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Comparative Aspects of *Theileria lestoquardi* and *Theileria annulata* Infections of Sheep and Cattle

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SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

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Theileria annulata and *T. lestoquardi* are apicomplexan protozoan parasites of domestic livestock in northern Africa, Asia and southern Europe, transmitted by ticks of the genus *Hyalomma*. This thesis deals with comparative aspects of infections of the two parasite species in sheep and cattle.

To validate an indirect fluorescent antibody test for diagnosis of *T. lestoquardi* infection, the antibody response was monitored in sheep infected with *T. lestoquardi* schizont infected cells cultivated *in vitro*. Post-infection sera showed a high level of cross-reactivity to *T. annulata* schizont antigen.

In a series of experimental infections, based on the inoculation of sporozoites from ticks, the infectivity and the pathogenicity of the two parasite species for sheep and cattle were compared. *T. lestoquardi* caused serious disease in sheep but failed to infect cattle. In contrast, *T. annulata* infected both cattle and sheep. But while it caused severe disease in cattle, infections of sheep were mild and piroplasms did not develop. *In vitro* infection of ovine and bovine peripheral blood mononuclear cells with sporozoites of the two parasite species confirmed the findings *in vivo*. Since *T. annulata* did not complete its lifecycle in the sheep, infection of this host is unlikely to play a role in the epidemiology of *T. annulata* infection in cattle.

Cross-immunity studies in sheep showed that these animals were partially protected against challenge infection with the heterologous parasite species. This provided further indication of a close relationship between these two parasites.

Parasite infected cell lines from the above infections were analysed for their expression of cell surface antigens. The two parasite species appeared to have infected macrophages and B cells in both sheep and cattle. Differences in expression of leukocyte subpopulation antigens were quantitative rather than qualitative. Consequently, neither similarities nor differences in the course of clinical infection of sheep with *T. lestoquardi* and of sheep and cattle with *T. annulata* could be explained on the basis of phenotypic characterization only.

Key words: tick-borne disease, tropical bovine theileriosis, malignant ovine theileriosis, protozoa, sub-tropics, antigens, susceptibility, cross-protection, flow cytometry.

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Abstract

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Papers I-IV

The thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Leemans, I., Hooshmand-Rad, P. and Uggla, A., 1997. The indirect fluorescent antibody test based on schizont antigen for the study of the sheep parasite *Theileria lestoquardi*. Veterinary Parasitology 69: 9-18.

II. Leemans, I., Brown, D., Hooshmand-Rad, P., Kirvar, E. and Uggla, A., 1999a. Infectivity and cross-immunity studies of *Theileria lestoquardi* and *Theileria annulata* in sheep and cattle: I. In vivo responses. Veterinary Parasitology 82: 179-192.

III. Leemans, I., Brown, D., Fossum, C., Hooshmand-Rad, P., Kirvar, E., Wilkie, G. and Uggla, A., 1999b. Infectivity and cross-immunity studies of *Theileria lestoquardi* and *Theileria annulata* in sheep and cattle: II. In vitro studies. Veterinary Parasitology 82: 192-204.

IV. Leemans, I., Fossum, C., Johannisson, A. and Hooshmand-Rad, P. Comparative studies on surface phenotypes of *Theileria lestoquardi* and *Theileria annulata* schizont infected cells. Parasitology Research. In press.

Abbreviations

BVDV	bovine viral diarrhoea virus
CD	cluster of differentiation
ELISA	enzyme-linked immunosorbent assay
EMEM	Eagles' minimal essential medium
FCM	flow cytometry
FDMEM	improved Dulbecco's minimal essential medium
FCS	foetal calf serum
GLP	good laboratory practice
GUTS	ground up tick supernatant
IFAT	indirect fluorescent antibody test
IFN	interferon
IL	interleukin
im	intramuscular
line	*Theileria* schizont infected cell line
mAb	monoclonal antibody
MMP	matrix metalloproteinase
NK cell	natural killer cell
NO	nitric oxide
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PCV	packed cell volume
sc	subcutaneously
te	tick equivalent
THK	*Theileria lestoquardi* (Kamalabad stock)
THL	*Theileria lestoquardi* (Lahr stock)
THS	*Theileria lestoquardi* (Shiraz stock)
TNF-α	tumor necrosis factor alpha
VLA	very late antigen
WBC	white blood cells

Introduction

Infections of livestock with tick-borne protozoan parasites are of great economic importance in large parts of the tropics and subtropics. In many countries in North Africa, in the Near and Middle East, in India and in other parts of Asia, infections of cattle with *Theileria annulata* are particularly important. Infection of exotic susceptible cattle and their crossbreds with this parasite most often results in severe or fatal disease and, consequently, improvement of livestock production in endemic areas is seriously hampered. *T. lestoquardi*, a much less studied parasite of sheep and goats that may cause severe theileriosis, particularly in the sheep, is also known to occur in some of these areas. Although both *T. annulata* and *T. lestoquardi* can become transmitted by the same vector tick, other aspects of the biology which these parasites may have in common and which may have implications for the understanding of the epidemiology and control of theilerioses have not been much studied. Besides the application of pesticides to control the vector ticks, control of the disease in cattle and to a certain extent also in sheep, depends on vaccination. These vaccines are based on *in vitro* grown parasite infected cells, and, although efficient, they have several shortcomings. A better understanding of the comparative biology and immunology of *T. lestoquardi* and *T. annulata* and the infections they cause, may contribute to a better use of existing control methods against these important pathogens and to the development of safe novel vaccines.

Background

The genus Theileria

Taxonomy

Together with parasites of the closely related genus *Babesia*, the tick transmitted obligate intracellular parasites of the genus *Theileria* form the family Theileriidae in the order Piroplasmida (Levine, 1985). This order belongs to the phylum Apicomplexa, subkingdom Protozoa. This phylum also contains other important parasitic genera such as *Plasmodium*, *Eimeria*, *Toxoplasma, Neospora*, *Sarcocystis* and *Cryptosporidium*. Historically, the first *Theileria* and *Babesia* species were described as *Piroplasma* species (reviewed in Neitz & Jansen, 1956). Infections caused by *Theileria* and *Babesia* are, even nowadays, often referred to as piroplasmoses because of the pear-shaped parasitic stages that can be found in the erythrocytes of a wide variety of mammal host species. Since the first description by Koch (1898) of *Theileria* piroplasms in blood of diseased cattle around Dar es Salaam, the taxonomy of *Theileria* species has been, and still is, the subject of controversy (Neitz & Jansen, 1956; Uilenberg, 1981a; Irvin & Morrison, 1987).

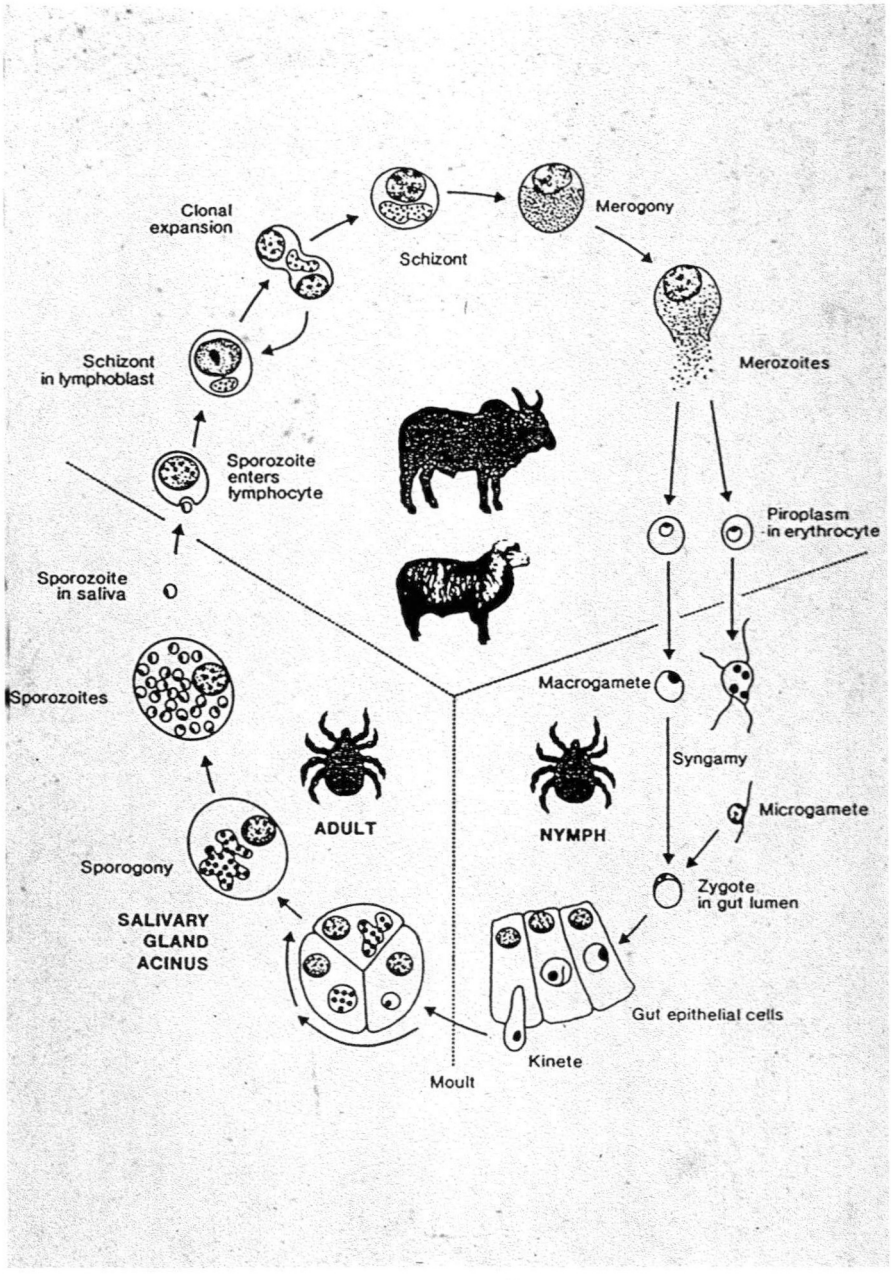

Figure 1. The life cycle of *Theileria* sp. (Adopted from Norval et al., 1992).

The status of some species such as *T. annulata* and *T. parva* has since long been established, but speciation of other *Theileria* parasites, including those of low pathogenicity in cattle and sheep and goats, is more complex. At genus level, the piroplasmids have also been the subject of changes as far as their classification is concerned. Recently, based on biological data, morphological features, biochemical properties and molecular biological relationships, *Babesia equi*, a piroplasmic parasite of equids, has been redescribed to belong to the genus *Theileria* (Katzer et al., 1998; Mehlhorn & Schein, 1998).

Life cycle

The *Theileria* species can infect a wide range of domestic and wild animals but are of particular importance in domestic livestock. The parasites have a typical apicomplexan lifecycle involving several differentiation and multiplication stages in their mammalian hosts and their vector ticks (Figure 1).

Transmission to the vertebrate host starts during feeding of an infected ixodid tick with the injection of sporozoites from its saliva into the host. Within minutes, these sporozoites enter their host cells (Fawcett et al., 1982; Jura et al., 1983), most often peripheral blood mononuclear cells (PBMC). Once inside the host cell, the host cell membrane that initially surrounds the parasite rapidly disintegrates, and, unlike many other intracellular protozoan parasites, the *Theileria* parasite then lies free in the host cell cytoplasm, where it differentiates into a multinucleated schizont. The presence of the schizont stimulates the host cell to undergo blastogenesis and proliferation. During division of the transformed host cell, the schizont also divides and is thus distributed to both daughter host cells, finally resulting in a clonal expansion of the parasite infected cells (Hulliger et al., 1964; DeMartini & Moulton, 1973; Irvin et al., 1982). After some time, depending on the *Theileria* species involved, but usually eight to ten days after infection in the case of *T. annulata,* micro-schizonts can be observed, and soon unicellular merozoites will develop. The merozoites, which are set free following disruption of the host cells, then invade erythrocytes where they transform into piroplasms. Again depending on the parasite species involved, these erythrocytic stages may multiply by schizogony (Conrad et al., 1985). The parasite infected erythrocyte is the stage that is infective to ticks.

In the tick, sexual reproduction and further multiplication and maturation occur (Schein et al., 1975; Gauer et al., 1995), finally giving rise to infective sporozoites in the salivary glands of the next tick stage. For a review of the development of *Theileria* parasites in ticks see Mehlhorn and Schein (1984, 1993).

Unlike transmission of *Babesia* species, which often is transovarial, transmission of *Theileria* species in the tick is always transstadial, i.e. after moulting of the tick stage that initially took up the infection (larva or nymph), the parasites are transmitted to a new host upon feeding of the next tick instar (nymph or adult). Generally, specific *Theileria* species are transmitted by specific tick species

(Mehlhorn & Schein, 1993). Consequently, the distribution of a particular parasite species is directly related to the distribution range of its vector tick(s).

In some *Theileria* species, the exo-erythocytic stage is very short and, consequently, schizonts are few and multiplication of the parasite mainly occurs during the piroplasmic stage, whereas in other species multiplication predominantly takes place during the schizont stage. Generally, those *Theileria* species in which the schizont stage of the lifecycle is very pronounced are considerably more pathogenic than those species in which the schizont stage is short (Uilenberg, 1981b). A major characteristic that distinguishes *Theileria* species from the true *Babesia* species is that the latter develop exclusively in erythrocytes (Mehlhorn et al., 1994).

Theileria species of cattle

To date, at least six *Theileria* species are known to infect cattle (Sergent et al., 1945; Neitz, 1957; Uilenberg, 1981a, b). Of these, *T. parva* and *T. annulata* are by far the most important since they both give rise to severe disease which in susceptible cattle usually results in death.

Theileria parva is believed to be originally a parasite of the African buffalo (*Syncerus caffer*). It causes an important and often fatal lymphoproliferative disease of cattle, called East Coast fever in eastern, central, and southern Africa as well as January disease in Zimbabwe, and Corridor disease when directly derived from buffalo (Norval et al., 1992). The main field vector tick is *Rhipicephalus appendiculatus. T. annulata*, on the other hand, is a parasite transmitted by ticks of the genus *Hyalomma*, causing tropical theileriosis in northern Africa, the Near and Middle East, India and other parts of Asia (Robinson, 1982). Because of their importance, *T. parva* and *T. annulata* have been extensively studied and more recent comprehensive reviews have been published dealing with different aspects of *T. parva* (Irvin & Morrison, 1989; Norval et al., 1992; Nene et al., 1993; Morrison & McKeever, 1998), *T. annulata* (Pipano, 1989; Tait & Hall, 1990; Mehlhorn, et al., 1994; Ahmed et al., 1999) or both (Irvin, 1987; Irvin & Morrison, 1987; Dolan, 1989; Boulter & Hall, 2000).

The other species infecting cattle, e.g. *T. mutans*, *T. taurotragi* and *T. velifera*, which occur in Africa, and parasites belonging to the cosmopolitan *T. buffeli/orientalis/sergenti* group are usually of low pathogenicity. However, the parasites of the last group are considered to be a problem in certain areas in Asia, predominantly in Korea and Japan, where they are usually referred to as *T. sergenti* (Minami et al., 1981), an invalid name (Morel & Uilenberg, 1981). Recent reports have also come on bovine fatalities in the United States caused by parasites belonging to this group (Chae et al., 1999). The main importance of these low or non-pathogenic species is that they, since they could not be differentiated from *T. annulata* and *T. parva*, for a long time have complicated the understanding of the latter parasites. The presence of the non-pathogenic *Theileria* species continues to complicate the picture of field studies on *T. parva*

and *T. annulata*, such as the interpretation of epidemiological data or the assessment of intervention programmes.

Theileria species of sheep and goats

In sheep and goats four species of *Theileria* have been recognised (Uilenberg 1981a). Three distinctive species are non-pathogenic, while only one species, *T. hirci* (syn. *T. lestoquardi*), is considered to be pathogenic to sheep and goats. This parasite has been described from northern Africa, southern Europe, the Middle East (Neitz, 1957; Uilenberg, 1981a) and India (Sisodia, 1981) and has been shown to be transmitted by *Hyalomma anatolicum anatolicum* ticks (Hooshmand-Rad & Hawa, 1973b).

However, Morel and Uilenberg (1981) considered that the name *T. hirci* was invalid for the pathogenic *Theileria* species of sheep and goats and have proposed the name *T. lestoquardi*. This name has now been generally accepted by the international scientific community and will be used throughout this thesis when reference is made to the pathogenic *Theileria* species of sheep and goats transmitted by *H. a. anatolicum* ticks. Otherwise, the names given by the authors of the various reports have been maintained.

Recent reports from China on outbreaks of theileriosis with high mortality in sheep and goats (Luo & Yin, 1997), where a tick referred to as *Haemaphysalis quinghaiensis* was implicated as the vector, suggest that there, in addition to *T. lestoquardi*, may exist one more *Theileria* species that is pathogenic to sheep and goats. Lately, analysis of the ribosomal small subunit RNA gene sequence of the parasite from China was shown to be different from the sequence of an Iranian *T. lestoquardi* isolate (Schnittger et al., 2000).

Of the three non-pathogenic *Theileria* parasites of sheep and goats (Uilenberg, 1981a), *T. separata* was described as a non-pathogenic parasite of sheep in eastern and southern Africa transmitted by *Rhipicephalus evertsi* (Uilenberg & Andreasen, 1974; Uilenberg & Schreuder, 1976; van Vorstenbosch et al., 1978). Piroplasms of this species are morphologically different from those of the other species (Uilenberg & Andreasen, 1974; Young & Mchinja, 1977). The identity and the validity of the names of the two other species, *T. ovis* Rodhain, 1916, and *T. recondita* Lestoquard, 1929, are less clear. *T. ovis* has been described from sheep and/or goats from Africa, Europe and Asia (reviewed in Neitz, 1957; Uilenberg, 1981a), whereas the name *T. recondita*, in some of the early literature used synonymously with *T. ovis*, has also been used for a different non-pathogenic *Theileria* species from sheep in western Europe (Enigk, 1953). This last parasite is analogous with the parasite in China transmitted by *Haemaphysalis* ticks (Friedhoff & Liebisch, 1978; Alani & Herbert, 1988a, b).

Compared to the vast body of literature available on *Theileria* infections of cattle, reports dealing with *Theileria* infections of sheep and goats are relatively few. Mostly, parasites have become identified as *T. lestoquardi* (syn. *T. hirci*) when

piroplasms in combination with disease or mortality were observed. More seldom, diagnosis was based on the detection of schizonts. Generally, parasites were considered to be *T. ovis* when piroplasms were demonstrated in the absence of disease. This species has been described from sheep and goats and sometimes other hosts such as mouflon and red deer, and different tick species and genera have been considered to be involved in transmission of what has been called *T. ovis* infection (reviewed by Neitz, 1957; Uilenberg, 1981a). However, according to the last author, the identity of the parasite, and thus the validity of the name *T. ovis,* is not clear for all reports of non-pathogenic small domestic ruminant theilerioses described as such in the literature. Moreover, confusion with any of these *Theileria* species with other piroplasms of sheep and goats, notably *Babesia* species, or the possibility of mixed infections, can sometimes not be excluded (Yakimoff, 1929; Khayyat & Gilder, 1947; Hashemi-Fesharki & Uilenberg, 1981).

Parasite maintenance and characterization

In the early days of *Theileria* research, parasites were maintained experimentally either through alternate passage between host animal and vector tick, as invariably used for *T. parva*, or through serial passage between cattle via the inoculation of infected blood or organ material, which was often used for *T. annulata* (Sergent et al., 1945; Norval et al., 1992). Later, schizont infected cells of several *Theileria* species including *T. annulata*, *T. parva* and *T. lestoquardi*, derived *ex vivo* from infected animals, could be successfully maintained at the laboratory as continuously growing cell cultures (Tsur-Tchernomoretz, 1945; Pipano 1974; Hooshmand-Rad & Hashemi-Fesharki, 1968; Malmquist et al., 1970; Hooshmand-Rad & Hawa, 1975). This technology, together with the development of techniques for cryopreservation and for *in vitro* transformation of bovine PBMC following incubation with *Theileria* sporozoites (Brown et al., 1973; Cunningham et al., 1973b), have greatly facilitated research on *Theileria.*

Several authors have reviewed the criteria used and the methods available for identification and characterization of different *Theileria* species (Irvin, 1987; Morzaria, 1989; Morzaria et al., 1995). They considered that often a combination of these methods is necessary to correctly identify a species. Criteria such as geographic distribution of the parasite and its vector tick(s), host animal and vector specificity, morphology of parasitic stages, pathogenicity, cross immunity, serology, and biochemical and molecular characteristics have been helpful in the identification and discrimination of *Theileria* parasites of cattle.

For the pathogenic *Theileria* species of cattle, it has been demonstrated that there are differences in virulence between different field isolates (Sergent et al., 1945; Robinson, 1982; Pipano, 1989). The existence of antigenic diversity among isolates is also well known, particularly in the case of *T. parva* (Morrison et al., 1995). It has been demonstrated that most field isolates consist of a mixture of genetically distinct populations (Conrad et al., 1987 & 1989; Ben Miled et al., 1994).

Tropical theileriosis of cattle

T. annulata is an economically important parasite of cattle transstadially transmitted by ticks of the genus *Hyalomma*, principally by *H. a. anatolicum* and *H. detritum*. In susceptible cattle it causes tropical theileriosis, or Mediterranean fever, a disease of high mortality. The parasite is widespread and has been found in most of the areas where its tick vectors occur (Robinson, 1982). Although a parasite of cattle, *T. annulata* can also infect and complete its lifecycle in the Asian water buffalo (*Bubalus bubalis*), but usually causes subclinical infection only (Neitz, 1957; Gautam, 1978; Uilenberg, 1981b; Pipano, 1989). Infection of other bovid hosts such as the American bison (*Bison bison*) and the yak (*Bos grunniens*) have been recorded in animals kept in captivity (Barnett, 1977). However, apart from the Asian buffalo, no other reservoir hosts are described. Sheep have been considered refractory to *T. annulata* infection (Sergent et al., quoted in Lestoquard, 1926; Sergent et al., 1945; Neitz, 1957; Robinson, 1982).

Clinical signs and pathology

The outcome of infection of cattle with *T. annulata* may range from severe disease leading to death within two to three weeks after infection to non-clinical cases. The final outcome depends on a variety of interrelated factors of which parasite virulence, host susceptibility and infective dose are but a few (Robinson, 1982). Local livestock from areas where *Theileria* infections are endemic, usually are less susceptible to disease and the infection may go unnoticed (Giesecke & Wiesenhütter, 1965; Eichler, 1967; Gautam, 1978 & 1981). On the other hand, severe disease is often observed in highly susceptible exotic stock, notably in imported animals and their crossbreds (Gautam, 1978).

In its severe form, the disease is characterized by the onset of high fever approximately one week after infection. This coincides with enlargement of the lymph node draining the site of tick attachment or, in the case of experimentally induced infections, of parasite inoculation. Subsequently, generalized lymphadenopathy develops and the animal shows further signs of generalized disease such as inappetence, listlessness, respiratory distress, ocular and nasal discharge, constipation often followed by diarrhoea, rapid loss of body condition and body weight. Anaemia, leukopenia and icterus are also characteristic features of theileriosis. Both schizonts and piroplasms are numerous and piroplasm parasitaemia may vary but often rises to over 50% infected erythrocytes in fatal cases. Death usually occurs within 2-4 weeks from the onset of infection. Animals that survive infection will recover, but full recovery may take several weeks and animals remain carriers of the infection (Pipano, 1989). Detailed accounts on clinical signs have been given by many authors (reviewed in Sergent et al., 1945; Neitz, 1957; Uilenberg et al., 1981b; Pipano et al., 1981; Preston et al., 1992a).

At necropsy of fatal cases, the carcass is usually emaciated, anaemic and often icteric. The main macroscopical lesions are hyperplasia and oedema of lymph nodes, splenomegaly, a yellowish enlarged liver, petechiae and ecchymoses of the epi- and endocardium, in the skin and on mucosae and serosae, grey-white foci on the cortex of the kidney and sometimes the liver. Haemorrhagic ulceration of the abomasum is a characteristic feature. The lungs are frequently oedematous (Dschunkowsky & Luhs, 1904; Gill et al., 1977; Uilenberg, 1981b; Pipano, 1989). Histopathologically, the lymph nodes show depletion of lymph follicles and other organs show varying degrees of degenerative change (Gill et al., 1977; Gautam, 1981).

Diagnosis

None of the clinical symptoms or pathological features are pathognomonic for theileriosis and, for a definite diagnosis, detection of the parasite is needed. Traditionally, the parasites have been demonstrated in Giemsa stained lymph node and liver biopsies or blood smears (Figure 2), or in impression smears prepared from different organs following necropsy (Sergent et al., 1945). More recently, immunocytochemistry (Forsyth et al., 1997, 1999) and molecular biological techniques (d'Oliveira et al., 1995; Kirvar et al., 2000) have been used for parasite detection. The latter techniques have also been applied to demonstrate infection in ticks (de Kok et al., 1993; d' Oliveira et al., 1997; Kirvar et al., 2000). In addition, several serological tests have been developed to demonstrate circulating antibodies against the parasite. The indirect fluorescent antibody test (IFAT), either based on schizont or piroplasm antigen (Pipano & Cahana, 1969; Burridge et al., 1974), and different ELISA tests are the most widely applied (Gray et al., 1980; Irvin, 1987; Kachani et al., 1992; Ilhan et al., 1998; Gubbels et al., 2000).

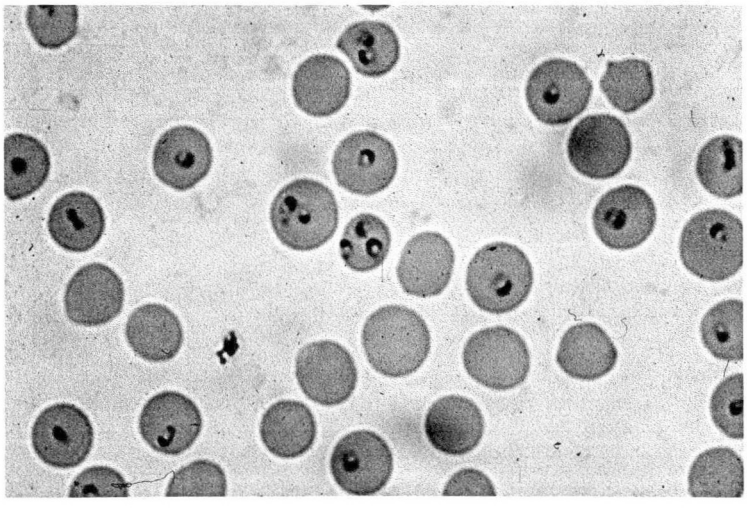

Figure 2. *T. annulata* piroplasms in Giemsa stained blood smear.

Pathogenesis and immune response

The clinical syndrome in tropical theileriosis of cattle as well as the accompanying pathology are mainly associated with the schizont stage of the parasites (Pipano, 1989), but the piroplasm stage of *T. annulata* is considered to contribute to the development of the haemolytic anaemia which is a characteristic feature of the later stages of the disease (Uilenberg 1981b).

Infection of the host by *T. annulata* initially results in hyperplasia of the local lymph node draining the site of inoculation of the sporozoites (Uilenberg, 1981b; Campbell & Spooner, 1999). Hyperplasia of the local lymph node is followed by an expansion of the schizont infected cell population and their distribution throughout the body (Gill et al., 1977; Irvin & Morrison, 1987; Forsyth et al., 1997, 1999). The last authors observed high levels of expression of CD2, CD11b and very late antigen-4 (VLA-4) on the surface of parasite infected mononuclear cells disseminated in virtually all analysed organs. They speculated that expression of these molecules, which are known to play a role in cellular adhesion and transmigration of leukocytes, could have contributed to the expansion of the parasite infected cells into the tissues of the host. Other authors suggested that expression by parasite infected cells of matrix metalloproteinases (MMPs), enzymes which degrade components of the extracellular matrix, could also contribute to metastasis of infected cells (Baylis et al., 1992, 1995; Adamson & Hall, 1997; Adamson et al., 2000).

The main pathogenic effects of *T. annulata* infection are observed during the schizont multiplication phase (Pipano, 1989). The pathological lesions observed were shown to be directly related to the presence of high numbers of parasite infected cells, suggesting that these cells themselves contribute to the pathology (Forsyth et al., 1999). Also, it is generally thought that the immune response elicited by infection with *T. annulata* plays a role in the development of the pathology (Preston et al., 1983; Ahmed et al., 1989, Tait & Hall, 1990; Kachani & Spooner, 1992).

In vitro studies have shown that *T. annulata* preferentially infects bovine monocytes or macrophages but can also infect B cells (Glass et al., 1989; Spooner et al., 1989; Campbell et al., 1994; Sager et al., 1997, 1998a, b). Studies of Forsyth et al. (1997, 1999) indicated that *T. annulata* infects monocytes *in vivo* also. An *ex vivo* derived *T. annulata* infected B cell line has been described as well (Moreau et al., 1999). *In vitro* studies on *T. annulata* infected cattle lines revealed that these cells were able to produce type 1 interferon (IFN) but not IFN-γ (Entrican et al., 1991; Preston et al., 1993). Several cytokines, and TNF-α in particular, were shown to enhance the proliferation of schizont infected cells *in vitro* (Preston et al., 1992b). In a later study these authors showed that macrophages exposed to *T. annulata* infected cells produced TNF-α and that exposure of lymphocytes to parasite infected cells induced the former to synthesize IFN-γ (Preston et al., 1993). The same authors demonstrated

spontaneous IFN-γ production in cultures of macrophages, PBMC or lymph node cells from a lethally infected calf. They suggested that cytokine production is enhanced during acute lethal tropical theileriosis. Production of IFN-γ during the initial stages of *T. annulata* infection was demonstrated by cannulation of the efferent lymph duct draining the site of parasite inoculation (Nichani et al., 1999), while it was shown by immunocytochemistry that schizont infected cells produced IFN-α_1 and TNF-α (Forsyth et al., 1999). Also, it has been demonstrated that nitric oxide (NO) has an inhibitory effect on establishment and proliferation of schizont infected cells *in vitro* (Richardson & Preston, 1994) and that NO is produced by calves undergoing bovine tropical theileriosis (Visser et al., 1995; Richardson et al., 1998). It is thought that the cytokines, and other products such as NO, produced by and in response to parasite infected cells contribute in controlling the infection or in facilitating the progress of the disease (reviewed by Preston et al., 1999; Boulter & Hall, 2000).

Infection with *T. annulata* induces a specific immune response that may result in clinical recovery followed by resistance to reinfection with the homologous stock and usually also with a heterologous stock (Sergent et al., 1945; Gill et al., 1980; Gautam, 1981; Pipano, 1989; Brown, 1990; Preston et al., 1992a). However, clinical cure is not the result of elimination of the parasite since the host remains a carrier of schizont infected cells (Sharma and Brown, 1981) as well as of piroplasm infected erythrocytes (Sergent et al., 1945; Neitz, 1957).

Infection with *T. annulata* is followed by a production of specific antibodies which have been detected by a variety of diagnostic tests (Burridge et al., 1974; Dhar & Gautam, 1977; Gray et al., 1980; Hooshmand-Rad & Hashemi-Fesharki, 1981). Like in *T. parva* infection (Muhammed et al., 1975), these antibodies are not considered to play a role in the development of protective immunity, since passive transfer of circulating antibody to susceptible cattle did not prevent nor mitigate the clinical results of *T. annulata* infection (Dhar & Gautam, 1978; Pipano, 1989). Also, no relation was observed between the level of antibody production and the degree of protection (Pipano et al., 1977; Kachani et al., 1992).

However, *in vitro* studies indicated that antibodies played a role in the blocking of sporozoite invasion into their host cells (Gray & Brown 1981; Preston & Brown, 1985; Williamson et al., 1989; Knight et al., 1998), and such antibodies have recently been demonstrated to give partial protection in immunization trials with a recombinant sporozoite surface antigen (Boulter et al., 1995, 1998, 1999). Promising results are also reported in vaccine studies with a structurally and immunologically similar sporozoite surface protein from *T. parva* (reviewed in McKeever & Morrison, 1998).

Several studies have shown that protective immunity against *T. annulata* infection is predominantly cell mediated and directed against the schizont infected cells (Ahmed et al., 1989; Boulter et al., 1995). It is thought that the main effector cells are macrophages but that natural killer (NK) cells and

cytotoxic lymphocytes also play a role in the immune response (reviewed by Ahmed & Mehlhorn, 1999; Preston et al., 1999).

Treatment and control

Treatment of *T. annulata* infection is based on the application of curative theilericidal drugs (McHardy & Morgan, 1985; Singh et al., 1993). However, these chemotherapeutic agents are expensive and only effective when applied at an early stage of infection (Dhar et al., 1988; Hashemi-Fesharki, 1991).

Besides the use of acaricides to decrease parasite transmission by the tick vectors, the control of *T. annulata* infection in many countries involves the use of a vaccine containing live attenuated *T. annulata* schizont infected cells (Pipano, 1977; Brown, 1990; Singh, 1990; Tait & Hall, 1990). Continuous propagation *in vitro* of *T. annulata* schizont infected cells leads to attenuation of the parasite and inoculation of such cells into susceptible cattle results in mild clinical reaction with only rare schizonts in the lymph nodes and liver (Tsur et al., 1964, quoted in Pipano, 1977). Attenuated cells, when used to immunize susceptible cattle, confer protection against challenge infection with *T. annulata* (Pipano et al., 1973). This technology has now replaced the traditional method of immunization through the inoculation of blood from animals undergoing acute *T. annulata* infection with naturally mild stocks (Sergent et al., 1945; Pipano et al., 1969). On the basis of attenuated *T. annulata* schizont infected cells successful vaccination schemes have been developed resulting in a satisfactory level of immunity to field challenge (Pipano, 1977, 1995).

Despite their effectiveness, such live attenuated vaccines have several shortcomings, including the high costs involved in production, the long time needed to reach attenuation, the need for a well functioning cold chain, the need for re-immunization in the absence of natural tick challenge in the field, and the potential risk of transmission of contaminating pathogens that could be present in the culture (Tait & Hall, 1990; Pipano, 1995; Musoke et al., 1996; Boulter & Hall, 2000). A method of immunization based on non-infective immunogens would overcome most of these shortcomings. Promising results have so far been obtained with recombinant antigens of the sporozoite and merozoite stages of the parasite while protective schizont antigens still need to be identified (reviewed in Boulter & Hall, 2000).

Malignant theileriosis of sheep and goats

Malignant theileriosis of sheep was first described in detail by F. E. Mason, pathologist at the Veterinary Pathological Laboratory in Cairo, Egypt, in the 1914 Annual Report of the Ministry of Agriculture (Littlewood, 1915). He quoted Mohamed Effendi Askar, Veterinary Inspector of Cairo District: 'With reference to the two Sudanese sheep who died on August 28 and September 2, 1914, I beg to state the following: '..... Bodies resembling Koch's blue bodies were found in the haemorrhagic infarcts in the supra-scapular and other lymph glands. .....'

Mason continues: 'This form of piroplasmosis in sheep has not hitherto been recorded by other observers, ...' Because of the presence of schizont infected cells in smears of spleen and lymph nodes as well as because of the smallness of the piroplasms, it was distinctive from the piroplasmosis caused by what was later considered to be *Babesia ovis*. The disease caused by this parasite was already well known at that time.

After the first report on the disease in sheep from Sudan, a similar disease has been recorded as being of common occurrence in Egyptian sheep (Littlewood, 1916). The parasite was first described as *T. ovis* (du Toit, 1918), then *T. hirci* (Dschunkovsky & Urodschevich, 1924) and eventually as *T. lestoquardi* (Morel & Uilenberg, 1981). It has been reported from sheep in Algeria (Lestoquard, 1924, 1926), Transcaucasia (Yakimoff, 1929), Turkey (Baumann, 1939), Iraq (Khayyat & Gilder, 1947), Iran (Mazlum, 1970), India (Raghvachari & Reddy, 1956); from goats in Serbia (Dschunkovsky & Urodschevich, 1924), Greece (Cardassis & Margaritis, 1964) and India (Vashistha & Mathur, 1987; Banerji et al., 1990), and from sheep and goats in India (Sasmal et al., 1983) and lately from China (Luo & Yin, 1997). The infection is reported to be common in Iran and Iraq (Hooshmand-Rad, 1974, 1985; Hawa et al., 1981; B. Latif, personal communication), causing an important disease in lambs during spring, summer and early autumn depending on the geographical zone (Hooshmand-Rad, 1974). Most reports from other countries deal with case reports of outbreaks of disease. The parasite has not been reported from Lebanon, (Pigoury, 1937), Syria (Köhler et al., 1967; Eichler, 1968) or Israel (Pipano, 1991), while infections of sheep with *T. ovis* and *B. ovis* are common in these countries.

Whereas one can assume that the parasites described from northern Africa and Iraq and Iran are the same pathogen as the one originally described by Littlewood, it is unclear whether the reports from elsewhere on malignant theileriosis of sheep and goats all deal with the same parasite (Brown et al., 1998). Many reports deal with clinical disease outbreaks in sheep and/or goats, of which the causal agents was considered to be *T. lestoquardi* (syn. *T. hirci)* merely on the basis of the combination of severe disease with the presence of piroplasms and, sometimes, schizonts (Raghvachari & Reddy, 1959; Sasmal et al., 1981; Sisodia & Gautam, 1983).

Ticks of the genus *Hyalomma* (Figure 3) had been suspected to be involved in the transmission of *T. lestoquardi* (Mazlum, 1970), and their role as vector tick was unequivocally demonstrated when nymphs of *H. a. anatolicum* that were fed on an infected sheep, after having moulted into adults, were shown to be able to transmit the infection to susceptible sheep (Hooshmand-Rad & Hawa, 1973b). In a recent outbreak of sheep theileriosis in Sudan, all ticks collected were *H. a. anatolicum* (Tageldin et al., 1992), and in a later report *H. a. anatolicum* ticks from that country were demonstrated to transmit *T. lestoquardi* to sheep (Morzaria & Latif, 1994, quoted in Osman, 1997). However, other tick species, such as *Rhipicephalus bursa*, either found during outbreaks or known to

commonly feed on sheep and goats, have also been suspected to be involved as vectors (Dschunkovsky & Urodschevich, 1924; Baumann, 1939). In India, both nymphs and adults of a laboratory reared stock of *H. a. anatolicum* ticks were able to transmit a pathogenic *Theileria* species of sheep. The same parasite stock was also transmitted by *Rhipicephalus* ticks collected in the field which were not identified beyond genus (Sisodia & Gautam, 1983).

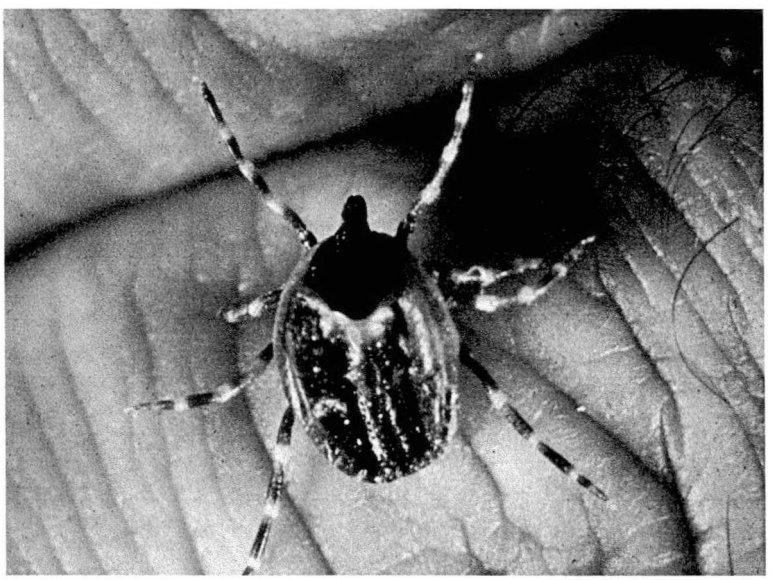

Figure 3. Adult female *Hyalomma* sp. tick (Photo: Heinz Mehlhorn).

Reports differ as to whether *T. lestoquardi* infections can be easily transmitted mechanically from one animal to the next through the inoculation of infected blood as is the case for *T. annulata* infections of cattle (Sergent et al., 1945). Whereas several researchers in India were successful in the transfer of infection to susceptible sheep (Raghvachari & Reddy, 1959; Sisodia & Gautam, 1983), researchers working on the Iraqi/Iranian parasites have reported on difficulties in mechanically transmitting the parasites. They demonstrated that reactions to inoculations varied, with some animals showing mild or severe disease, whereas others remained uninfected (Hawa et al., 1981), while in another study sheep showed a serological response to schizont antigen in IFAT upon inoculation of *T. lestoquardi* infected blood, but no clinical reactions were observed nor parasites detected (Hawa et al., 1976). Similar results were also reported by Hooshmand-Rad (1985), who considered that inoculation of infected blood was not sufficiently reliable to be used as challenge infection for the evaluation of the development of immunity in vaccination trials.

Reports are contradictory regarding the possibilities to experimentally infect goats with the sheep parasite. Hooshmand-Rad & Hawa (1973a) were unable to demonstrate infection in a goat inoculated with a suspension of ovine liver cells containing high levels of schizont infected cells, while sheep inoculated with the same material became infected and schizonts and piroplasms were easily detected. Since there are no published reports that *T. lestoquardi* affects goats in Iraq and Iran, these authors considered that, in this area, the parasite is not an important pathogen in this animal species. In India, some researchers were unable to transmit infection through the inoculation of infected blood from sheep to goats, including goats that were splenectomized (Raghvachari & Reddy, 1959; Sisodia & Gautam, 1983), whereas others were able to do so in goats that had been treated with dexamethasone (Sasmal et al., 1983).

Clinical signs and pathology

Several authors have given more or less detailed accounts of the symptoms of acute malignant theileriosis in sheep and/or goats (Lestoquard, 1926; Baumann, 1939; Neitz, 1957; Raghvachari & Reddy, 1959; Hooshmand-Rad & Hawa, 1973a) and considered that these were very similar to those of cattle suffering from acute tropical theileriosis. The symptoms include high fever, listlessness, anorexia and emaciation, diarrhoea or constipation, enlarged superficial lymph nodes and pale and icteric mucous membranes. As with the clinical signs, the pathological features described in malignant theilerioses of sheep and goats are very similar to those described for tropical theileriosis of cattle (Neitz, 1957; Hooshmand-Rad en Hawa, 1973a; Sisodia, 1981; Sisodia & Gautam, 1983; Tageldin et al., 1992). However, typical haemorrhagic ulcers of the abomasum, as seen in *T. annulata* infection of cattle, were notably absent (Hooshmand-Rad & Hawa, 1973a).

Diagnosis

As with *T. annulata*, diagnosis of *T. lestoquardi* infection is based on the demonstration of parasitic stages in blood or organ smears. As mentioned earlier, the causal agent has often been identified only on the basis of the presence of piroplasms in combination with clinical disease or pathological findings. An IFAT using a schizont antigen has been described which was used for follow up of immunization but no details were given about its specificity (Hawa et al., 1981).

Pathogenesis and immune response

Theileria lestoquardi is considered to be very pathogenic to sheep, and high morbidity and mortality rates have been reported, even in indigenous stock (Hooshmand-Rad & Hawa, 1973a; Hooshmand-Rad, 1974). Little is known about the mechanisms involved in the pathogenesis of the disease, and the immune response to *T. lestoquardi* has not been the subject of specific studies. However, it is known that animals that survive infection are resistant to challenge infection.

Treatment and control

Little is known about the efficacy of the theilericidal drugs used for treatment of *T. annulata* and *T. parva* infection of cattle against *T. lestoquardi* infection in sheep and goats. Although they are likely to be effective (Hashemi-Fesharki, 1997), such drugs may be too expensive for treatment of these animal species.

Following the successful cultivation of *T. lestoquardi* schizont infected ovine cells (Hooshmand-Rad & Hawa, 1975) and the discovery that the parasite, like *T. annulata*, becomes attenuated but maintains its immunogenicity following prolonged culture *in vitro*, immunization of sheep with such cells has been carried out successfully in Iraq (Hawa et al., 1981). Immunization is widely applied in Iran and has been shown to provide good protection against virulent field isolates (Hooshmand-Rad, 1985; Hashemi-Fesharki, 1997).

The present study

Outline

The experimental infections that formed the starting point for this thesis were carried out to study the development of immunity in sheep following inoculation with live attenuated *T. lestoquardi* schizont infected cells and to elaborate and evaluate diagnostic tools for use in clinical and epidemiological studies.

The objective of the subsequent experiments was to study similarities and differences between *T. lestoquardi* of sheep and *T. annulata* of cattle. A series of experiments was undertaken to compare the infectivity of *T. lestoquardi* and *T. annulata* sporozoites, the natural infective stages of the parasites, for sheep and cattle; to compare the pathogenicity of the two parasite species for each host species; and to study the development of cross protection.

Finally, attempts were made to explain similarities and differences in pathogenicity by phenotypic characterization of parasite infected cells.

Comments on materials and methods applied

In the following, a brief description is given of the materials and methods used throughout these studies. More detailed information can be found in the accompanying papers (I-IV).

Parasites (I-IV)

For the description of the different parasite populations used throughout this thesis, the terminology proposed by Irvin and Morrison (1987) for characterization of such populations has been used:

- Isolate: viable organisms isolated on a single occasion from a field sample into experimental hosts or culture systems, or by direct preparation of a stabilate.
- Stock: all populations of a parasite derived from an isolate without any implication of homogeneity or characterization; populations comprising a single stock include cell lines and tick stabilates, and subsequent parasite preparations derived from them.
- Stabilate: a sample of organisms preserved alive on a single occasion.

Different *Theileria* stocks were used in the experiments described in papers I-IV. They were either used as schizont infected cell cultures (lines) or as cryopreserved sporozoite stabilates of ground up tick supernatant (GUTS).

Four different stocks of *T. lestoquardi,* isolated from naturally infected sheep from different localities in Iran, were used in these studies. The THS1, THS2 and THK stocks, originating from Shiraz and Kamalabad, have been maintained *in vitro* as lines (Hooshmand-Rad et al., 1993). The THS1 stock was used as an attenuated high passage line. This line has been used in Iran for vaccination purposes (Hooshmand-Rad, 1985). Lines of these three stocks were employed for the preparation of antigen slides for use in IFAT (paper I). The THS1 line was also used for inoculation experiments of sheep (paper I).

The *T. lestoquardi* (Lahr) stock used for the experimental infections of cattle and sheep or their PBMC described in papers II-III, had not previously been analysed in detail. This stock originates from a naturally infected sheep suffering from acute theileriosis in the Lahr province, Iran (Hooshmand-Rad, personal communication). A cryopreserved sporozoite stabilate of GUTS prepared according to Brown et al. (1973) from a laboratory colony of experimentally infected adult *H. a. anatolicum* ticks was used in the experiments described. The ticks used for preparation of the stabilate were prefed on rabbits for a period of three days (Kirvar et al., 1998b). Ovine lines which resulted from the experimental infections (papers II-III) were used for the phenotypic characterizations of the infected cells in paper IV.

A cryopreserved sporozoite stabilate of *T. annulata* (Ankara) was used for the experimental infections of cattle and sheep or their PBMC (papers II-III). This stock, first described by Schein et al. (1975), has been widely used for experimental studies of *T. annulata* infections of cattle (Brown et al., 1998). Bovine and ovine lines resulting from these infections provided the material for the phenotypic characterizations in paper IV.

Lines of a *T. annulata* stock, isolated from a naturally infected bovine in Iran and subsequently maintained *in vitro* (Hooshmand-Rad, personal communication), and a *T. parva* (Pugu) stock, originating from a bovine field isolate from Tanzania (Uilenberg et al., 1977), were used for preparation of antigen slides for use in IFAT (paper I).

Inoculation of animals and initiation of cell cultures (I-III)

For the experiment described in paper I, six one-year-old male sheep were inoculated by subcutaneous (sc) or intramuscular (im) injection of 1.5 x10^6 *T. lestoquardi* schizont infected cells of the attenuated THS1 line in phosphate buffered saline (PBS). This was a slightly higher dose than the one used for field vaccination of sheep in Iran (Hooshmand-Rad, 1985).

For the series of experiments described in paper II, a total of 14 male sheep and eight female calves were inoculated according to Brown (1979) with infective sporozoites through sc injection of cryopreserved *T. annulata* or *T. lestoquardi* tick stabilates. The use of supernatant of ground up pre-fed infected ticks was originally described for the initiation of *in vitro* infections of PBMC with *T. parva* (Brown et al., 1973), but is now also widely applied for the establishment of *Theileria* infections *in vivo*. As doses of sporozoites can be quantified, predictable and reproducible infections can be induced (Cunningham et al., 1973a; Brown, 1979; Preston et al., 1992a).

Parasite infected cell cultures (Figure 4) were initiated in two ways. Firstly, *in vitro*, as described by Brown (1987) by incubation of ovine or bovine PBMC with sporozoite stabilates of GUTS. Secondly, *ex vivo*, from lymph node biopsy material or PBMC from sheep or cattle undergoing *Theileria* infections induced through the inoculation of sporozoites or of schizont infected cells (Brown, 1987). Throughout this thesis, the lines that resulted from these incubations are referred to as *in vitro* or *ex vivo* lines, respectively.

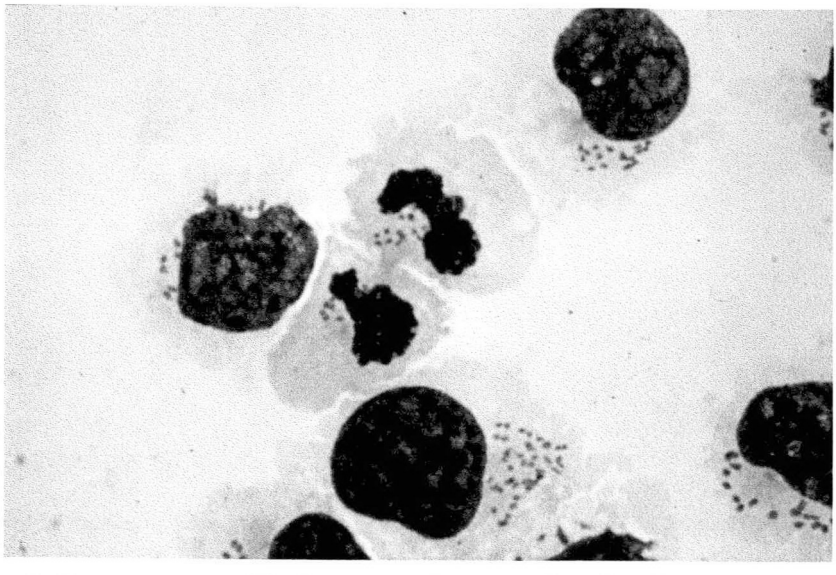

Figure 4. *In vitro* grown *Theileria* sp. schizont infected cells (Giemsa staining) (Photo: Duncan Brown).

The dosage of the *T. lestoquardi* stabilate that was used for the establishment of the infections *in vivo* and *in vitro* was based on the results of a limited number of infections of sheep and goats and on titration experiments *in vitro* of ovine PBMC as reported by Brown et al. (1998). On the basis of these results, for the stabilate batch used, a dose of 4.5 tick equivalents per animal (te/animal) was extrapolated which was expected to result in moderate to severe reactions in susceptible sheep. A *T. annulata* stabilate dose of 1 te/animal was determined based on the experience of one of the authors of papers II-III in the use of stabilates for the establishment of *T. annulata* infection in cattle (Brown et al., 1998).

Laboratory procedures (I-IV)

The *T. lestoquardi* infected cell line (THS1) used for inoculation of the sheep described in paper I as well as the lines that resulted from these inoculations were cultured in Eagles' minimum essential medium (EMEM) containing 0.12 g penicillin and 0.1 g streptomycin/l and 10% foetal calf serum (FCS). For all later cultures Dulbecco's medium with additives (FDMEM; SVA, Uppsala, Sweden) was used and supplemented as for EMEM. For initiation of the *ex vivo* or *in vitro* cultures described in paper III 20% FCS was added to the medium.

After the implementation of good laboratory practice (GLP) routines at the Department of Parasitology, only bovine viral diarrhoea virus (BVDV) negative batches of FCS were used for culture media and all cell cultures were regularly screened for presence of contaminating BVDV and *Mycoplasma* species infections. During the time the experiments described in paper I were carried out, such procedures had not yet been implemented.

Monitoring of infection (I-III)

Sheep and cattle used in papers I-II were monitored for the establishment and development of infection with *T. lestoquardi* or *T. annulata* through regular analysis of various clinical, parasitological, haematological and serological parameters routinely used in *Theileria* research (Anonymous, 1989; Preston et al., 1992a). Clinical reactions were classified as inapparent, mild, moderate or severe as defined in a workshop on East Coast fever immunization held in 1988 (Anonymous, 1989).

To increase sensitivity of parasite detection, lymph node biopsy material of the animals described in papers I-II was regularly cultured and monitored for the presence of schizont infected cells. Establishment and maintenance of these cultures as well as of the cultures of PBMC that were inoculated *in vitro* with tick stabilate (paper III), were carried out as described by Brown (1987).

In sheep that had survived primary infection with *T. lestoquardi* or *T. annulata* the development of cross protection was analysed in challenge infections with sporozoite stabilates of the heterologous species (paper II). For evaluation of

challenge infections the same parameters were analysed as for primary infections. Moreover, the identity of the *Theileria* parasites in lines that established *ex vivo* from these challenge infections, was determined by a PCR assay using a *T. lestoquardi* and a *T. annulata* specific primer pair (Kirvar et al., 1998a, 2000) (paper III). The same PCR assay was also applied to confirm that no laboratory cross contamination between lines had inadvertedly occurred during handling.

Immunolabelling (IV)

Several of the lines described in paper III were phenotypically characterized (paper IV). The expression of leukocyte cell surface antigens by *Theileria* schizont infected cells was first characterized on the *T. lestoquardi* and *T. annulata* infected sheep lines that established after infection *in vitro* of PBMC using predetermined optimal dilutions of each monoclonal antibody (mAb) and flow cytometry (FCM).

The initial panel of mAbs was selected to identify the major subpopulations of sheep leukocytes. These were the same mAbs that were originally used to characterize *T. lestoquardi* infected sheep lines (Hooshmand-Rad et al., 1993). Later, some more mAbs were included that had become available since then, i.e. a mAb directed against a marker on B cells (VPM30) and two mAbs against markers on myeloid lineage cells including monocytes/macrophages (VPM64 and VPM65).

Based on the results of the initial characterizations of *in vitro* lines, *ex vivo* derived *T. annulata* infected sheep and cattle lines and *T. lestoquardi* infected sheep lines were analysed. For these experiments, the panel of mAbs was extended further with some mAbs directed to markers on bovine and ovine B cells (CC21 & VPM13) as well as to markers on myeloid lineage cells (IL-A15 & IL-A24). Antibodies directed against the same antigens had been shown to be of interest for the characterization of *T. annulata* infected cattle lines (Forsyth et al., 1997; Sager et al., 1997 & 1998a). All the mAbs that were included in the final panel had been characterized during one of the three workshops held so far on ruminant leukocyte antigens (Howard, 1991; Naessens & Howard, 1993; Naessens & Hopkins, 1996) and most are commercially available.

Results and discussion

Indirect fluorescent antibody test (I-II)

An indirect fluorescent antibody test (IFAT) based on *T. lestoquardi* schizont infected cells was described (Hawa et al., 1976) soon after it had been shown that *T. lestoquardi* schizont infected cells, like those of *T. annulata*, could be grown in cell culture (Hoosmand-Rad & Hawa, 1975). This IFAT was later used to follow sheep in immunization studies (Hawa et al., 1981). However, no details were presented on its specificity and sensitivity. The studies described here were carried out as a first step in the validation of IFAT, based on *T. lestoquardi*

schizont infected antigen, as a diagnostic assay. Its performance characterisitics were analysed using sera from known *T. lestoquardi* infected sheep, sera from negative control animals as well as control sera positive for related pathogens (paper I). Antibodies raised in sheep that had become infected through inoculation of *T. lestoquardi* schizont infected cells, specifically reacted to the intracellular schizonts and early first appearance of antibodies following infection as well as prolonged duration of the antibody response suggested that the IFAT based on *T. lestoquardi* schizont infected cells could be a valuable tool for epidemiological studies. No cross reaction was observed with ovine *Babesia* and *Toxoplasma* positive sera but limited levels of cross reaction were found with ovine *T. ovis* and *T. separata* and with bovine *T. annulata* positive sera. On the other hand, very high levels of cross reaction were observed with *T. lestoquardi* positive sera on bovine *T. annulata* schizont antigen. Cross reaction between *T. parva* and *T. lestoquardi* did occur but was less pronounced. It was concluded that *T. lestoquardi* when analysed by IFAT has diagnostic antigens in common with the two pathogenic *Theileria* parasites of cattle and that *T. lestoquardi* is antigenically closer related to *T. annulata* than to *T. parva*. Recently, sequence analysis of the small subunit ribosomal RNA gene and of a gene coding for a sporozoite surface antigen has confirmed that *T. lestoquardi* phylogenetically is closer related to *T. annulata* than to *T. parva* (Katzer et al., 1998; Skilton et al., 2000).

In later studies described in this thesis (papers II-III), sheep were shown to be susceptible to infection with *T. annulata* and, consequently, natural infection of sheep with this parasite cannot be excluded. Given that *T. annulata* and *T. lestoquardi* have a similar geographical distibution, sheep in these areas could thus be infected with any of the two or with both parasite species. Combining this possibility with the existence of cross reactive epitopes on *T. lestoquardi* and *T. annulata* schizonts, as described above, makes it very unlikely that a schizont based IFAT will become a useful instrument for epidemiological studies of *Theileria* infections of sheep.

The IFATs applying *T. lestoquardi* schizont infected ovine cells and *T. annulata* schizont infected bovine cells as antigen, were used in the study of the sporozoite induced infections described in paper II. A positive reaction in IFAT was consistently associated with detection of parasitic stages, while absence of a response in IFAT correlated well with a failure to detect parasites in the animals. Thus, despite the limited applicability of IFAT as an epidemiological tool, it remains a sensitive instrument for the follow up of experimental *Theileria* infections.

Infectivity (I-III)

T. lestoquardi *schizont infected cells*

Our attempts to infect six naive sheep with an attenuated *T. lestoquardi* schizont infected cell line were successful (paper I). Schizont infected cells were detected

in low numbers in Giemsa stained smears of lymph node biopsies of some sheep and infection was confirmed in three sheep through the establishment *in vitro* of schizont infected lines. Positive results by IFAT of post inoculation sera of all sheep provided indirect evidence for infection in the remaining sheep also. Piroplasms were never demonstrated in any of the animals. This was not unexpected since the THS1 stock that was used for inoculation was a vaccine stock that had become attenuated during prolonged *in vitro* culture, and was known not to produce piroplasms (Hooshmand-Rad, 1985).

A point of major interest was that during a routine control in line with the introduced GLP procedures, the THS1 stock that had been used for inoculation was found to be contaminated with BVDV (unpublished results). The source of contamination could be traced back to a batch of FCS that was in use at the time of the experiments described in paper I. All lines that resulted from the experimental infections were shown to be infected with BVDV. Using an ELISA detecting antibodies to BVDV (Juntti et al., 1987), all sheep that were inoculated with *T. lestoquardi* infected cells had seroconverted while the control sheep remained sero-negative (results not shown). Retrospectively, it must therefore be concluded that the experimental animals were, inadvertedly, inoculated with BVDV simultaneously with *T. lestoquardi*. Although we believe that the infection with BVDV had no bearing on the performance of the sheep sera in IFAT, our experiences, once again, highlight the risks associated with the use of live cell culture derived material as immunizing agent (Pipano, 1995).

Comparing the chromosome pattern of the male parent line used for inoculation with a line that was established upon re-isolation from a female sheep, we were able to show that schizonts had been transferred from the donor cells to cells of the recipient (unpublished observations). In this respect *T. lestoquardi* of sheep resembled *T. annulata* of cattle, i.e. immunization of cattle with allogeneic *T. annulata* infected cells resulted in the easy establisment of infection and the transfer of the parasite from the cells of the donor animal to those of the recipient (Brown, 1981; Innes et al., 1992). This is in marked contrast with the situation in *T. parva*, where sporozoites preferentially infect T cells and B cells *in vitro* (Baldwin et al., 1988), and where in *in vivo* infections transfer of the parasite from the donor cell line to the cells of the recipient animal only occurs very ineffectively (reviewed in Brown, 1981). Consequently, immunization with *T. parva* schizont infected cells has not proved satisfactory for infecting with, or immunizing against, *T. parva* (Brown et al., 1978; Brown, 1981).

Little is known about the mode of transfer and subsequent dissemination of *T. annulata* infected cells in the body of the recipient host, but it has been speculated that the cell type which can become infected by *T. annulata* and support its further development, is of major importance (reviewed in Boulter & Hall, 2000). It is thought that upon inoculation of cattle with *T. annulata* schizont infected cells, schizonts will be taken up by monocytes and macrophages through phagocytosis and infection can become established. Monocytes and macrophages

have been shown to be the preferential host cells *in vitro* for *T. annulata* sporozoites (Glass et al., 1989; Spooner et al., 1989, Campbell et al., 1994; Sager et al., 1997, 1998a, b). It is of interest to note that we found indications that *T. lestoquardi* is able to infect sheep monocytes/macrophages (paper IV).

T. lestoquardi *sporozoites*

In the experiments described in paper II, a total of ten sheep was successfully infected by inoculation of *T. lestoquardi* sporozoite stabilate. Both schizonts and piroplasms were demonstrated in all sheep and they all seroconverted as deemed by IFAT and lines were established easily *ex vivo* from lymph node biopsies from all animals. For six of these sheep *in vitro* infection of their PBMC was attempted and shown to be successful (paper III).

In contrast, infection of cattle or their PBMC with *T. lestoquardi* was not demonstrated (papers II-III). Neither schizonts nor piroplasms were ever detected in six calves inoculated with the same dose of sporozoites that provoked infection in sheep. No changes related to the sporozoite inoculation were observed in any of the clinical and haematological parameters analysed. Sera of all calves remained negative to schizont antigen when tested in IFAT, and in none of the cell cultures derived from lymph node biopsies taken from these calves were schizont infected cells ever observed. Signs of proliferation of schizont infected cells were not seen in cultures of bovine PBMC inoculated *in vitro* with sporozoites of *T. lestoquardi,* and schizonts were never observed in Giemsa stained cytospin smears taken regularly from these cultures.

Apart from the preliminary experiments on susceptibility of sheep to infection with *T. lestoquardi* sporozoites that formed the basis for the studies described here (Brown et al., 1998), papers II-III are the first full reports on the successful establishment of *T. lestoquardi* infections of sheep induced through the inoculation of sporozoite stabilate. Our findings that cattle do not become infected with *T. lestoquardi* are in agreement with two other reports on attempts to infect cattle with this parasite which were both unsuccessful. Raghvachari & Reddy (1959) failed to transmit *T. lestoquardi* (then *T. hirci*) from sheep to cattle by blood inoculation, whereas Brown et al. (1998) were not successfull in infecting cattle with a tick derived sporozoite stabilate of *T. lestoquardi* (Lahr stock). Contrary to the non-susceptibility of cattle, goats were shown to be susceptible to *T. lestoquardi* infection when inoculated with sporozoites (Brown et al., 1998). Previous attempts to infect a goat with a suspension of schizont infected cells from the liver of a sheep suffering from an acute *T. lestoquardi* infection had failed (Hooshmand-Rad & Hawa, 1973a).

Knowledge of the host specificity of different *Theileria* species is essential for a greater understanding of the epidemiology of theilerioses. Most studies to determine the susceptibility of various host species to different *Theileria* species have been carried out in eastern and southern Africa where many of the wild Bovidae are known to carry *Theileria* piroplasms in their erythrocytes

(Grootenhuis & Young, 1981). For many of these parasites, the tick vector, host range and pathogenicity are little known. The host range of different *Theileria* species will depend on the ability of sporozoites to enter and transform cells of different hosts. In *in vitro* studies, it has been shown that several steps could be distinguished in the process of infection by sporozoites: i) attachment of the sporozoite to the host cells involving receptors for sporozoite binding; ii) infection, i.e. the entry of the sporozoite into the host cell with subsequent development into a schizont; and iii) transformation, i.e. the changes induced in the infected host cell by the parasite leading to the establishment of continuously growing schizont infected lines (Brown, 1979; Stagg et al., 1983).

So far, the molecules on the host cell that participate in sporozoite invasion have not yet been identified, but it has been shown that MHC class I molecules are essential for binding and entry of *T. parva* sporozoites, and that CD45R may play a similar role (Shaw et al., 1991 & 1995; Syfrig et al., 1998). Another study showed that in the rare event of infection of bovine alloreactive cytotoxic T cells by *T. annulata* sporozoites, susceptibility to infection seemed to be influenced by increased levels of MHC class II expression (Innes et al., 1989b). Partial degradation of cell surface antigens by proteinase K was shown to enhance the susceptibility of bovine lymphocytes to invasion by *T. parva* sporozoites (Syfrig et al., 1998). Interestingly, these authors not only confirmed previous observations that normal goat PBMC could not become infected with *T. parva* sporozoites (Stagg et al., 1983), but demonstrated that treatment of such cells with proteinase K rendered them susceptible. The authors speculated that treatment of cells by proteinase K, in removing many surface proteins, had exposed receptor(s) for *T. parva* sporozoites that were normally inaccessible (Syfrig et al., 1998).

We did not specifically study the early process of *T. lestoquardi* sporozoite attachment and entry to bovine cells, and thus the possibility could not be excluded that sporozoites in fact had entered host cells while their subsequent transformation apparently could not be supported. Also, in our experiments, attempts to establish cultures were carried out without the presence of feeder layer cells. In other studies, such cells were shown to enhance the growth of *T. annulata* infected bovine cells during the early stages of cultivation (Steuber et al., 1986). However, since *T. lestoquardi* schizont infected cells were never detected in any of the bovine cultures it was concluded that *T. lestoquardi* sporozoites were unable to infect and transform cattle cells. Thus, since sheep cells easily became infected with *T. lestoquardi* sporozoites, sheep cells had receptors for binding and entry of these sporozoites, while such receptors were likely not to be present or accessible on cattle cells, or otherwise, that other processes needed for *T. lestoquardi* sporozoite entry had not become activated in cattle cells.

T. annulata *sporozoites*

Inoculation of cattle as well as incubation *in vitro* of their PBMC with sporozoites of *T. annulata* always resulted in infection (papers II-III). Eight calves, six of which had been inoculated previously with *T. lestoquardi* sporozoites, developed severe theileriosis. Both schizonts and piroplasms were demonstrated and *T. annulata* schizont infected lines rapidly established *ex vivo* from all calves. Seroconversion was demonstrated in five of the seven calves that survived longer than 14 days. Continuously growing cell lines became easily established following inoculation *in vitro* of PBMC of six calves with *T. annulata* sporozoites (paper III).

Susceptibility of sheep to sporozoites of *T. annulata* was analysed both *in vitro* and *in vivo*. In seven out of eight cultures of PBMC inoculated *in vitro* with *T. annulata* sporozoites schizonts were seen and *T. annulata* lines established. Schizonts were rarely seen in Giemsa stained cytospin smears of lymph node biopsy material of four sheep infected by inoculation of sporozoites and a finding of major interest was that in none of the sheep were piroplasms ever observed. However, infection was finally demonstrated in all four sheep since *T. annulata* infected lines established from *ex vivo* derived cultures. Also, three weeks after inoculation, antibodies to schizont antigen were detected in all four sheep. Apparently, although the cells infected by *T. annulata* sporozoites were able to support their transformation into schizonts, they did not support their further development into merozoites. One could speculate that the host cell type that was infected with *T. annulata* had an influence on the ability of *T. annulata* to complete its lifecycle in sheep. This speculation could not be confirmed in the phenotypic characterization experiments described in paper IV.

To my knowledge, this is, apart from the study by Brown et al. (1998), the only report on successful experimental infection of sheep with sporozoites of *T. annulata*. Previously, the sheep was considered to be refractory to infection with this parasite. Reports on the non-susceptibility of sheep to *T. annulata* date back as far as the early 1920's when blood transmission of *T. annulata* infected cattle to sheep was shown to be unsuccessful (Sergent et al., quoted in Lestoquard, 1926). A failure to transmit *T. annulata* to sheep with infective *H. excavatum* ticks has been reported, but no further experimental details were given (Neitz, 1957).

However, *in vitro* studies carried out many years later showed that PBMC of sheep could become infected by *T. annulata* sporozoites and transformed into continuously growing cell lines (Steuber et al., 1986; Entrican et al., 1991). Interestingly, in sheep inoculated with autologous *T. annulata* schizont cells that had become infected *in vitro*, no piroplasms were detected while these did develop in cattle inoculated with the *T. annulata* schizont infected ovine cells (Steuber et al., 1986). Thus, these results support our findings that *T. annulata*, in not producing piroplasms, is unable to complete its lifecycle in the sheep.

Equally, piroplasms were not observed in goats that had become infected by *T. annulata* sporozoites (Brown et al., 1998).

Different results between authors regarding the susceptibility of different host species for different *Theileria* species may have been caused by differences in the way of inoculation and the material used for it, i.e. mechanical transmission of schizont infected cells present in organs and/or blood of animals undergoing acute infection versus 'tick transmission' of infective sporozoites, with the latter apparently being more effective. As described earlier, successful establishment of infection by mechanical transmission may depend on the efficiency with which schizonts from the donor animal can be transferred to a cell type of the host that can support its further development.

Another reason for inconsistent results between different research groups could be that they, in fact, may be working with different parasite species. Recently, workers in China have reported on an outbreak of severe theileriosis affecting sheep and goats and assigned *T. lestoquardi* (syn. *T. hirci*) as the causal agent (Luo & Yin, 1997). Later studies based on sequence analysis of the ribosomal small subunit RNA gene revealed a closer relationship of this parasite with the bovine *T. buffeli/orientalis/sergenti* group than with *T. lestoquardi* (Schnittger et al., 2000) and makes it likely that the Chinese parasite is different from *T. lestoquardi*. These findings fit well with the observation that the Chinese parasite is transmitted by *Haemaphysalis* ticks (Luo & Yin, 1997). Ticks of this genus are known to transmit parasites of the *T. buffeli/orientalis/sergenti* group (Uilenberg, 1981a) and have also been shown to transmit *T. recondita* of sheep in Wales (Alani & Herbert, 1988a). Thus, there is a need for the development of reliable tests for correct parasite identification.

Pathogenicity (I-II)

In the six sheep that had become infected through the inoculation of attenuated *T. lestoquardi* schizont infected cells (paper I), the only clinical signs observed were slight fever for some days and enlarged lymph nodes at the side of inoculation (results not shown). Schizont infected cells were first detected in low numbers in Giemsa stained smears of lymph node biopsies taken ten to twelve days after inoculation and piroplasms were never demonstrated in any of the animals. Thus, previous findings by Hooshmand-Rad (1985) that sheep immunized with an attenuated stock of *T. lestoquardi* schizont infected cells develop mild symptoms only, were confirmed.

Clinical symptoms varied in the sheep following *T. lestoquardi* infection induced by sporozoite inoculation (paper II). While one animal showed mild and another moderate reactions, reactions in the other eight sheep were considered severe and four animals died or had to be killed to avoid suffering. In all sheep, the prescapular lymph node at the side of stabilate inoculation increased in size from d5-d6 onwards, while fever started several days later (Figure 5). First day of parasite detection was uniform, with schizont infected cells appearing in the

draining lymph node by d7-d11 and piroplasm infected erythocytes by d10-d12. Schizont infected cells were demonstrated in lymph node biopsy smears and were sometimes, but rarely, also seen in peripheral blood slides. First schizont detection in lymph node biopsies coincided with the development of fever. Maximum piroplasm levels ranged from 5-32% and dropped suddenly in the third to fourth week of infection. White blood cell (WBC) counts and packed cell volume (PCV) values diminished in all animals and leukopenia and anaemia developed in most sheep. All sheep lost body condition and a variety of other symptoms such as listlessnes, ruminal atony and diarrhoea were seen in most animals during the second and third week of infection. Icterus that lasted for several days was noticed in the PCV samples.

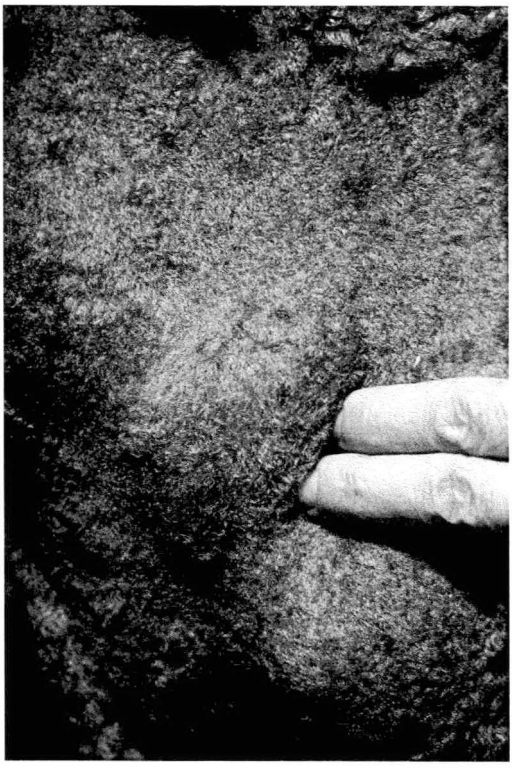

Figure 5. Enlarged prescapular lymphnode in sheep inoculated with *T. lestoquardi* sporozoites (Photo: Bengt Ekberg).

The four sheep that died were necropsied (results not shown). All demonstrated anaemia and icterus and generalized muscle degeneration. The most common other findings were hyperplastic splenitis and generalised lymphadenitis (Figure 6), diffuse reactive hepatitis, focal interstitial lymphocytic nephritis and catarrhal or catarrhal-haemorrhagic (gastro-)enteritis. Mononuclear cell infiltrates were seen in several organs. All sheep showed a catarrhal or fibrinopurulent pneumonia from which *Mycoplasma ovipneumoniae* and sometimes *Pasteurella haemolytica* were isolated.

Our findings on disease developement, appearance of schizont infected cells and piroplasms as well as the post mortem findings in *T. lestoquardi* infections of sheep are in agreement with other reports on mechanically or tick induced infections (Neitz, 1957; Raghvachari & Reddy, 1959; Hooshmand-Rad en Hawa, 1973a; Sisodia, 1981; Sisodia & Gautam, 1983; Brown et al., 1998).

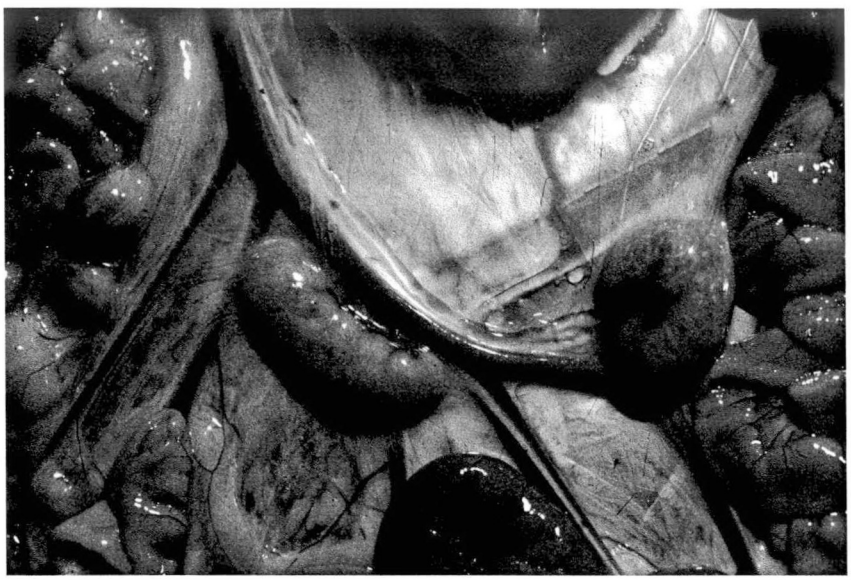

Figure 6. Enlarged mesenteric lymph nodes in sheep inoculated with *T. lestoquardi* sporozoites (Photo: Bengt Ekberg).

In *T. annulata* infected cattle disease reactions were uniform (paper II). Eight calves, six of which had been inoculated previously with *T. lestoquardi* sporozoites, developed severe theileriosis resulting in the necessary killing of five animals. Both schizonts and piroplasms were demonstrated and *T. annulata* schizont infected lines established very easily *ex vivo* from all calves. No difference was observed in any of the clinical and haematological parameters analysed between the two naive animals and the ones that had been inoculated previously with *T. lestoquardi*. Also, time to first appearance of parasitic stages and duration and level of piroplasm parasitaemia were the same in both groups.

The five calves that were killed were necropsied (results not shown). Carcasses were anaemic and icteric and the most prominent findings were a generalised lymph node hyperplasia and an enlarged spleen. Intestines, lungs and lymph nodes were oedematous. Haemorrhages and petechiae were present in conjunctival mucosa and kidneys and under the epicardium. Localised lymphocytic infilatrations were seen in several organs including the skin. Erosions of the buccal mucosa were observed as well as ulcers in the abomasum. The animals also had an acute fibrinopurulent pneumonia, but no specific bacterial infection was demonstrated in two animals analysed.

On the other hand, very mild reactions were observed in four sheep that were inoculated with the same dose of *T. annulata* sporozoites that provoked severe disease in cattle (paper II). In the sheep, an increase in the size of the prescapular lymph node at the side of inoculation and slight fever for a number of days, were the only signs of infection. In some of the sheep schizonts were rarely demonstrated in cytospin smears of lymph node biopsy material but were finally demonstrated in all animals in cultures initiated from these biopsies. Piroplasms were never observed.

It has been demonstrated in experimental infections of cattle with graded doses of *T. annulata* sporozoites that the response to infection is influenced by the dose of sporozoites used for inoculation (Preston et al., 1992a). The time to appearance of the different parasitic stages, the time and severity of pyrexia and the degree of anaemia as manifested by PCV were shown to be dose dependent. The clinical, parasitological and haematological responses observed in the studies described here in cattle undergoing acute *T. annulata* infection as well as their pathology were well in line with the findings of other authors who applied potentially lethal doses of sporozoites to establish experimental *T. annulata* infections in cattle (Preston et al., 1992a; Forsyth et al., 1999). Since sheep, when inoculated with a dose of *T. annulata* sporozoites that was lethal to cattle, underwent very mild infection only, it can be concluded that *T. annulata* is a parasite of low pathogenicity to sheep.

Cross protectivity (II-III)

In sheep that survived *T. lestoquardi* infection, indications were found for some degree of cross protection upon challenge with *T. annulata* sporozoites (paper II). One sheep showed mild symptoms with slight fever for a few days, whereas infection in three more sheep virtually passed unnoticed. Reactions in four sheep that underwent a primary *T. annulata* infection were mild in three and not apparent in one sheep. However, whereas a substantial increase in size of the prescapular lymph nodes was seen in these animals, similar changes were only observed in the one challenged sheep that showed fever. This was the only challenged sheep from which a continuously growing *T. annulata* schizont infected line could be established from one sampling occasion, while from three of four sheep undergoing primary *T. annulata* infection, such lines established from more than one occasion. Schizont infected lines established from two more challenged sheep, but PCR analysis revealed that these lines were infected with *T. lestoquardi*. Since no *T. annulata* schizont infected cells were ever detected in three of the challenged sheep, and antibody levels remained at preinfection levels when tested by IFAT with *T. annulata* and *T. lestoquardi* antigens, it is likely that in these animals the *T. annulata* infection did not establish at all or only to a very limited extent. Transformation and establishment *in vitro* of *T. annulata* infected lines from PBMC of all four challenged sheep did not succeed (paper III). This contrasted with the successful establishment *in vitro* of lines from PBMC taken from two naive sheep. Other authors have reported on difficulties in establishing

T. annulata infected cultures *in vitro* with PBMC from immune cattle (Preston & Brown, 1985). They demonstrated that early infection levels in cultures from immune animals were markedly lower than infection levels in cultures initiated with PBMC from susceptible cattle. Also, PBMC of immune cattle have been shown to inhibit the growth *in vitro* of already established cultures of *T. annulata* infected cells (Ahmed et al., 1989). Results from a later study indicated that cytokines, e.g. IFN-α, IFN-γ, TNF-α and IL-2, could inhibit the proliferation of early *T. annulata* infected cultures *in vitro* (Preston et al., 1992b).

Likewise, indications were found for partial cross protection between *T. lestoquardi* and *T. annulata* in sheep that underwent *T. lestoquardi* challenge infection after previous *T. annulata* infection (paper II). However, previous *T. annulata* infection did not prevent the establishment of *T. lestoquardi* in sheep, since both schizonts and piroplasms were demonstrated in all four animals. But while reactions in control sheep undergoing primary *T. lestoquardi* infections were severe in three and mild in one animal, reactions in all four challenged sheep were classified as mild. Apart from moderate enlargement of the lymph node on the side of inoculation in all sheep and slight fever in two of them, the animals remained clinically normal. However, leukopenia developed in three of the challenged sheep and anaemia in two. In one of the sheep WBC counts and PCV values fell to levels also seen in sheep undergoing primary *T. lestoquardi* infection. In both groups, schizonts and piroplams were first detected around the same time, but maximum piroplasm levels were lower in the challenged sheep than in the animals undergoing primary infection. Not all attempts to establish *ex vivo* lines from the challenged sheep were successful and often it took more time for cultures from these animals to develop into continuously growing lines compared to cultures from sheep undergoing primary infection. Although schizont infected cells were sometimes seen in cultures from biopsy material of the contralateral lymph nodes, only one of these cultures could be maintained as a continuously growing line. All lines from challenged sheep that established from lymph node biopsy material from the side of inoculation were identified by PCR as infected with *T. lestoquardi*, whereas the only line that established from a biopsy of the contralateral lymph node was shown to be infected with *T. annulata*.

Very little information is available on the development of cross protectivity between different *Theileria* species of sheep, although cross protection between *T. lestoquardi* (syn. *T. hirci*) and *T. ovis* is considered to be absent (Lestoquard, 1926; Raghvachari & Reddy, 1959). However, experimental details confirming such accounts are scarce (Raghvachari & Reddy, 1959). Since sheep were generally considered refractory to *T. annulata* infection, cross protection studies involving *T. lestoquardi* and *T. annulata* have not been carried out previously. On the other hand, it is well known that in cattle cross protection between different bovine *Theileria* species does not develop (Irvin, 1987; Morzaria, 1989). Cross protectivity studies of cattle involving studies on different stocks of the same parasite species or vaccine studies revealed that protected animals showed no or a

less severe clinical response to challenge infection, had longer prepatent periods (length of time until schizonts were seen in lymph node smears) and parasite infected cells could not be demonstrated or were only detectable at lower levels (Preston et al., 1983; Ahmed et al., 1989; Innes et al., 1989a; Boulter et al., 1995). Similar observations were made in the experiments described here, indicating that in sheep partial cross protection had developed between *T. lestoquardi* and *T. annulata*. In studies analyzing the response of cattle that had recovered from a *T. annulata* infection to subsequent challenge infection with a heterologous stock of *T. annulata* sporozoites, it could be demonstrated that the level of protection was related to the severity of the primary infection, which in its turn depended on the size of the initial dose of sporozoites (Preston et al., 1992a). In the studies described here we also found some indication for a relationship between severity of reaction to primary infection and response to challenge infection, since the sheep reacting stongest to *T. lestoquardi* challenge infection was the only one that had not shown a temperature reaction to primary *T. annulata* infection.

Cell surface phenotypes (II- IV)

Cell lines that were re-isolated from the sheep and cattle that underwent the experimental infections described in paper II were characterized using polyclonal antibodies and mAbs to leukocyte cell surface markers and FCM (paper IV). These characterization experiments were carried out in an attempt to analyse i) if the observed differences in the course of the disease in sheep infected with *T. lestoquardi* or *T. annulata* and ii) if the similarities between *T. lestoquardi* infections of sheep and *T. annulata* infections of cattle, could be explained by similarities or discrepancies in the cell surface phenotypes of the parasite infected cells.

It is generally accepted that the diffences between infections of cattle with *T. annulata* and *T. parva* in disease development and ensuing pathology, can be at least partially explained by different cell types becoming infected with these parasites (Irvin & Morrison, 1987; Innes et al., 1992; Boulter & Hall, 2000). As mentioned earlier, *in vitro* studies had demonstrated that *T. annulata* preferentially infects monocytes and macrophages but can infect B cells also, and, only under exceptional circumstances and at low efficiency, T cells as well (Spooner et al., 1989; Innes et al., 1989b; Sager et al., 1997), while *T. parva* preferentially infects T cells and B cells (Baldwin et al., 1988). On the other hand, the majority of cells infected *in vivo* by *T. parva* are T cells (Emery et al., 1988). More detailed indications that the infection of T cells, rather than B cells, contributes to the pathogenicity of *T. parva,* comes from a study in which experimental infection of cows with autologous B cells that had become infected *in vitro* by sporozoites gave rise to a self limiting infection whereas infection with autologous parasitized T cells resulted in severe disease (Morrison et al., 1996).

In the experiments described here, *T. lestoquardi* and *T. annulata* infected sheep lines established *ex vivo* as well as *T. annulata* infected cattle lines all expressed the non-lineage specific markers CD45, MHC class I and MHC class II. There were only minor differences between lines in the proportions of stained cells, and within lines in staining intensity. Expression of antigens normally associated with myeloid lineage cells, i.e. CD11b (Gupta et al., 1993), CD14, CD16 and the antigen detected by mAb IL-A24 (Naessens & Hopkins, 1996), was demonstrated in lines of each type. Compared to expression of the non-lineage specific antigens, there was more variation in expression of the myeloid lineage markers in proportions of reacting cells within and between lines. With a few exceptions, staining intensity was comparatively low, particularly in the sheep lines. Expression of sIg, a characteristic of B cells, was demonstrated in several of the *T. lestoquardi* infected sheep lines and in three out of four *T. annulata* infected sheep lines by the use of a polyclonal and a monoclonal antibody. Cell lines that expressed sIg also reacted to mAb VPM30, a mAb detecting an antigen expressed on B cells (Naessens & Howard, 1991). In *T. annulata* infected cattle lines, expression of B cell antigens, including sIg, CD21 and the antigen detected by VPM30, was observed in at least one line. In several lines of each type low proportions of cells reacting to the mAb identifying the T cell antigen CD8 were seen. Apart from the expression of CD16 in all *T. lestoquardi* infected sheep lines, which was not detected in *T. annulata* infected sheep lines and not looked for in *T. annulata* infected cattle lines, differences between lines were quantitative rather than qualitative. Irrespective of the type of line, heterogeneous responses were seen within lines of the same type with the antibodies identifying lineage specific markers, i.e. individual lines were seen to react to mAbs identifying B cell markers as well as to those identifying monocyte/macrophage markers. Similar results were obtained with *T. lestoquardi* and *T. annulata* infected lines that established *in vitro*.

It is well known that phenotypic alterations occur on cattle cells that become infected *in vitro* with *T. annulata* or *T. parva*. While non-lineage specific markers generally continue to be expressed, changes have been observed in the expression of lineage specific markers. *T. parva* infected T cells continue to express T cell markers and sometimes acquire expression of additional T cell markers (Baldwin et al., 1988), while expression of sIg has been reported to become downregulated in both *T. parva* and *T. annulata* infected B cells (Baldwin et al., 1988; Spooner et al., 1989; Glass et al., 1989). However, other authors have reported a continued, though sometimes diminished, expression of B cell markers on *T. annulata* infected bovine cells (Sager et al., 1998a; Moreau et al., 1999). Expression of several markers on monocytes and macrophages, including CD14, is generally considered to decrease following infection with *T. annulata* (Spooner et al., 1989; Glass et al., 1989; Sager et al., 1997), and expression of some of these markers has been shown to become upregulated after treatment of the cells with a theilericidal drug (Sager et al., 1997 & 1998a). The generally low levels of expression of lineage specific markers observed in the studies described here,

suggested that such a downregulation had occurred also in sheep cells infected with *Theileria* parasites.

Assuming that reactivity of cells with antibodies identifying myeloid lineage markers suggested a myeloid origin of these cells, while reactivity with antibodies identifying B cell antigens, was indicative of B cell origin, we considered it likely that *T. annulata* had infected monocytes/macrophages and B cells in cattle as well as in sheep and also that *T. lestoquardi* had done so in sheep. Consequently, we concluded that *T. lestoquardi* and *T. annulata* were both able to infect cells of different lineage *in vivo* as well as *in vitro*. However, the low level of expression of the lineage specific markers by a proportion of cells only, precluded any definite conclusions on any possible difference between *T. lestoquardi* and *T. annulata* in their preference for infection of a certain cell type. Consequently, we were unable to ascribe the similarities and differences in pathogenicity of *T. lestoquardi* and *T. annulata* for sheep and cattle to a difference in cell preference.

Summary of findings and concluding remarks

The studies described here have focussed on comparative aspects of the cattle parasite *T. annulata* and the much less studied corresponding parasite of sheep, *T. lestoquardi*.

When analysed by IFAT based on schizont antigen, *T. lestoquardi* and *T. annulata* were shown to have more diagnostic antigens in common with each other than with *T. parva* of cattle, or with *T. ovis* and *T. separata* of sheep. Recent reports in the literature on molecular analysis also support a closer phylogenetic relationship of *T. lestoquardi* with *T. annulata* than with *T. parva*.

Inoculation of sheep with *T. lestoquardi* sporozoites resulted in the establishment of infection and the subsequent development of clinical theileriosis which very much resembled acute tropical theileriosis of cattle infected with *T. annulata*. *T. annulata* infected sheep but caused mild infections only and no piroplasms were detected. In contrast, T. *lestoquardi* did not infect cattle.

Thus, *T. lestoquardi* is capable of causing severe disease in sheep. *T. annulata*, on the other hand, is a pathogenic parasite of cattle that may cause mild or inapparent infection of sheep, but fails to complete its vertebrate cycle in this animal species. Consequently, infection of sheep with *T. annulata* plays no role in the epidemiology of bovine tropical theileriosis.

Infection of sheep with *T. lestoquardi* or *T. annulata* seemed to confer partial protection to infection or to the clinical consequences of infection with the heterologous species. Thus, *T. lestoquardi* and *T. annulata* are likely to share protective antigens in addition to diagnostic antigens. It would be interesting to analyse if *T. annulata* schizont infected bovine or ovine cell lines would confer protection against *T. lestoquardi* infection of sheep.

In vivo susceptibility of sheep and cattle to infection with *T. annulata* or *T. lestoquardi* corresponded to susceptibility to infection of their PBMC by the sporozoites of these parasites *in vitro*.

Given the extensive cross-reactivity of *T. lestoquardi* and *T. annulata* antigens in IFAT, in combination with the fact that sheep can become naturally infected with *T. lestoquardi* as well as with *T. annulata*, the IFAT applying schizont infected cells as antigen source is not sufficiently specific to discriminate between infections of sheep with *T. lestoquardi* and *T. annulata*. For epidemiological studies of malignant ovine theileriosis, diagnostic tests need to be developed which can reliably distinguish both pathogens.

Cell surface phenotypes of *T. annulata* infected bovine or ovine lines and of *T. lestoquardi* infected ovine lines were shown to be very similar. The cell surface phenotypes of these lines, whether derived from *in vitro* infection or *ex vivo*, suggested that both parasites infected macrophages and B cells of their respective host species. In general, expression of leukocyte subpopulation specific markers was low, suggesting a downregulation of cell surface antigens. Differences in cell surface phenotypes of these lines were too small to explain the observed differences in the course of clinical infection of sheep with *T. lestoquardi* or *T. annulata*. Likewise, similarities in the course of clinical infections of sheep infected with *T. lestoquardi* and of cattle with *T. annulata* could not be directly related to similar expression of cell surface antigens.

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