966. The Hoespli phenomenon in schistosomiasis. Comparative pathology and immunopathology. *Am. J. Trop. Med. Hyg.*, 15, 886-895.
- Warren, K. S., 1973. The pathology of schistosome infections. *Helminthol. Abstr. Ser. A.*, 42, 591-633.
- Warren, K. S. and Domingo, E. O., 1970. Granuloma formation around *Schistosoma mansoni*, *S. haematobium* and *S. japonicum* eggs. Size and rate of development, cellular composition, cross-sensitivity, and rate of egg destruction. *Am. J. Trop. Med. Hyg.*, 19, 292-304.
- Warren, K. S., Domingo, E. O. and Cowan, R. B. T., 1967. Granuloma formation around schistosome eggs as a manifestation of delayed type hypersensitivity. *Am. J. Pathol.*, 51, 735-756.
- Weinstock, J. V., 1992. The pathogenesis of granulomatous inflammation and organ injury in schistosomiasis: interactions between the schistosome ova and the host. *Immunol. Invest.*, 21, 455-475.
- Welsh, M. D., Cunningham, R. T., Corbett, D. M., Girvin, R. M., McNair, J., Skuce, R. A., Bryson, D. G. and Pollock, J. M., 2005. Influence of pathological progression on the balance between cellular and humoral immune responses in bovine tuberculosis. *Immunology*, 114, 101-111.
- Wilson, M. S., Mentink-Kane, M. M., Pesce, J. T., Ramalingam, T. R., Thompson, R. and Wynn, T. A., 2007. Immunopathology of schistosomiasis. *Immunol. Cell Biol.*, 85, 148-154.

- Woolhouse, M. E. J. and Hagan, P., 1999. Seeking the ghost of worms past. *Nat. Med.*, 5, 1225-1227.
- Wynn, T. A. and Cheever, A. W., 1995. Cytokine regulation of granuloma formation in schistosomiasis. *Curr. Opin. Immunol.*, 7, 505-511.
- Xiao, S. H., Friedman, P. A., Catto, B. A. and Webster, L. T., 1984. Praziquantel-induced vesicle formation in the tegument of male *Schistosoma mansoni* is calcium dependent. *J. Parasitol.*, 70, 177-179.

8 Acknowledgements

The work in this thesis was performed at the Division of Pathology, Pharmacology and Toxicology, Department of Biomedical Sciences and Veterinary Public Health, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), with the support of former and present heads of the department: Lennart Jönsson, Martin Wierup and Leif Norrgren, and in collaboration with the Danish Centre for Experimental Parasitology and DBL-Centre for Health Research and Development, Faculty of Life Sciences, University of Copenhagen.

Financial support was provided by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (Formas) and the Danish National Research Foundation.

I would like to express my sincere gratitude to co-workers, friends and family who have all in different ways contributed to this work. I wish to thank:

Ronny Lindberg, my main supervisor, for welcoming me into this project, thus introducing me to the world of science as such, and to the scientific field of schistosomosis in particular. Thank you for guidance, and for all the effort that you have put into reading and revising manuscripts. I am truly grateful for having had the opportunity to be taught by you in scientific thinking and writing.

Jesper Monrad, my co-supervisor, for letting me use material from your excellent experiment in my work, for setting up a new experiment regarding to our wishes, and for teaching me about parasitological techniques.

Maria Hurst, my co-supervisor, for the important contribution you made to this work by joining our group towards the end, bringing with you your specific knowledge and experience into the project.

Maria V. Johansen, my co-author, for help with the serological study and for always being supportive and ready to give valuable comments on my work. Thank you also for hospitality during my visit in Copenhagen.

Niels Ørnbjerg, my co-author, for your commitment in the preparation of the first paper.

Jonas Tallkvist, my co-author, for teaching me how to run PCRs and for all other help with the PCR study.

Els Meeusen, **Maija-Leena Eloranta** and **Caroline Fossum**, for valuable advice on issues related to immunology, and **Els** also for generously sharing antibodies with us.

Briitta Ojava, **Ulla Hammarström**, **Åsa Gessbo** and **Agneta Boström** for skilful technical assistance in histology and immunohistochemistry, and **Agneta** also for teaching me how to perform immunohistochemistry, and for your patience with an endless number of re-runs.

Katarina Näslund, for kindly teaching me how to run the ELISA.

Birgitta Vegerfors, for excellent help with statistics.

Gunnel Erne and **Agneta Lind**, for great library service.

Anne-Sofie Lundquist and **Birgitta Berthas** for kind help with practical matters.

Lena Olsén, my 'Excel-guru', for always taking the time to work out solutions to my questions.

Eva Tydén and **Helena Öhrvik**, for valuable help and advice with the analysis of PCR results, and **Eva** also for help with figures etc. during the final preparation of this thesis.

Karolina Larsson, for the beautiful drawing of the *Schistosoma bovis* life cycle.

All former and present **PhD-students** and **residents** at the department, for all kinds of help, and for sharing the everyday joys and sorrows ;-)) of conducting research studies.

All other **staff-members** at the Division of Pathology, Pharmacology and Toxicology, for creating a pleasant work atmosphere.

My **friends**, for providing good times, laughs and support throughout these years.

My **relatives with families** for caring about me. Especially the **Sörén family** for help in various ways, such as taking care of children, photographing goats, help with the cover picture, etc., and my **Mother** for constant support and encouragement. I can always count on you.

Fredrik, for all your love and support throughout the years, and for always keeping a positive attitude, no matter what. It makes life so much easier!

Stella and **Fanny**, for brightening my days, just by being who you are! ♥