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Titre de la thèse : **Rôle des éosinophiles dans la régulation des populations d’*Haemonchus contortus* chez le mouton.**

Soutenue le 11 juin 2007 devant le jury composé de :

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Getachew Terefe
June 2007, Toulouse (France)

Dedicated to my mother

Abebech Adinew

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INTRODUCTION

Le parasitisme gastro-intestinal est une des plus sérieuses contraintes de l'élevage des petits ruminants dans le monde. Son importance économique est due aux pertes de production engendrées lors de fortes infestations, aux coûts de la chimioprophylaxie et parfois, à la mort des animaux. Parmi les parasites du tube digestif des moutons, *Haemonchus contortus*, ver hématophage de la caillette, est le plus important compte tenu de sa pathogénicité et de sa grande distribution géographique (régions tropicales, subtropicales et tempérées du globe).

Le contrôle des nématodes parasites en général, et d'*H. contortus* en particulier, repose sur les traitements anthelminthiques. Cependant, ces molécules ne sont pas toujours disponibles et surtout, leur coût restreint leur utilisation dans les pays en développement. A l'inverse, lors d'utilisation trop intensive de ces molécules, l'acquisition d'une résistance aux anthelminthiques par de nombreuses espèces pose des problèmes de plus en plus sérieux aux éleveurs (van Wyk *et al.,* 1997 ; Eddi *et al.,* 1996). Ces deux éléments soulignent l'importance de promouvoir des stratégies de lutte alternatives au seul emploi de molécules chimiques. En dépit d'avancées importantes réalisées jusqu'à maintenant, l'efficacité de méthodes alternatives comme la vaccination ou la sélection d'animaux résistants est limitée par notre méconnaissance des interactions hôtes-parasites et des mécanismes immunitaires mis en jeu par les hôtes pour réguler les populations parasitaires.

Des études *in vitro* et *in vivo* ont montré, dans les modèles rongeurs, que les infestations par des nématodes gastro-intestinaux entraînent une réaction immunitaire adaptative de type Th2 (Hogan *et al.,* 2000 ; Mckenzie *et al.,* 1998 ; Urban *et al.,* 1991 ; Sher *et al.,* 1990). Les cytokines sécrétées par les lymphocytes T CD4+ TH2 engendrent une mastocytose, une éosinophilie sanguine et tissulaire ainsi que la production d'anticorps spécifiques (Gause *et al.* 2003 ; Grencis 2001 ; Else and Finkelman, 1998). Cependant, les rôles respectifs de ces mécanismes effecteurs restent controversés (Behm and Ovington, 2000). La destruction des parasites par les éosinophiles chez la souris est suggérée par de nombreuses études *in vitro* (Shin *et al*., 2001 ; Strote *et al.,* 1990 ; Butterworth, 1984 ; Kazura and Grove, 1978) ou par des études *in vivo* de mobilisation sélective de ces cellules dans des chambres de diffusion contenant des larves de parasites (Brigandi *et al.,* 1996 ; Rotman *et al.,* 1996). De plus, des

études *in vivo* utilisant des souris Il-5$^{-/-}$ ou Il-5 transgéniques suggèrent que les éosinophiles sont impliqués dans la résistance de l'hôte même s'ils ne sont certainement pas les seuls acteurs de cette résistance et si les résultats diffèrent selon les parasites (Klion and Nutman, 2004 ; Behm and Ovington, 2000 ; Meeusen and Balic, 2000).

Chez le mouton, les données ne sont pas aussi nombreuses que dans les modèles murins. Toutefois, récemment, il a pu être montré que les infestations par des nématodes gastro-intestinaux s'accompagnent de réactions immunitaires de type Th2 mettant en jeu les mêmes mécanismes effecteurs cellulaires et humoraux (Lacroux *et al.,* 2006 pour le modèle *H. contortus* et Pernthaner *et al.,* 2006, 2005 pour le modèle *Trichostrongylus colubriformis* de l'intestin grêle). Ici aussi, les éosinophiles sont recrutés en grand nombre dans le sang et dans les tissus infestés (Balic *et al.,* 2006 ; Stear *et al.,* 2002 ; Rothwell *et al.,* 1993). Les éosinophiles de mouton sont capables d'immobiliser des larves infestantes d'*H. contortus* en présence de sérum d'animaux immuns ou de complément comme cela a été démontré *in vitro* par Rainbird *et al.,* 1998. Cependant, leur rôle protecteur *in vivo* est moins clair et bien moins documenté que dans les modèles murins. Des études histologiques montrent que les éosinophiles sont, en nombre, les principales cellules recrutées dans les muqueuses abomasales d'animaux infestées (Balic *et al.,* 2000) et qu'ils sont retrouvés à proximité ou en contact de larves mortes dans les tissus (Balic *et al.,* 2006). L'absence de moutons génétiquement modifiés (analogues des souris IL-5 $^{-/-}$ ou IL-5 transgéniques) limite l'étendue des recherches. Dès lors, la démonstration de l'implication *in vivo* des éosinophiles dans la résistance aux strongles gastro-intestinaux chez le mouton repose le plus souvent sur des corrélations entre la résistance à une infestation et l'intensité d'une réponse éosinophilique sanguine ou tissulaire. Des variations de cette réponse sont décrites selon la race et l'âge des animaux, la fréquence de l'exposition aux helminthes, l'espèce parasite en cause et parfois aussi selon le protocole d'infestation expérimentale utilisé (Amarante *et al.* 2005 ; Stear *et al.* 2002 ; Thamsborg *et al.* , 1999 ; Woolaston *et al.,* 1996 ; Dawkins *et al.,* 1989). En bilan, l'analyse de la littérature disponible chez le mouton ne permet pas de dégager une conclusion définitive sur le rôle des éosinophiles dans la régulation des populations de strongles gastro-intestinaux. Ceci justifie de nouvelles recherches pour comprendre le rôle exact de chaque composante de la réponse immune. Comprendre ces mécanismes contribuera à la mise au

point de nouveaux vaccins et/ou à la production de lignées résistantes de moutons dans un objectif de développement de méthodes alternatives à la chimiothérapie.

Les travaux de cette thèse ont été réalisés dans le but de mieux comprendre l'implication des éosinophiles dans la résistance du mouton à *H. contortus*. Compte tenu de l'hyperéosinophilie sanguine et tissulaire constamment observée lors d'infestations naturelles ou expérimentales, nous avons fait l'hypothèse que cette cellule joue un rôle majeur dans la régulation des populations de ce nématode. Des études in vitro et *in vivo* ont été menées afin de savoir :

i) si les éosinophiles de moutons sont effectivement capables de tuer des larves infestantes d'*H. contortus in vitro*. Ceci a été confirmé après implantation chirurgicale dans la caillette de larves ayant été en contact avec des suspensions cellulaires enrichies en éosinophiles,
ii) si l'augmentation ou la réduction préalable de la réponse éosinophilique de l'hôte a un effet sur la résistance de celui-ci lors d'infestation expérimentale par *H. contortus*,
iii) quels sont les facteurs affectant la réponse éosinophilique de l'hôte lors d'infestations par *H. contortus*.

Ce manuscrit est composé de quatre grandes parties : la première partie est consacrée à une revue bibliographique sur le parasite et la réponse immunitaire de l'hôte lors d'infestation par un nématode gastro-intestinal ; la seconde partie présente le matériel et les méthodes utilisés ; la troisième expose les principaux résultats obtenus, enfin, la quatrième discute les résultats et trace les principales perspectives de ce travail.

INTRODUCTION

Gastrointestinal parasitism is arguably the most serious constraint affecting sheep production worldwide. Economic losses are caused by decreased production, costs of prophylaxis and treatment, and death of the infected animals. The blood-feeding nematode, *Haemonchus contortus,* is among the most important gastrointestinal (GI) parasites of sheep. This parasite, besides its pathologic significance, has a very important position from epidemiological point of view in that it is a parasite of tropical, subtropical and temperate regions of the world.

The control of nematode parasites in general and *H. contortus* in particular traditionally relies on anthelmintic treatment. However, anthelmintics are not always available or affordable. At the other extreme where anthelmintics are used intensively, the development of drug resistance by many species of parasites poses a serious problem to the animal breeder (van Wyk *et al*., 1997; Eddi *et al*., 1996) collectively demanding for other control strategies that are less dependent on chemoprophylaxis. In spite of the enormous advances so far registered, the development of effective alternative control strategy (vaccine, selected breeding etc.) is hampered by limitations in properly defining and understanding of the nature of the host-parasite interaction and the underlying immune mechanisms.

In vitro and *in vivo* studies in several rodent species show that Th2-polarized immune responses characterize gastrointestinal parasitic infections (Hogan *et al*., 2000; Mckenzie *et al*., 1998; Urban *et al*., 1991; Sher *et al*. 1990). The cytokines secreted by Th2 cells promote mastocytosis, eosinophilia and the production of antibodies (Gause *et al*., 2003; Grencis, 2001; Else and Finkelman, 1998). However, the precise roles of these effector mechanisms remain controversial (Behm and Ovington, 2000). Eosinophil mediated killing of several helminth species is suggested in a number of studies in mice, both *in vitro* (Shin *et al*., 2001; Strote *et al*., 1990; Butterworth, 1984; Kazura and Grove, 1978) and in diffusion chambers containing parasite larvae (Brigandi *et al*., 1996; Rotman *et al*., 1996). On the other hand, *in vivo* works using IL-5 knockout or IL-5 transgenic mice suggest that eosinophils definitely involve but are

not essential elements in the hosts' resistance to helminth parasites (Klion and Nutman, 2004; Behm and Ovington, 2000; Meeusen and Balic, 2000).

In sheep, from the limited data so far available, it can be deduced that immunity to gastrointestinal nematode infections is governed by a similar Th2 type immune response with characteristic effector cells and antibodies (Lacroux *et al*., 2006; Pernthaner *et al*., 2006, 2005; Gill *et al*., 2000). Here also, eosinophils are the most consistently mobilised cells in both blood and tissues (Balic *et al*., 2006; Stear *et al*., 2002; Rothwell *et al*., 1993). In one *in vitro* study, it was demonstrated that eosinophils are capable of immobilizing *H. contortus* infective larvae in the presence of immune serum and/or complement (Rainbird *et al*., 1998). However, information on the protective role of these cells *in vivo* is much less clear than what have been documented in laboratory animals. Histological abomasal tissue studies show that eosinophils are the major cells recruited into the abomasal mucosa and were found on and around dying tissue dwelling stages of *H. contortus* (Balic *et al*., 2006, 2000). Lack of genetically modified sheep (gene knockout or transgenic) targeted to reduce or enhance specific cytokines and effector cell (such as IL-5, eosinophils) has limited the advance in such studies *in vivo*. Demonstration of the role of eosinophils in the resistance to helminth infections in sheep is often based on statistical correlations that exist between resistance/susceptibility to infection and the magnitude of eosinophil responses. However, such correlations are subject to variations inherent to experimental animals and study protocols (Amarante *et al*., 2005; Stear *et al*., 2002; Thamsborg *et al*., 1999; Woolaston *et al*., 1996; Dawkins *et al*., 1989). Hence, analysis of the reports so far available in sheep does not seem to allow a specific conclusion on the role of eosinophils in the resistance to helminth infections. This calls on further study to elucidate the potential values of the different immune mechanisms involved in sheep infected by GI nematodes. Understanding these mechanisms is believed to contribute to the designing and production of effective vaccines and/or in the development of resistant lines or breeds of animals that can be used as alternative control strategies against GI parasitism.

Hence, the works in this thesis were initiated with the objective of exploring the role of eosinophils in the immune response of sheep to *H. contortus* infection. Our hypothesis was that the consistent expression of eosinophilia at high levels, especially in resistant breeds/lines, in blood and tissues during abomasal parasitism could have an important role in the resistance of sheep to *H. contortus*.

To achieve our objective, *in vitro* and *in vivo* experimental methods have been employed to see:

- If sheep eosinophils are capable of killing *H. contortus* infective larvae *in vitro*. This was supported by intra-abomasal administration of larvae incubated with eosinophil-enriched blood leucocytes as a confirmatory procedure
- If enhancing or reducing blood eosinophilic responses has an effect on the resistance of sheep during infection with *H. contortus*
- Factors affecting eosinophilic responses in the natural resistance of sheep to *H. contortus*

This thesis is divided into four major parts: Part I is devoted to literature review that deals with the general overview of the parasite studied and the immune response of hosts against nematode infections. Part II deals with the materials and methods employed to achieve our objectives. Part III presents the results obtained at the end of each study. These are mainly organized in the form of published and unpublished documents. Finally, we discuss our findings in part IV with some perspectives indicating future areas of concern.

PREMIERE PARTIE

REVUE BIBLIOGRAPHIQUE

Cette partie comprend une description du parasite étudié, *Haemonchus contortus* et des mécanismes immunitaires que l'hôte oppose au parasitisme par ce nématode. Dans la première section, la biologie et l'action pathogène du parasite sont décrites. La section 2 donne une vue générale des mécanismes immunitaires mis en jeu contre les parasites gastro-intestinaux. Comme la biologie et l'effet de l'éosinophile constituent le coeur de ce travail, la section 3 leur est entièrement consacrée.

PART I. LITERATURE REVIEW

This part is concerned mainly with the description of the parasite under study, *H. contortus,* and the different immune mechanisms involved. In section 1, the biology of the parasite and pathologies associated to infection with the parasite are reviewed. Section 2 gives an overview of the general immunological mechanisms against pathogens and Th2 polarised immune effector systems in gastrointestinal parasitism. As eosinophils occupy the core of our study, their biology and role in GI parasitism is treated separately in section 3.

1. HAEMONCHUS CONTORTUS

The majority of gastrointestinal strongyles of ruminants belong to the family Trichostrongylidae. The genus *Haemonchus* is in the sub-family of Haemonchinae and consists of four main species in domestic ruminants, namely, *H. contortus* (in ovine and caprine), *H. placei* and *H. similis* (in bovine) and *H. longistipes* (in dromedary).

1.1. Morphology

An adult *H. contortus* measures about 15 to 30 mm long, the male being shorter than the female. The morphological characteristics of *H. contortus* (Figure 1) are a mouth capsule with a single dorsal lancet and two prominent cervical papillae in the oesophageal area. The male parasite is characterised by its copulatory bursa formed of two large lateral lobes and a small asymmetrically positioned dorsal lobe. Together with the two chitinous spicules, which are inserted in the female genital opening during copulation, this part of the worm is important for identification. The females have a reddish digestive tube filled with ingested blood, spirally surrounded by two white genital cords (ovaries). They have a sharply pointed slender tail and a vulva with or without anterior vulval flap (Morales and Pino, 1987). The eggs are of strongyle type with a diameter between 70 and 85 µm (Kassai, 1999; Bussiéras and Chermette, 1988).

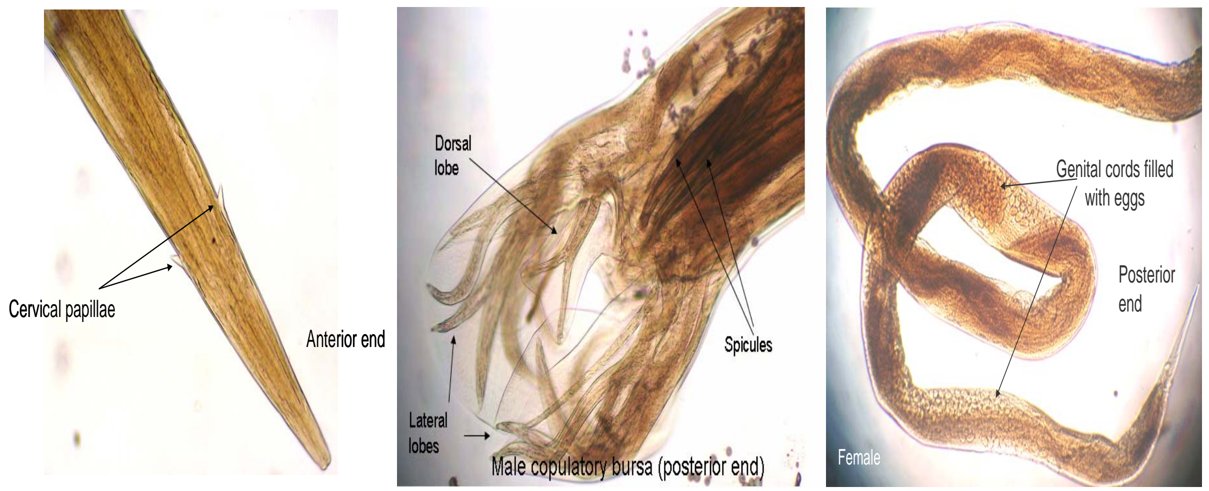


Figure1. Morphology of male and female *H. contortus* (Photos: G. Terefe)

1.2. Life cycle

The life cycle of *H. contortus* consists of free-living stages on the pasture and parasitic stages within the host's abomasum (Figure 2). Eggs produced by the female are passed out in the faeces. A mature female *H. contortus* can produce 5000-7000 eggs per day (Coyne *et al*., 1991a & b). The free living stage starts when embryonated eggs hatch into first-stage larvae (L1), which in turn moult into second-stage larvae (L2) shedding their protective cuticle in the process. The L2 develops into the third-stage larvae (L3), but retains its cuticle of L2 stage. Nematode cuticle is generally composed of highly cross-linked structural proteins (mainly collagen), lipids and carbohydrates. The moulting process involves the cyclical synthesis of new structural proteins and the digestion of the cuticle-anchoring proteins during ecdysis aided by protease enzymes such as cysteine proteases (Page, 2001). The first two larval stages usually feed on bacteria and organic materials, but the L3 sealed off from the environment by the retained cuticle of the L2, cannot feed and must survive on the stored nutrients acquired in the early stages. The time required for the eggs to develop into infective larvae depends mainly on temperature and moisture content of the immediate environment. Under optimal conditions (high humidity and warm temperature), the developmental process requires about 7-10 days while in cooler temperatures the process may be prolonged.

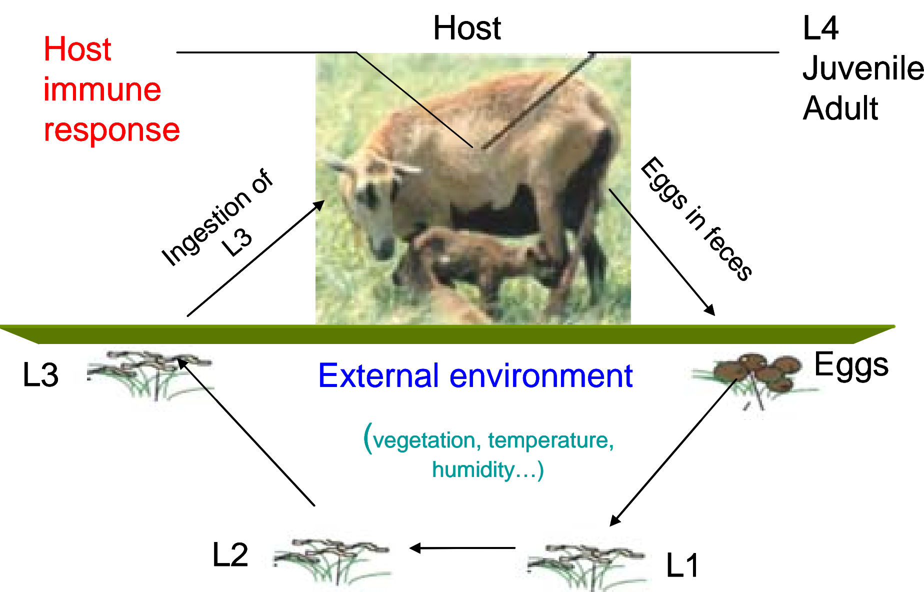


Figure 2. Schematic representation of the life cycle of *H. contortus* in sheep

Sheep are infected by ingesting the L3. Most larvae are picked up during grazing and pass to the abomasum. This infective larval stage sheds its L2 sheath once it reaches the rumen. This might be initiated by the parasite through secretion of an exsheathing fluid and by components of ruminal bicarbonate buffer system (Page, 2001; Gamble *et al*., 1989). However, not all ingested larvae arrive at the abomasum exsheathed. Dakkak *et al*. (1981) have demonstrated by studying the kinetics of passage of *H. contortus* L3 through the rumino-omasal orifice that some of the larvae arriving at the orifice were not exsheathed. Exsheathed L3 of *H. contortus* then penetrates the gastric pits of the abomasum and within 1-4 days moult to the next larval stage (L4). The L4 remains in the mucous membrane for about 7-11 days before emerging as late L4 into the lumen and evolves into the immature (L5) and then adult stages (Rahman and Collins, 1990). The pre-patent period is about 3 weeks in sheep when the internal and external conditions are favourable. Otherwise, the L4 may enter into a phase of arrested development or hypobiosis temporarly (Waller *et al*., 2004; Miller *et al*., 1998; Urquhart *et al.,* 1996; Reinecke *et al*., 1989). Hypobiosis is usually recognised by the presence of large numbers of larvae at the same stage of development in animals withheld from infection for a period longer than that required to reach that particular larval stage. Hypobiosis may avoid the potential danger on the viability of excreted eggs and development of larvae. The nature of the stimulus for arrested development and the subsequent maturation of the larvae is still a matter of debate. It might be an environmental stimulus (extreme temperatures and low relative humidity) received by the free-living infective stages prior to ingestion by the host or it can be an evolutionary adaptation by the parasite to avoid adverse climatic conditions for its progeny by remaining sexually immature in the host until more favourable conditions return. It may also be caused by enhanced immune status of the animal (Gibbs, 1986). Hypobiosis usually follows the onset of cold autumn/winter conditions in the northern hemisphere or very dry conditions in the subtropics and tropics (Waller *et al*., 2004; Gatongi *et la*., 1998). Furthermore, Jacquiet *et al*. (1995) also demonstrated that in some extreme climatic conditions such as in the desert areas of Mauritania, adult *H. contortus* were capable of surviving for up to 50 weeks in the host, which guaranteed the perpetuation of the species from one wet season to another.

1.3. Epidemiology

H. contortus is a species most commonly encountered in small ruminants. The disease caused by this parasite, haemonchosis, is the most frequently observed gastro-intestinal problem in tropical and sub-tropical regions of the world (Achi *et al.,* 2003; Nginyi *et al.* 2001; Ankers *et al.,* 1998; Tembely *et al*., 1997; Fabiyi, 1987). As for any disease process, the parasite is in a dynamic interaction with its environment and the host, the outcome of which depends on various intrinsic and extrinsic factors.

1.3.1. Intrinsic factors (the parasite)

H. contortus is a highly prolific, blood-feeding parasite with various strategies to escape adverse climatic conditions and immune reactions of the host. Its ability to produce large number of eggs (Coyne *et al*., 1991a & b) during its lifetime provides *H. contortus* with an advantage over other parasites in that it can easily contaminate grazing areas and may survive in its small ruminant hosts through frequent and rapid reinfections. Variations in the degree of infectivity of different *H. contortus* isolates have been documented. A comparison in infectivity between *H. contortus* isolates from France with those from French West Indies (Guadeloupe) in two breeds of sheep, namely the Barbados Black Belly and the INRA 401, has shown that the latter (sympatric isolate) established better than the former (allopatric isolate) in the Black Belly (Aumont *et al*., 2003) suggesting that it is important to take into account parasite genetic diversity in different agro-ecological zones.

1.3.2. Extrinsic factors

1.3.2.1. The environment

Climate and vegetation

Factors including temperature, rainfall, humidity and vegetation cover influence patterns of parasite development (Tembely *et al*., 1997; Coyne *et al*., 1992; Krecek *et al*., 1992). In most tropical and sub-tropical countries, environment temperatures are permanently favourable for larval development. The ideal temperature for larval development of many nematode species in the microclimate of the pasture or vegetation is between 22 and 26 °C

while the best humidity is close to 100% (Krecek *et al*., 1992). Desiccation from lack of rainfall kills eggs and larvae rapidly and is the most lethal of all climatic factors (Onyali *et al*., 1990). A pasture larval assessment in Ghana (Agyei, 1997) and the use of tracer lambs in Kenya (Wanyangu *et al*., 1997) revealed that very few or no *H. contortus* infective larvae were available during dry periods while numbers of larvae were high in the rainy seasons and shortly after. Below 5°C, movement and metabolism of L3 is minimal favouring prolonged survival as these larvae are enclosed in a double sheath and thus unable to feed to continuously renew their energy (Urquhart *et al*., 1996).

Nutrition

There is substantial evidence for a beneficial role of a good plane of nutrition in the resistance or resilience of sheep to GI nematode infections. According to Coop and Kyriazakis (2001), nutrition can influence the development and consequences of parasitism in three different ways: (1) it can increase the ability of the host to cope with the adverse consequences of parasitism (resilience), (2) it can improve the ability of the host to contain and eventually to overcome parasitism (resistance) by limiting the establishment, development and fecundity of the parasites and/or (3) it can directly affect the parasite population through affecting the intake of certain antiparasitic compounds. Highly metabolizable protein diets have been shown to augment resistance of Ile de France and Santa Ines lambs against *H. contortus* (Bricarello *et al*., 2005). Well-fed animals can withstand the harmful effects of GI parasitism, can remain reasonably productive and may require less anthelmintic treatments when compared with undernourished animals (Knox *et al*., 2006). The major problem in this respect is that haemonchosis is more prevalent in regions where animal feed resources are very scarce and/or improperly managed and therefore insufficient to satisfy the demand throughout the year.

1.3.2.2. Nature of the host

Host breed

Though it is still not clear how natural selection might shape patterns of immunoresponsiveness in terms of type and strength of response, different breeds of sheep

express different susceptibility to gastro-intestinal parasitic infections. In this respect, the Santa Ines (Amarante *et al*., 2004), Barbados Black Belly (Aumont *et al*., 2003; Gruner *et al*., 2003) and Texel (Good *et al*., 2006) breeds of sheep are known to be more resistant to infection with *H. contortus* compared with Suffolk and Ile de France, INRA 401 and Suffolk breeds respectively. This is evidenced by reductions in faecal egg count (FEC) and/or worm number, slower worm development and reduced fecundity. Genetic variations in the resistance to *H. contortus* within sheep flocks have also been demonstrated and used in breeding schemes in Australia (Woolaston and Baker, 1996; Albers and Gray, 1987).

Host age, sex and reproductive status

In addition to genetic factors, animals of different ages and sex respond differently to parasitic infections under similar management conditions. Young animals are generally more susceptible to parasitic diseases than adults. It is believed that lower resistance to disease in young ruminants is partly due to immunological hyporesponsiveness, and is not simply a consequence of their not having been exposed sufficiently to pathogens to develop immunity (Colditz *et al*., 1996; Manton *et al*., 1962). Innate immunity, often age-related, is also considered important in many cases. This may be due to physico-chemical differences in the gut environment in adult compared with young hosts (Mulcahy *et al*., 2004). The contribution of the stress associated with weaning of young lambs to delaying the development of protective immune responses to *H. contortus* and *Trichostrongylus colubriformis* has been documented (Watson and Gill, 1991).

On the other hand, previous exposure to *H. contortus* infection could result in enhanced resistance to subsequent infections. Improved resistance to *H. contortus* was reported in second infections in Rhön and Merinoland (Gauly *et al*., 2002), Black Belly and INRA 401 (Aumont *et al*., 2003) and St. Croix and Dorset (Gamble and Zajac, 1992) lambs. This may be due to the alteration of immunological and physicochemical mechanisms that while incapable of controlling the primary infection is nevertheless able to influence the challenge infection.

In a recent study, it was reported that male lambs excreted significantly higher number of faecal eggs, carried higher number of *H. contortus* worms and were more anaemic than their female counterparts (Gauly *et al*., 2006). Earlier studies with *H. contortus* showed that castration enhanced the resistance of male lambs to the extent that FEC were lower than those

of female lambs (Shaw *et al*., 1995) suggesting the existence of hormone related influences.

The phenomenon of the peri-parturient rise (PPR) in nematode egg output is also of great importance in the epidemiology of GI nematodes of sheep. This is due to a temporary loss of acquired immunity to infection at around the time of parturition and during lactation. Tembely *et al*. (1998), Romjali *et al*. (1997) and Woolaston (1992) reported that the PPR in FEC started 2 to 4 weeks before lambing and continued into lactation in the post-parturition period. The increased susceptibility may be due to pregnancy, increased milk production and related nutritional factors. Ovariectomized ewes that received 20 days of pre-inoculation exposure to progesterone and prolactin during infection were found to have greater number of nematodes (Fleming and Conard, 1989) indicating the involvement of hormones that are linked to pregnancy and lactation in the PPR in FEC. The relaxed immunity during lactation also places the ewes at a greater risk of parasitic disease and production loss. A significant reduction in haematocrit and serum albumin concentration in post-parturient ewes that had been experimentally infected with *H. contortus* has been reported (Thomas and Ali, 1983). Furthermore, the PPR in FEC could also contribute to increased pasture contamination. Hence, animals in such a physiological state require special attention if losses are to be avoided.

1.4. Clinical manifestations and diagnostic methods

Haemonchosis in sheep may be classified as hyperacute, acute, or chronic. In the hyperacute form, death may occur within one week of heavy infection without significant signs. This form of the disease is very rare and appears only in highly susceptible lambs. The acute form is characterised by severe anaemia accompanied by oedema (“bottle jaw”). Anaemia is also characteristic of the chronic infection, often of low worm burdens and is accompanied by progressive weight loss (Urquhart *et al*., 1996). The chronic form is the most commonly observed during natural infections. The lesions are associated to anaemia resulting from blood loss. With the exception of the L3, all other stages of development feed on blood. *H. contortus* is known to produce calcium and a clotting factor binding substance known as calreticulin (Suchitra and Joshi, 2005), enabling the parasite to feed easily on host blood and in so doing cause haemorrhagic lesions. At *post mortem*, the abomasum appears oedematous

with petecheal haemorrhages, occasional nodular developments and a rise in pH (Scott *et al.*, 1999, Lawton *et al.*, 1996).

The clinical signs, mainly anaemia, oedema and loss of weight in association with reduced haematocrit values might be characteristic of heamonchosis in sheep. South African researchers have also developed a visual colorimetric assessment (FAMACHA) of the level of anaemia caused by parasitic infections in sheep (van Wyk and Bath, 2002). However, all these signs can be shared by a number of parasitic and non-parasitic diseases and hence must be supported by other diagnostic methods. In this regard, demonstration of parasite eggs in faecal material can prove the presence of infection and is the most commonly used diagnostic method. Nevertheless, this method does not always reveal the presence of the parasite during low level of parasitic burden and pre-patent periods (Borgsteede, 2000, Eysker & Ploeger, 2000), requiring repeated examinations. Host resistance to GI helminths also delays egg laying (Silverman and Patterson, 1960) and a change in female worm size affects its fecundity (Stear and Bishop, 1999). Hence, egg counts do not necessarily reflect the number of worms present. Other methods like measurement of parasite-specific antibodies can be used as supplementary diagnostic tools (Gomez-Muñoz *et al.,* 1999; Schallig *et al.*, 1995a). In general, a more accurate diagnosis lies on the utilisation of all available information regarding the epidemiology, clinical manifestations and laboratory diagnostic methods.

1.5. Pathophysiology of abomasal parasitism

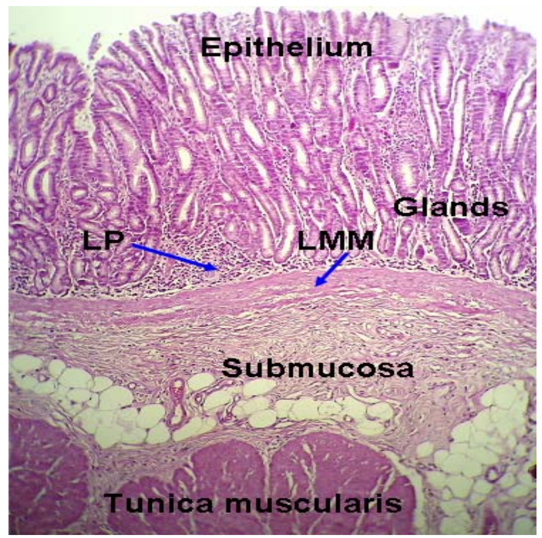
1.5.1. The abomasum and pathological changes associated with helminth infections

The abomasum is the glandular compartment of the ruminant stomach. Its wall is made up of four major layers: mucosa, submucosa, muscularis and serosa. The mucosal layer is further divided into three (Figure 3). The lamina epithelialis mucosa is lined by mucus-secreting simple columnar epithelium. The lamina propria (LP) mucosa is a connective tissue layer just under the epithelium infiltrated with variable number of leucocytes, macrophages etc. In addition, gastric glands penetrate variable distance into this layer. The lamina muscularis mucosa is a thin smooth muscle layer separating the LP and the submucosa (Banks, 1986).

Three types of gastric glands define the mucosa of abomasum at three different regions. The proximal, (cardiac) region contains branched tubular coiled cardiac glands lined by mucus-secreting cuboidal or columnar cells. The body (fundus) region contains long branched tubular fundic glands with mucus neck cells, acid-secreting parietal cells and pepsinogen-secreting chief cells (zymogen cells) (Banks, 1986). The secretory products of mucus neck cells may protect the fundic gland from proteolytic and hydrolytic activities of gastric enzymes (pepsin, rennin, and gastric lipase) and hydrochloric acid secreted by the chief cells and parietal cells respectively. The caudal (pylorus or antrum) gland region is endowed with short, simple or branched tubular glands where mucous secreting cells predominate. Nematodes common in the abomasum of sheep are *H. contortus* and *Teladorsagia circumcincta*. Late fourth-stage larvae emerge from the fundic glands and develop into adult. However, possible migration of these worms (late L4 to adults) towards the pyloric region has also been suggested (Dash, 1985; Rahman and Collins, 1990).

Studies of field parasitism and experimental larval infections identified the morphological and physiological effects of abomasal nematodes: nodule development, mucous cell hyperplasia, superficial epithelial damage and eventual tissue restitution; reduced acid secretion and increased serum gastrin and pepsinogen concentrations (Simpson, 2000). Glands with developing larvae are often lined by a flat epithelium containing few secretory cells and reduced number of parietal and chief cells. Fundic mucosal tissues are thicker due to mucous cell hyperplasia and marked accumulation of inflammatory cells such as lymphocytes and eosinophils (Scott *et al.*, 2000, 1998), which sometimes result in nodule development (Figure 4). Hyperplasia in ostertagiosis is thought to largely be confined to the vicinity of parasitized glands and appears morphologically as nodules. Hyperplasia within nodules affects the full mucosal thickness, with the chief cells and acid secreting parietal cells being replaced by a population of immature cells phenotypically closer in appearance to mucous cells (Scott *et al.*, 1999). In contrast, *Haemonchus* species larvae emerge earlier from the gastric glands and are therefore, ambulatory over the abomasal surface. Hyperplasia is therefore, usually generalized in haemonchosis and is thought to occur in the more superficial layers of the epithelium, so that parietal and chief cell numbers are not necessarily affected (Hunter and Mackenzie, 1982). After parasites emerge into the lumen, there is more widespread mucosal hyperplasia and parietal cell loss. Reduction in the number of chief cells

may result from the failure of immature mucus neck cells to mature to chief cells in the absence of the correct signal from parietal cells (Simpson, 2000). Along with the rapid decrease in abomasal acid secretion, the parietal cells develop dilated canaliculi and/or degenerative changes typical of necrosis (Scott *et al*., 2000).



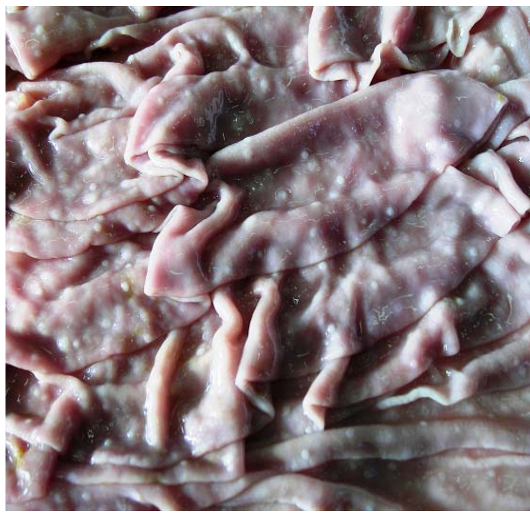


Figure 3 (Left). Abomasal histology: The mucosal layer constitutes the lamina propria (LP), the lamina muscularis mucosa (LMM) and the epithelial layers. Hematoxylin/carbol-chromotrope stain. (Photo: G. Terefe).

Figure 4 (Right). Nodule development in the abomasal mucosa (fundus) of sheep repeatedly infected with *H. contortus* (Photo: G. Terefe)

The cellular response to abomasal nematodes involves the accumulation of inflammatory cells such as mast cells, globule leucocytes, eosinophils (Figure 5) and lymphocytes (Lacroux *et al*., 2006; Perez *et al*., 2001). Cell pattern and time course of this cellular infiltration may vary according to host factors such as age, immune status, genetic predisposition, reproductive status and plane of nutrition (Huntley *et al*., 2004; Mckellar, 1993; Gamble and Zajac, 1992; Woolaston *et al.,* 1990; Salman and Duncan, 1985, 1984). Lymphocytes, eosinophils etc. began accumulating 1-2 days after adult parasite transfer and were present in large numbers after 8 days (Scott *et al*., 2000, 1998). Most studies of the cellular changes in the GI mucosa of sheep infected with nematode larvae rely on *post mortem* sampling of groups of animals at specified time during the infection. Sequential abomasal or intestinal

biopsy also offers the advantages of more frequent sampling than is practical with killing groups of sheep, and enables to study mucosal inflammatory responses throughout the course of the infection (Huntley *et al*., 2004; Pfeffer *et al*., 1996).

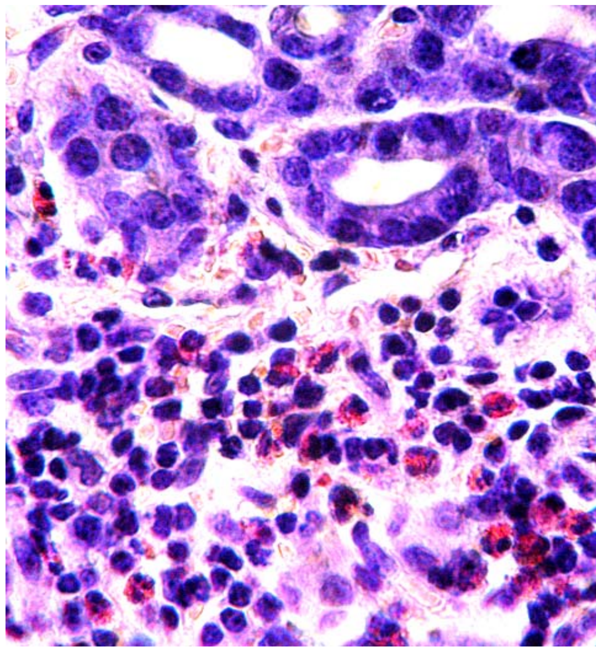


Figure 5. Eosinophils (pink-red) in the abomasal lamina propria of sheep infected with *H. contortus*. Hematoxylin/carbol-chromotrope stain (Photo: G. Terefe)

1.5.2. Abomasal Pathophysiology

The most obvious component of the mucosal defence system is the mucosal gel, which creates a gradient of pH from the epithelium to luminal contents and low paracellular permeability to ions (Lichtenberger, 1999). In the parasitized abomasum, gastric dysfunction and superficial epithelial damages caused by the presence of larvae in the gastric glands (stretching the glands) and the movement of adult worms that feed on the mucosal surfaces presumably compromise the protective barrier to diffusion and allow parasite and luminal chemicals access to host tissues. The pathophysiology of the abomasum during helminth infections is excellently reviewed in Simpson (2000) and Fox (1997). The marked changes in gastrointestinal secretions that accompany abomasal nematode infections in ruminants are well established and include a reduction in gastric acid secretion and an increase in circulating pepsinogen and gastrin levels. The abomasal hypoacidity may reduce pepsinogen activation and appears to be responsible for increased gastrin secretion in the initial phase of infection.

Increased serum pepsinogen concentration is attributed mainly to the increased back-diffusion of luminal pepsinogen through the more permeable mucosa (Prinz *et al*., 1999). An increase in serum gastrin concentration was observed following infection of sheep with *T. circumcincta* before any change in gastric pH suggesting that a change in abomasal pH is not the only factor that affects circulating gastrin levels (Fox, 1997). Abomasal secretion begins to change around the time of parasite emergence from the glands. Because of the timing, abomasal dysfunction has been attributed to tissue damage during emergence (Berghen *et al*., 1993). Recent studies involving transplantation of adult worms have demonstrated their importance in the disease process (Scott *et al*., 1998; Simpson *et al*., 1997; Lawton *et al*., 1996). It was found that after transplantation of adult *O. circumcincta* and *H. contortus*, abomasal pH and serum gastrin and pepsinogen become elevated within days. The rapidity of acid inhibition after adult parasite transfer and the equally rapid recovery when they are removed by anthelmintic treatment suggests that parasite chemicals may be responsible (Merkelbach *et al.,* 2002; Simpson *et al.,* 1999; Anderson *et al.,* 1985; Dakkak *et al*., 1985). Parasites may inhibit the parietal cells inadvertently by provoking inflammation or disrupting the protective mucosal defence system or, alternatively, by targeting these cells through excretory/secretory chemicals. Therefore, inhibition of acid secretion and loss of parietal cells appears to be a key event responsible for both the secretory dysfunction and the altered cellular composition of the gastric glands.

The contributions of the host and the parasite to the pathophysiology of abomasal parasitism may have quite different costs and benefits to each of them. The ability to inhibit acid secretion may allow colonization of a hostile acid environment, which also contains proteolytic activity capable of digesting an unprotected parasite (Simpson *et al*., 1999). Raising the pH may also enhance egg laying; e.g. for *H. contortus* it is maximal between pH 4 and 4.5 (Honde and Bueno, 1982). A curious feature of the inflammation caused by parasites is that the parasites may themselves actively recruit granulocytes through secreted chemotaxins. Eosinophil chemotactic factors have been found in a wide range of parasites including *Ostertagia ostertagi* and *H. contortus* (Wildblood *et al.,* 2005; Klesius *et al.,* 1989). Based on these circumstantial evidences, it is reasonable to assume that the parasite benefits from inhibition of acid secretion and recruitment of inflammatory cells. The other argument is that abomasal pathophysiology is not parasite-driven but is a host response to the presence of

the parasite aimed at its removal, but unfortunately, which damages host tissues in the process. For example, eosinophils are capable of extensive tissue damage through their oxidative bursts (Lacy *et al*., 2003; Capron, 1992). Hence, it is a matter of speculation whether the host, the parasite, or neither, benefits from the pathophysiology initiated by the presence of the parasite (Simpson, 2000). Indeed, immunological responses are often explained in terms of the costs (resources used and associated tissue damage) and benefits (final killing of the pathogen). If immunity proves to be costly, natural selection ought to favour enhanced resistance to pathogens or immunoresponsiveness only when it is beneficial. One aspect of this is the pattern of circulating blood eosinophils, which peaks within the first 2 to 3 weeks of *H. contortus* exposure, and then down-regulated to the base line level within one or two weeks despite the presence of adult parasites in the gut lumen (Amarante *et al*., 1999). This may be partly attributed to down-regulatory cytokines produced by regulatory T cells (Groux, 2001; Belkaid *et al*. 2006). The other feasible mechanism is that eosinophils, one of the most important inflammatory cells during GI helminth infections, are capable of degranulating selectively through piecemeal degranulation (PMD) or require a very close contact with the parasite surface for releasing their toxic products (Fondati *et al*., 2003; Daly *et al*., 1999). This may minimise cytotoxic damages inflicted by these cells.

1.6. Control of haemonchosis

The aim of most parasite control strategies is not to totally eliminate the parasites in livestock, but to keep the population under a threshold, above which it would otherwise inflict harmful effects on the host population (Larsen, 2000). The relative success or failure of any control strategy can be judged in terms of immediate and/or long-term objectives, the ultimate goals being increased production, minimising risks of drug resistance and addressing consumer and environment associated problems. Generally, nematode control strategies can be directed against the parasite in the host and/or in the environment.

1.6.1. Targeting the parasite in the host

1.6.1.1. Chemotherapy and chemoprophylaxis

Anthelmintic drugs are commonly used either for prophylactic purposes, in which the timing of treatment is based on a knowledge of the epidemiology, or for therapeutic purposes to treat existing infections or clinical outbreaks. Since the advent of modern anthelmintics, enormous advances have been made to make use of various preparations for different species of animals against a diverse spectrum of parasites. The relative success of these drugs depends on their ease of administration, persistency of action after administration and the period and/or frequency of application based on the epidemiology of the disease problem. In most cases, anthelmintics are administered *per os* in the form of a solution, paste or bolus but there are some, which can be given via other routes (parenteral etc...). In temperate areas, priority is usually given to strategic treatments rather than to a regular interval dosing with anthelmintics. Animals at risk, such as weaned lambs, are often treated during the first grazing season. In some arid areas where haemonchosis is a problem, first season animals are treated at least twice during the rainy season, four weeks after the onset of the rains and at the end of the rains (Urquhart *et al*., 1996). An additional treatment at the culmination of the wet season may sometimes be necessary. Various drugs have been shown to be successful (almost 100%) in eliminating *H. contortus* during their early periods of utilisation, and some still remain effective in different parts of the world (Table 1). Hence, in the traditional sense of chemotherapy-chemoprophylaxis, we have probably achieved the maximum effect of what is possible from excellent anthelmintics developed by the pharmaceutical industry since 1960, i.e. from thiabendazole through levamisole and morantel tartrate, to more advanced benzimidazoles and to the avermectins and milbemycins (Williams, 1997).

Table 1. List of some recommended drugs against haemonchosis in sheep (Bowman, 1999).

Chemical group	Anthelmintics	Prescribed dose
IMIDAZOTHIAZOLES	Levamisole	7.5mg/kg (Andrews, 2000)
BENZIMIDAZOLES	Albendazole	5mg/kg
	Fenbendazole	5mg/kg
	Oxfendazole	5mg/kg (Mckenna and Watson, 1987)
MACROCYCLIC LACTONES	Ivermectin	0.2mg/kg
(avermectins)	Moxidectin	0.2mg/kg
SALICYLANILIDES	Closantel	10mg/kg (Uppal *et al*., 1993)

For various reasons however, the efficacy of such valuable and very effective drugs is endangered. Their long-term utilisation, inappropriate handling and under-dosage may be some of the reasons for their reduced efficacy and for the increasing development of drug resistance. On the other hand, where these drugs are not easily accessible because of either economic reasons or scarcity of veterinary services, as in most parts of Africa, animals die as a result of acute haemonchosis or develop a chronic form of the disease resulting in marked loss of body weight and consequent reduced production. This adds another constraint to the already existing poor production performance of small ruminants in such regions.

1.6.1.2. Risk of drug resistance

Development of drug resistance by populations of *H. contortus* in sheep and goats to repeated applications of benzimidazoles, levamisole and ivermectin has already been demonstrated (Table 2). In most cases where resistance against various anthelmintics has been reported, closantel remained as the only efficient drug available signalling the urgent need to develop alternative measures. *H. contortus* strains resistant to one group of drugs may also be resistant to other groups of drugs, which suggest the existence of multiple resistance to the major anthelmintic drugs currently available. In one experimental study, Waruiru (1997) tested the efficacy of closantel, albendazole, levamisole and ivermectin against ivermectin resistant and susceptible isolates of *H. contortus* in sheep. A very impressive result was obtained where all these drugs were almost 100% effective against ivermectin susceptible isolates while only closantel proved efficacious on the ivermectin resistant strain. Further

alarming findings were also reported where such resistance in *H. contortus* was found to be inherited as either dominant or recessive traits. According to Le Jambre *et al*. (2000), a completely dominant autosomal trait governs the resistance of *H. contortus* larvae to avermectin while they suggested that in adult worms the expression of resistance was sex-influenced. On the other hand, resistance to levamisole and benzimidazoles has been reported to be inherited as an incomplete recessive autosomal trait (Sangster *et al*., 1998) and at least two genetic loci (in the beta-tubulin gene) are involved (Beech *et al*., 1994). Highly prolific species such as *H. contortus* with relatively short life expectancy of adult worms have a higher risk of developing diverse resistance-alleles due to spontaneous mutations than the less prolific *T. colubriformis* (Silvestre and Humbert, 2002).

Table 2. Some examples of drugs to which resistant strains of *H. contortus* in sheep and goats were reported in different countries.

	Country	**Anthelmintics**	**Reference**
Africa	Ethiopia	Albendazole, tetramizole, ivermectin	Sissay *et al*. (2006)
	South Africa	Almost all groups	van Wyk *et al*. (1997)
Europe	France	Benzimidazoles, levamisole	Chartier *et al*. (1998)
	G. Britain	Benzimidazoles	Coles (1998)
Asia	Malaysia	Benzimidazoles, levamisole, closantel, ivermectin	Chandrawathani *et al*. (1999)
South America	Argentina	Benzimidazoles, levamisole, ivermectin	Eddi *et al*. (1996)
	Uruguay	Benzimidazoles, levamisole, ivermectin	Nari *et al*. (1996)
Australia	Australia	Benzimidazoles, levamisole, ivermectin	Green *et al*. (1981) Le Jambre *et al*. (1995) http://www.wormboss.com.au

Therefore, if effective parasitic treatment with the existing drugs is to continue, more efficient and strategic dosing regimes must be practiced in order to enhance the efficacy or prolong the useful lives of the currently available anthelmintic compounds. Reduction of feed intake before oral anthelmintic treatment slows ruminant digesta flow and premature drug removal (Ali and Hennessy, 1995) whereas administering the normal dose over several hours rather than increasing the amount of drug (Sangster *et al*., 1991) prolongs availability. Improved drug delivery systems such as the use of chemicals or physical carriers (salts, oils

etc) that reduce drug absorption and metabolism and that can specifically direct large quantities of actives to the sites of parasite habitat must be adopted (Hennessy, 1997), but these should be cost effective.

1.6.1.3. Other treatment methods

Alternative treatment measures such as the use of copper oxide wire particles and medicinal plant extracts have long been used against parasitic diseases with varying apparent success. Briefly, administration of 2.5 to 5 gm of copper oxide wire particles in sheep was shown to reduce *H. contortus* faecal egg counts (Knox, 2002). However, besides its limited usefulness, the use of 4 gm of these wire particles in late pregnancy was reported to threaten the life of multiple born offspring (Burke *et al*., 2005). On the other hand, plant extracts such as condensed tannins, which are secondary tanniferous plant metabolites, have been found to reduce *H. contortus* faecal egg counts and the number of eggs per female worm in goats (Paolini *et al*., 2003) and the faecal egg counts, worm number and fecundity of *T. colubriformis* in sheep (Athanasiadou *et al*., 2000). Similarly, flower extracts of *Calotropis procera* have shown excellent anthelmintic activity against *H. contortus* in sheep (Iqbal *et al*., 2005). Despite the possible existence of a wide variety of plant species with the potential to control gastrointestinal parasites, the difficulty in the selection of potential candidates and extraction of the active ingredients essential for nematode killing without compromising the health of the animal is still a problem, which hinders the development of these resources.

1.6.2. The FAMACHA system

Anthelmintics are not always available to all livestock breeders. When they are easily accessible, their usefulness is increasingly hampered by the development of drug resistance. At present, there appears to be no new chemical class of anthelmintics with a new mode of action on the horizon, and hence the chemical actives that are currently available are all that we are likely to have for the foreseeable future (Hennessy, 1997). One recently discovered compound, emodepside, which inhibits pharyngeal pumping of nematodes via latrophilin-like receptors (Harder *et al*., 2003), is very expensive and is currently only in use for limited

animal species such as cats. Hence, every available option should be exploited to minimise anthelmintic usage.

The FAMACHA system developed by South African researchers could be one of the best methods for reducing the necessity of regular anthelmintic treatments against *H. contortus* infections of sheep. It is used in the management of *H. contortus* infection in sheep and goats, based on the clinical identification of developing anaemia in individual animals within a flock (van Wyk and Bath, 2002). Accordingly, trials over several seasons showed that most sheep under severe *H. contortus* challenge required no or only one treatment over a full summer season in contrast to the usual repeated medication of all animals in the flock. One important feature of the system is that it is easy to use at all levels of the farming community. The visual appraisal of anaemia is linked to an identification chart; therefore literacy is not a requirement and the system can be applied throughout the moist tropics/subtropics of the world where *H. contortus* is endemic (van Wyk and Bath, 2002; Waller, 1999). However, while applying the FAMACHA system, one should also take into consideration other important parasitic infections (such as fasciolosis, babesiosis etc.) that may often be accompanied by detectable anaemia. Apart from the use of chemical medications in livestock, other measures targeting mainly the environment but also the host are also in practice.

1.6.3. Targeting the microenvironment

1.6.3.1. Grazing strategy

Alternate grazing of different host species (Barger, 1997) and alternation of grazing and cropping are management techniques that can provide safe pasture and give economic advantage when combined with anthelmintics. Studies in the wet tropical climates of several Pacific Island countries showed that peak larval concentrations of *H. contortus* and *Trichostrongylus* species occurred on pasture about one week after contamination, but fell to barely detectable levels within 9 weeks (Banks *et al.*, 1990). Based on this, a rotational grazing system was designed which has resulted in a significant reduction in faecal egg counts as well as the number of anthelmintic treatments needed per year. However, in many parts of Africa, communal pastoral systems do not allow for regulated grazing as a means of lowering

exposure to infective larvae on pasture. Growing human populations and livestock densities coupled with the frequent drought in some regions necessitate unregulated animal movement in search of green pasture and drinking water (Abule *et al*., 2005). Exploitation of refugia through alternate grazing of cattle and sheep (Barger, 1997), or sheep and goats (Sissay *et al*., 2006) to reduce pasture levels of infective larvae or dilute populations of drug resistant strains of parasites, could be of great value in any management program.

1.6.3.2. Biological control: use of fungal spores

This is a method in which selected biological agents can be used to reduce the populations of parasites either on pasture or in the host and by so doing minimise the frequency of anthelmintic usage. One example of biological control against gastrointestinal nematodes is the use of some species of nematophagous fungi with the potential to reduce nematode larval populations on pasture by using these either as their main source of nutrients or as a supplement to a saprophytic existence. There are a number of reviews on this topic by Larsen (2006) and Waller and Faedo (1996). Two groups of such fungi have been identified: there are predacious fungi, which produce adhesive or non-adhesive nematode-trapping structures and endoparasitic fungi that infect nematodes or their eggs. Among the endoparasitic fungi, those reported to infect *H. contortus* are *Drechumeria coniospora* and *Harposporium anguillulae* while *Arthrobotrys oligospora* and *Arthrobotrys robusta* are predacious fungi of different species of *Haemonchus* (Larsen, 2000). A significant breakthrough in this area was reported by a number of studies using the species, *Duddingtonia flagrans* (Paraud *et al*., 2005; Chandrawathani *et al*. 2004; Fontenot *et al*., 2003; Waller *et al*., 2001). This predacious fungus produces three-dimensional sticky networks, which tightly traps free-living nematode larvae in the faeces ultimately resulting in their death. Unlike difficulties associated with the use of other species of fungi, several authors have reported the successful passage of *D. flagrans* chlamydospores in the faeces of sheep after oral drenching (Larson, 2000). Despite its appreciable degree of efficacy, this method of parasite control is still not widely applicable. This may be attributed largely to the requirement for continuous oral or in-feed dosing with fungal spores to achieve the desired level of efficacy (Paraud *et al*., 2005; Terrill *et al*., 2004). In addition, the chlamydospores have a relatively short shelf life (less than 1week) in a moist

environment, which enables the fungal spores to start to germinate and become vulnerable to degradation during their passage through the animal host (Larsen, 2006).

1.6.4. Improving host resistance

1.6.4.1. Vaccination

Control of gastrointestinal parasites by vaccination has been a long-term objective of many parasite research programs. Ideally, vaccines should have a high efficacy and be commercially viable for their proposed use in the livestock sector. A number of GI nematode proteins have been tested as potential vaccine products. In general, these molecules have been divided into two categories. Those termed 'natural antigens' or 'conventional antigens' are recognised by the host during an infection and are targets of the naturally acquired immune response whereas molecules which are normally not recognised, or which do not induce an immune response during a natural infection but which may serve as targets of the immune response generated against them, are termed 'concealed' or 'hidden' antigens (Klei, 1997). Natural antigens are constituted mainly of worm surface antigens or excretion/secretion products. Vaccines for *H. contortus* based on natural antigens can generate some level of protection, which although likely to significantly reduce pasture contamination, may not be sufficient to protect young lambs from severe haemonchosis (Newton, 1995).

The majority of concealed antigens of GI parasites described so far are components of epithelial cell surface membranes of the digestive tract of *H. contortus*. Antibodies directed against these molecules following immunisation and ingestion of blood by the parasites, have proven to be effective in reducing worm burdens (Smith, 1993; Jasmer and McGuire, 1991). In these early studies, a serum transfer experiment suggested that the effector mechanism was serum antibody, which bound to the brush border membrane of the parasites' intestinal cells, and sheep, which had acquired immunity to previous *H. contortus* exposure, did not recognise the gut membrane proteins suggesting that these are normally hidden from the host. A more comprehensive review on gut-associated membrane antigens is given by Knox and Smith (2001). One of these molecules, H11, is a 110 kDa integral membrane protein expressed on the intestinal microvilli of the parasitic stages of *H. contortus* and homologues have been

identified in *T. circumcincta* (Smith *et al*., 2001). This molecule has been cloned and characterised as an aminopeptidase localised in the brush border of the epithelial cells. The H11 vaccine is apparently effective in all age groups of sheep and against different isolates of *H. contortus* (Newton *et al*., 1995; Jasmer and McGuire, 1991).

Other gut associated antigens such as the 1000 kDa *Haemonchus* galactose-containing glycoprotein complex (H-gal-GP) and the 46 and 52 kDa (P46 and P52) glycoproteins have excellent efficacy in reducing FEC and worm burdens (Smith *et al*., 2000). The cDNAs encoding H11 as well as most of the components of H-gal-GP have been expressed in *E. coli* but, unfortunately, none of these recombinant proteins has been reported to be protective (Knox and Smith, 2001).

Regardless of promising results achieved over the years, especially in terms of vaccine efficacy, we are still waiting for the release of a commercial product. However, its haematophagous nature makes *H. contortus* more prone to gut-associated vaccines compared to other GI nematode parasites, the complex nature of its antigens, involving extensive glycosylation, has probably precluded their molecular cloning at a commercial level. In addition, the requirement for various adjuvants and repeated injections that could raise the cost of vaccination, have created considerable difficulties in the realisation of GI nematode vaccines (Knox and Smith 2001; Klei, 1997).

1.6.4.2. Breeding for resistance

The other promising angle, both for the developed and the developing livestock sector, is the selection of breeds or lines of sheep for parasite resistance. There is a sizable body of evidence for the existence of genetic variation in resistance to gastrointestinal nematode parasites both between and within breeds (Gray, 1997) and selection for parasite resistance has been successfully demonstrated in Australia and New Zealand (Bisset *et al*., 1996; Woolaston and Baker, 1996; Barger, 1989). The benefits of such selection arise from the effects of having fewer and less developed worms or greatly reduced faecal egg counts, which in turn leads to a reduced impact on production, a decreased requirement for chemical control and a reduced contamination of pasture by infective larvae. In this respect, although possibly lacking the productivity and performance capacity of their counterparts in temperate regions,

a number of indigenous tropical breeds of livestock have the genetic ability to tolerate or resist disease, a potential developed through natural selection. The long-term exposure to GI nematodes in endemic areas coupled with their adaptation to harsh environmental conditions and low levels of nutrition have allowed them to survive in the regions in which they exist. Some examples of relatively resistant breeds have already been given in this text, but it is likely that there are many others, as yet untested, breeds. Similarly, within populations of animals genetically determined differences in parasite resistance has been reported (Woolaston, 1992). Such animals may serve as a potential nucleus for selecting *Haemonchus*-resistant sheep. A number of markers such as FEC, worm burden, peripheral eosinophil count and antibody levels have been suggested to identify animals with increased resistance to infection (Stear *et al*., 2002; Douch *et al*., 1996), and the results using FEC as a marker are promising. However, as a selection trait, FEC has practical limitations and its use may incur production penalties through withholding drench treatment for prolonged periods. Furthermore, FEC can be influenced by the level and composition of a natural nematode challenge and the expression of the immune response (Douch *et al*., 1996). Moreno *et al*., (2006) have detected a QTL for resistance to *H. contortus* on ovine chromosome 5 in the INRA 401 x Barbados Black Belly back cross lines. The most likely location of this QTL corresponded to the IL-3/IL-4/IL-5 region. As these cytokines are characteristic of Th2 type immune responses (Lacroux *et al*., 2006), studies of both the cellular and humoral responses will be of paramount importance in the understanding of the mechanism of resistance (Balic *et al.,* 2000; Colditz *et al*., 1996; Gill, 1991). Hence, there is still much to be done in order to identify the best markers of resistance for use during selection processes.

2. IMMUNE RESPONSE AND GI PARASITISM

The mammalian body is susceptible to infection by many pathogens, which differ greatly in their sizes, lifestyles, the structure of their surfaces, and means of pathogenesis, which therefore require an equally diverse set of defensive responses from the host immune system. Not surprisingly, this has resulted in a complex and highly intricate system that has the ability to respond effectively to disease-causing organisms, as well as to distinguish these from harmless substances in the environment and from self-antigens. It is generally believed that immune defence mechanisms develop following a critical interaction between the two main parts of the immune system known as the innate and the adaptive immune system (Saalmüller, 2006).

2.1. Innate immunity

Innate immunity is a system that acts effectively to sense altered signals without previous exposure to a pathogen, conferring broad protection. It provides the first line of defence by detecting the immediate presence and nature of infection. The epithelial surfaces of the body keep pathogens out by preventing pathogen adherence, secreting mucus that may contain antimicrobial enzymes and peptides, and increasing intestinal propulsive activity (Castro and Arntzen, 1993; Ishikawa *et al*., 1993; Alizadeh *et al*., 1987; Miller *et al*., 1981; Sukhdeo and Croll, 1981). Putative mechanisms underlying the protective role of mucins against infectious agents also include the demonstration of trapping of parasites in the mucus and inhibition of parasite motility and feeding capacity (Rothwell, 1989; Miller, 1987). The innate response also consists of humoral factors (cytokines and complement) and cellular components (natural killer cells, macrophages, dendritic cells, eosinophils, mast cells, etc) (Saalmüller, 2006; Biron *et al.,* 1999). Bacteria, viruses and parasites that overcome the epithelial barrier are faced immediately by these components of the innate immunity. Cells of the innate immune system do not recognise specific epitopes, as is the case for the specific immune response, instead they recognise pathogen associated molecular patterns (PAMP) by molecules derived from Toll-like receptor family (Janeway and Medzhitov, 2002; Fraser *et al*., 1998) or pattern

recognition receptors (PRR) expressed especially on antigen-presenting cells (APC) such as macrophages and dendritic cells. Eosinophils were found to be involved in larval killing during innate immunity against *Strongyloides stercoralis* infection in mice (Herbert *et al*., 2000). Complement plays a key role in the innate immunity and represents an important link between adaptive and innate immunity in higher organisms (Morgan *et al*. 2005). An increase in the expression of complement receptor type 3 (CR3) on eosinophils after activation by schistosomes, and enhanced eosinophil-dependent antibody-mediated toxicity to the parasites was reported (Capron *et al*., 1987, 1984). However, the early innate immune system is sometimes evaded or overcome by many pathogens and does not lead to immunological memory.

2.2. Adaptive immunity

Adaptive or acquired immunity is initiated when an innate immune response fails to eliminate a new infection and antigens and activated APCs are delivered to the draining lymphoid tissues. The abilities to recognise all pathogens specifically and to provide enhanced protection against re-infection are the unique features of adaptive immunity. In an adaptive immune response, antigens are recognised by two distinct sets of highly variable receptor molecules: (1) the antigen-specific receptors on T cells (TCR) and (2) immunoglobulins that serve as antigen receptors on B cells (BCR).

2.2.1. Antigen presenting cells

Professional antigen presenting cells (APCs): macrophages, dendritic cells (DC) and B cells are uniquely specialised for both antigen acquisition and presentation, linking innate and adaptive immunity. APCs present different sets of antigens and may serve to activate T cells via co-stimulatory molecule (B7-1 and B7-2 (CD80 and CD86)) signals. During infection, APCs express PRRs that recognise differential PAMP on foreign agents. When these molecules bind their ligands on the pathogen, they stimulate the APCs to up-regulate major histocompatibility complex (MHC) and co-stimulatory molecules, providing these cells with strong antigen presentation and activation potential to T lymphocytes (Singh and Agrewala,

2006; Rodriguez-Sosa *et al*., 2002). DCs and macrophages need to process and transform antigens into peptides for presentation on MHC-II molecules as peptide-MHC class II complexes. Activated APCs secrete cytokines (Interleukin-1 (IL-1), IL-6, IL-8, IL-12, INF-α) and chemokines, through which they can help to initiate the development of an adaptive immunity (Saalmüller, 2006). For example, DCs are specialised in both the initiation and polarization of adaptive immunity (Banchereau *et al*., 2000; Moser and Murphy, 2000). They are activated via defined PRR/Toll-like receptors ligation (Barton and Medzhitov, 2002). If 'danger signals' are encountered by DCs in the form of PAMPs, inflammatory cytokines or signals from activated T cells, a process of classical 'maturation' is triggered and they transform from dedicated antigen collectors into specialised APCs (Perona-Wright *et al.,* 2006). DC migration towards naïve T cells in the draining lymph node is accompanied by a significant up-regulation and stabilisation of surface MHC, increased expression of key co-stimulatory molecules (B7-1 and B7-2) and an enhanced readiness to secret T cell stimulatory cytokines. They can present antigens via both MHC-I and MHC-II (de Jong *et al*., 2006; Dilioglou *et al*., 2003; Robson *et al*., 2003). Thus, they can activate directly both $CD8^+$ and $CD4^+$ T cells. B cells are capable of producing various immunoglobulin isotypes. Unlike the other two, B cells possess native antigen receptors, surface immunoglobulins. Special type of B cells expressing CD25 surface molecules having high level of surface immunoglobulins (but lacking the ability to produce antibody) were described in human subjects. These $CD25^+$ B cells express high levels of CD80 and CD86 and act as antigen presenting cells via MHC-II (Brisslert *et al.,* 2006; Yoon *et al*., 2005). In order to express co-stimulatory molecules, B cells need to be activated (Galdiero *et al*., 2005; Yoon *et al.,* 2005; Lapointe *et al*., 2003; Lankar *et al*., 1998) by T cells or other mechanisms.

Evidences from *in vitro* studies show that other cells (non-professional APCs) such as intestinal and abomasal epithelial cells and eosinophils can also process and present antigens to T cells (Padigel *et al*., 2006; Shi *et al*., 2000; Del Pozo *et al*., 1992; Bland and Whiting, 1989; Gorrell *et al*., 1988; Bland and Warren, 1986).

Nevertheless, while the mechanism by which viral, bacterial or protozoal pathogens interact with and activate APCs are increasingly understood, much less is known about how these cells react to more complex organisms such as helminths. Helminths are multicellular organisms capable of elaborating a plethora of surface and excretion/secretion antigenic

products, and it is not yet clear which of its antigens are taken by APC and which one of them elicit protective immune responses (Onah and Nawa, 2000). Ekkens *et al*. (2002) and Gause *et al*. (1996) have demonstrated in mice primed with *Heligmosomoides polygyrus* that IL-4 production and memory Th2 effector cells can develop in the absence of B7-1/B7-2, CD28 interactions suggesting the presence of other alternative pathways. On the other hand, the importance of B7 interactions during *N. brasiliensis* infection in mice has been shown in studies where CTLA-4Ig treatment (a chimeric fusion protein that blocks interaction between CD28 and B7 molecules) resulted in reduced generation of type 2 cytokines, although it did not alter worm expulsion (Harris *et al*., 1999).

2.2.2. $CD4^+$ T cells

Alpha-Beta (αβ) T cell Precursors emigrate from the bone marrow to the thymus and produce the predominant intermediary $CD4^+CD8^+$ T cells. These double positive T cells subsequently give rise to the T helper (Th) cells expressing $CD4^+$ and cytotoxic T cells expressing $CD8^+$ molecule and migrate to the periphery (Kimura and Nakayama, 1999; Ismaili *et al*., 1996; Fowlkes and Schweighoffer, 1995; Shortman, 1992; Scollay *et al*., 1980). Activated $CD8^+$ T cells are very effective at destroying target cells especially virus-infected cells and tumour cells. $CD4^+$ T cells are upregulated especially during parasitic infections.

The peripheral $CD4^+$ T cell pool is highly heterogeneous and may exist in three states; naïve, effector or memory depending on their history of exposure. Naïve $CD4^+$ (T helper) cells have never been exposed to specific antigens and are characterised by CD62L (MEL-14, L-selectin), the CD45 high-molecular weight isoform and low CD44 expression. When a circulating $CD4^+$ T cell encounters its specific foreign antigen presented by APCs in the form of classical MHC class II complex in peripheral lymphoid tissues, it is induced to proliferate and its progeny then differentiate into effector cells (Th1 and Th2 type cells). Effector $CD4^+$ lymphocytes are recently antigen-activated and have activation markers including CD69, CD25 (IL-2 receptor α) and high CD44 surface molecule. Some of the proliferating $CD4^+$ lymphocytes differentiate into memory cells, ready to respond rapidly to the same pathogen if it is encountered again (Seder and Ahmed, 2003; Tough *et al*., 2000; Desbarats *et al*., 1999; Mosmann and Sad, 1996).

The discovery in mice that $CD4^+$ T cells can be segregated into two distinct T helper subsets, Th1 and Th2, based on their cytokine secretion profiles (Mosmann and Coffman, 1989) provided the basis for subsequent understanding of the underlying T cell immune mechanisms controlling resistance to GI nematode infections. A third type of Th cell known as regulatory T cell (Treg), which includes type 1 T regulatory cells (Tr1) and Th3 cells has also been described (Belkaid *et al*., 2006; Groux, 2001). The decision on which fate the progeny of a naïve $CD4^+$ T cell will follow is made during the clonal expansion that takes place after the first encounter of APCs with antigens in the innate immune response. Naïve T cells require two distinct signals for activation (Grewal *et al*., 1997). The first signal comes from the engagement of the TCR with the MHC-II complex peptides on the APCs and the second signal is provided by the engagement of co-stimulatory (B-7) molecules such as the Th1 promoter CD80 and the Th2 prompter CD86 on the surface of APC via interaction with CD28 and CD152 on the surface of T cells (Freeman *et al*., 1995).

The principal early event that leads to Th1 differentiation is the production of IL-12 and INF-γ by macrophages and DCs responding to antigens or direct infection (Trinchieri, 1995). Polarised Th1 cells produce IL-12, INF-γ and lymphotoxin, which promote antibody-dependent cellular cytotoxicity supporting delayed-type hypersensitivity reaction (Mosmann and Coffman, 1989). Stimulation of naïve $CD4^+$ T cells by T cell receptor ligation in presence of IL-4 leads to Th2 differentiation (Coffman and von der Weid, 1997). Besides naïve T cells themselves, mast cells, basophils, eosinophils and rare population of natural killer cells (NK 1.1^+ $CD4^+$ T cells) are believed to be the initial sources of IL-4 (Coffman and von der Weid, 1997; Hameg *et al*., 1999). Polarised Th2 cells produce IL-3, IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (Kunzendorf *et al*., 1998; Mosmann and Coffman, 1989) and have important effects on immunoglobulin isotype switch as well as differentiation, mobilisation and activation of characteristic effector cells such as eosinophils and mast cells. A large body of evidence suggests that Th1 dominated immune response inhibits the Th2 dominated arm and vice versa (Mosmann and Sad, 1996; Pearce *et al*., 1991; Mosmann and Coffman, 1989).

The immune system has also evolved multiple mechanisms to ensure protective immunity in the absence of immune pathology. There is now compelling evidence that $CD4^+$ T cells that specialise in the suppression of immune responses play a critical role in the immune regulation (Belkaid *et al*., 2006; Groux, 2001). Induction of Treg cell is generally governed by

the activation status of DC (immature or semi-mature) and the cytokine secretion (mainly IL-10 or transforming growth factor –β (TGF-β)) by innate cells such as macrophages and DCs (Groux *et al*., 2004; Mills and McGuirk, 2004; McGuirk and Mills, 2002). Upon activation, Tr1 cells secrete high levels of IL-10 with or without IL-5, IL-13 or TGF-β but little or no IL-2, IL-4 and IFN-γ while Th3 cells secrete high levels of TGF-β (Mills and McGuirk, 2004). The demonstration that Treg cells can suppress the cytokine secretion and proliferation of both Th1 and Th2 cells (McGuirk and Mills, 2002; Cottrez *et al*., 2000) has raised the possibility that certain pathogens may promote the induction of Treg cells in order to subvert protective immune responses. For example, infection of IL-10-deficient mice is associated with enhanced parasite-specific immune responses and pathogen clearance from the host. However, this sterilising cure results in a loss of memory, which confers resistance to re-infection by the same parasite (Mills and McGuirk, 2004).

2.3. Th2-polarized response during GI parasitic infections

Laboratory models of intestinal nematode infections have played an important role in developing our understanding of the immune mechanisms that operate against gastrointestinal parasites. Several studies with different nematode species have demonstrated that protective immunity against GI nematodes is T cell dependent. Recent studies have concentrated on four main species of nematode parasites, *Nippostrongylus brasiliensis, Trichinella spiralis, Heligmosomoides polygyrus* and *Trichuris muris* in the mouse (Behm and Ovington, 2000; Finkelman *et al*. 1997). Each parasite has its own unique life cycle characteristics and hence the type and intensity of the immune response directed against specific parasite may depend on a variety of factors such as the type of APC and co-stimulatory molecules, the nature and dose of parasite antigen, and perhaps more importantly the immediate cytokine environment the T cell experiences at the time of antigen presentation (Artis and Grencis, 2001). Depletion of $CD4^+$ but not $CD8^+$ cells by mAb treatment at the time of *N. brasiliensis* infection in mice blocked spontaneous elimination of adult worms (Katona *et al*., 1988) signalling the specificity of $CD4^+$ T cells during helminth infections. The type of helper T cell response that develops following infections with such nematode parasites is also said to be critical to the outcome of parasitic infection (Bancroft *et al*., 2001; Constant and Bottomly, 1997; Grencis,

1996). If helper T cells of the Th2 type gain ascendancy (IL-4 being the prime stimulator), then a protective immune response ensues mediated by Th2 type cytokines and the effector mechanism they control. In contrast, if an inappropriate Th1 type response predominates, the ability to expel infection is compromised (Bancroft *et al.,* 2001; Khan *et al*., 2001; Urban *et al*., 1996). In general, extracellular helminths preferentially trigger Th2 dominated response. Infection of mice with nematode parasites such as *H. polygyrus* results in the development of a type 2 immune response that is characterized by a marked elevation of type 2 cytokines, blood eosinophilia, intestinal mucosal mastocytosis, and IgG1 and IgE (Urban *et al*., 1992).

Cytokines are critical mediators of any immunological response and in the case of helminth infections display a distinct allergic or Th2 profile. IL-3, IL-4, IL-5, IL-13 and granulocyte/macrophage-colony stimulating factor (GM-CSF) are the principal cytokines in the Th2 polarised immune responses. The immunological hallmarks of infection with parasitic helminths, namely eosinophilia, mastocytosis and increased IgE synthesis, all appear to be induced by cytokines from the Th2 subset. The *in vivo* manipulation of specific Th2 cytokines using anti-cytokine monoclonal antibodies or mouse strains with targeted deletions in cytokines and/or cytokine receptor genes has given great insight into the importance of individual cytokines and the responses they control in contributing to host resistance. Treatment of mice with anti-IL-3 and anti-IL-4 mAb resulted in an 85% decrease in the mastocytosis (Madden *et al*., 1991) and a 95% decrease in IgE response (Finkelman *et al*., 1989) observed during N. *brasiliensis* infection. Protective immunity to a secondary infection and concomitant immunity to multiple infections with *H. polygyrus* are abrogated by injection of mAb that either neutralises soluble IL-4 or blocks IL-4 receptors (Urban *et al*., 1991) while exogenous IL-4 causes rapid reduction in worm fecundity and parasite establishment (Urban *et al*. 1995). Both normal and parasite induced eosinophilia are dependent on the expression of IL-5 (Hogan *et al*., 2000; Sher *et al*., 1990) and IL-4 (Chen *et al*., 2004). IL-5 also acts as potent stimulator and survival factor for eosinophils (Yamaguchi *et al*., 1988). IL-13 also is incriminated for worm expulsion and mucosal goblet cell hyperplasia (Mckenzie *et al*., 1998).

In ruminants, although nematode parasites inhabiting the GI tract are generally considered luminal dwellers, both larval and adult phases appear to induce definite immunological responses. In the case of GI nematodes of sheep and cattle, the exsheathed L3 enters the abomasal glands or intestinal subepithelial spaces and moults into the L4 while the immature

and adult parasites reside on the surface of the GI mucosa where they feed on blood or mucus secretions. Hence, in both occasions, the different developmental stages have the opportunity to interact with systemic as well as local immune responses.

However, information on the Th1/Th2 polarization in ruminants is very scant and their association to the expression of resistance is often incompletely described. Treatment of lambs with anti-$CD4^+$ monoclonal antibody has indicated a pivotal role for $CD4^+$ T cells in the expression of resistance to *H. contortus* (Peña *et al*., 2006; Karanu *et al*., 1997; Gill *et al*., 1993a). Enhanced reactivity of lymphocytes in calves infected with *Cooperia oncophora* coincided with a significantly increased frequency of $CD4^+$ cells form peripheral blood and lymph nodes (Kanobana *et al*., 2003). The $CD4^+$:$CD8^+$ cell ratio in the abomasal mucosal biopsy of *Trichostrongylus axei*-infected sheep increased during the course of infection compared to that of the controls (Pfeffer *et al*., 1996). This was followed by a significant increase in blood eosinophilia and total IgG responses as well as number of mucosal inflammatory cells (mast cells/globule leucocytes and eosinophils). These responses may suggest the development of a Th2-like immune mechanism. In addition, significant increases in Th2 cytokines (IL-4, 1L-5, IL-10, IL-13) during GI parasitic infections were reported in a number of studies. Pernthaner *et al*. (2005) have witnessed increased expression of IL-5, IL-13, but also the Th1 cytokine tumour necrosis alpha (TNFα) genes in intestinal lymph cells of sheep selected for enhanced resistance to nematodes during infection with *T. colubriformis.* In *O. ostertagi*-infected calves, significant decrease in Th1 cytokines and an increase in the Th2 cytokines (IL-4, IL-5, IL-10 and IL-13) in the abomasal lymph node were demonstrated (Claerebout *et al*., 2005), but no association was observed between the levels of these parameters and protection. Very recently, Lacroux *et al*. (2006) have revealed the development of unequivocal Th2-type immune response in *H. contortus*-infected INRA 401 breed of sheep. This was characterised by elevated IL-4, IL-5, and IL-13 cytokines followed by higher numbers of effector cells in the abomasal mucosa. Gill *et al*. (2000) have also shown that cells isolated from abomasal and mesenteric lymph nodes of *H. contortus*-infected resistant line lambs expressed higher level of IL-5 and parasite-specific IgG1 and IgE production when stimulated with the T-cell mitogen (concanavalin A) or larval parasite antigen. In this study, abomasal tissues from autopsied resistant lambs were found infiltrated with higher densities of mast cells and eosinophils than in random-bred lambs. Altogether,

these studies tend to support the conclusion that a Th2-polarized immunity develops during gastrointestinal helminth infections in sheep even though the protective role of such polarized immunity is not consistently demonstrated.

2.4. Role of effector cells and Antibody

As we have seen earlier, parasitic infections seem to invariably be accompanied by the recruitment of Th2 type effector cells (mast cells/globule leucocytes and eosinophils) and increased production of immunoglobulin isotypes (IgG, IgA, IgE). Indeed, mast cells and eosinophils have long been regarded as key effector cells of immune response to helminth parasites in the gastrointestinal tract (Sasaki *et al*., 1993; Tuohy *et al*., 1990), each of them also regulating the development of the other (Piliponsky *et al*., 2001; Takafugi *et al*. 1998; Okayama *et al*., 1997; Henderson *et al*., 1980). Though their collective action can not be underestimated as can be explained in the following topics, there exist many contradicting reports questioning the protective values of individual cell types in several experimental models of GI parasitism. As a major target of this thesis, eosinophils will be treated in a separate section while we discuss mast cells and antibodies in the following subtopics.

2.4.1. Mast cells and globule leucocytes

Mast cells exhibit properties that allow their categorisation as a cell involved in innate immunity including phagocytosis, activation through pattern-recognition receptors, and location at surfaces that face the external environment. A role in acquired immunity is similarly evidenced by their ability to bind parasite-specific IgE, which then leads to mast-cell activation after exposure to parasite antigen or the parasite itself (Prussin and Metcalfe, 2003).

Mast cells are first recognised in the tissues of various organs based on the unique metachromatic staining characteristics of their secretory granules. They contain granules rich in acidic proteoglycans that take up basic dyes. Subsequent studies discriminated two distinct mast cell subpopulations in the rodent intestine (Irani *et al*., 1989; Befus *et al*., 1985; Enerback, 1966). The population, with safranin-positive granules containing tryptase and chymase, which resides in the submucosal connective tissue was termed as connective tissue

mast cell (CTMC). The granules of CTMC stain even after formalin fixation. The other population, which resides in the mucosal epithelial surface, was termed as mucosal mast cell (MMC). These mast cells, containing only tryptase, were safranin-negative and lost their staining after formalin fixation of the tissues. Despite the sharp histochemical and functional contrasts between MMCs and CTMCs, extensive experimental evidences support a common lineage for these cells (Arinobu *et al*., 2005; Sonoda *et al*., 1986). Globule leucocytes are derived from mast cells that have been activated and degranulated (Murray *et al*. 1968). They are found within epithelia and characteristically contain large acidophilic granules or globules.

Mast cells originate from $CD34^+$ pluripotent stem cells in the bone marrow (Kitamura *et al*., 1993; Gurish and Boyce, 2006). Their precursors circulate in blood and lymphatics, and home to tissues. These cells are ordinarily distributed throughout normal connective tissues, where they are often situated adjacent to blood and lymphatic vessels, near or within nerves and beneath epithelial surfaces such as those of the respiratory and GI systems and the skin (Gurish and Boyce, 2006; Galli, 1990). There, they survive and mature under the influence of stem cell factor (SCF) produced locally by stromal cells including fibroblasts and endothelial cells. Mast cells in tissues may have their survival, maturation and biologic expression influenced by such cytokines as IL-3, IL-4, IL-5 and IFN-γ (Prussin and Metcalfe, 2003). They exert their biological effects by releasing preformed and *de novo* synthesised mediators upon stimulation. Classically, mast cell activation involves the binding of IgE to the high affinity IgE receptors (FcεRI) on mast cells. On activation, mast cells release inflammatory mediators such as leukotrienes (Oliveira *et al*., 1997), proteases (tryptase and chymase) (Krishnaswamy *et al*., 2002), histamine, (Huntley *et al*., 1992; Metcalfe, 1984), platelet activating factor (PAF) and cytokines such as IL-4, IL-5 and IL-13 (Bischoff, 1996; Okayama *et al*., 1995), which perpetuate the Th2 response. The biological activity of mast cells can be regulated by eosinophil granule proteins. Activation of mast cells by major basic protein, eosinophil peroxidase and eosinophil cationic protein up-regulates the release of histamine and pro-inflammatory cytokines (Piliponsky *et al*., 2002, 2001; Zheutlin *et al*., 1984; Henderson *et al*., 1980).

Although their protective role in GI parasitism remains inconsistent, a prominent mastocytosis is observed during gut nematode infections and is controlled by a variety of Th2

type cytokines (Anderson *et al*., 1990; Hamaguchi *et al*., 1987). Treatment of mice with anti-IL-3 and anti-IL-4 mAbs results in 85% decrease in the mastocytosis observed during a primary *N. brasiliensis* infection but does not prevent worm expulsion (Madden *et al.,* 1991). Likewise, W/Wv mice, which have a defect in the stem cell factor receptor *c-kit*, and are thus deficient in intestinal mast cells, are still able to expel a *N. brasiliensis* infection (Crowle and Reed, 1981). In contrast, there is compelling evidence that the mast cell plays a major role in the resistance to a primary infection of mice with *T. spiralis* (Tuohy *et al*., 1990) and *H. polygyrus* (Wahid *et al*., 1994).

Mucosal mastocytosis, including intra-epithelial globule leucocytes, is also a common phenomenon during GI helminthiasis in sheep. Huntley *et al*. (1995, 1992) studied the mucosal responses of sheep and/or goats infected with *H. contortus*, *Trichostrongylus vitrinus* or *T. circumcincta*. These infections were found to be accompanied by increases in the numbers of MMCs and levels of associated mast cell protease, and values were higher among animals with lower worm burdens. Also, Amarante *et al.* (2005, 1999), Macaldowie *et al.* (2003) and Bisset *et al*. (1996) have reported negative correlations between MMC numbers and various parasitological parameters in resistant lines/breeds of sheep. In situations where mast cells appear to play an important role in the resistance to infection, the exact mechanism by which they do so has not been clearly identified. It is likely that they contribute to a non-specific inflammatory response within the gut through the secretion of inflammatory mediators, which may promote increased permeability of gut epithelium and eosinophil recruitment to tissues, and the release of various cytokines which involve in the perpetuation of Th2 responses (Shakoory *et al*., 2004; Scudamore *et al*., 1995; Tuohy *et al*., 1990; Perdue *et al*., 1989; Moqbel *et al*., 1987).

2.4.2. Immunoglobulins and parasite antigens

The response of sheep against GI nematodes also involves production of parasite-specific immunoglobulins (Harrison *et al*., 2003; Show *et al*., 1998; Pfeffer *et al*., 1996). In mammals, there are five types of antibody: IgA, IgD, IgE, IgG and IgM produced by plasma cells (Schallig, 2000). IgG1, IgA and IgE are the three immunoglobulin isotypes frequently incriminated during GI nematode infections in sheep. In general, increases in serum and

mucus antibodies against primary and secondary infections with GI nematodes including *H. contortus* were observed (Gómez-muñoz *et al.*, 1998; Douch *et al.*, 1995; Schallig *et al.*, 1995b). However, a direct relationship between the serum antibody levels and the immune status of sheep is questioned (Emery *et al.*, 2000; Gómez-muñoz *et al.*, 1999). For example, in an attempt to generate immunity against *T. colubriformis* and *H. contortus* in young lambs by trickle infection with viable nematodes, McClure *et al*. (1998) have found that the lambs were partially protected against *T. colubriformis* but not against *H. contortus*. Protection against *T. colubriformis* was correlated with local mast cells and increased worm-specific antibody titres. One problem with studying the local response is the *in vivo* inaccessibility of the abomasum for experimental sampling. The development of the technique to cannulate the gastric lymph duct, which contains efferent lymph from the ovine stomach made it possible to monitor local immune responses to abomasal nematodes. Following infection, the number of IgA-, IgG- and IgM –containing cells in the abomasum of sheep was increased and peak values were found 21 and 28 days after infection (Gill *et al*., 1992; Smith *et al*., 1987). IgA containing cells were the most frequently observed cell types followed by IgG1, suggesting an important role for IgA and IgG1 in the local immune response against haemonchosis. Resistant (Santa Ines) and susceptible (Suffolk and Ile de France) breeds have mounted similar intensities of *H. contortus*-specific IgA and cellular responses, but these are negatively correlated to FEC and worm burdens (Amarante *et al*., 2005). Recently, Domínguez-Toraño *et al*. (2003) have underlined the importance of IgG in sheep vaccinated against *H. contortus*.

In situations where protection is achieved, the mechanism by which antibodies contribute to immunity against GI nematodes is not completely clear. The antibodies could have a direct effect on the parasite by neutralising or inactivating vital metabolic enzymes of *H. contortus* (Gill *et al.* 1993b). Smith *et al*. (1987, 1985) have observed negative correlations between the magnitude of the gastric lymph IgA response of *T. circumcincta*-infected sheep and worm length suggesting that IgA antibodies could interfere with worm's ability to feed. A more general role for IgG and IgA antibodies by participating in hypersensitivity reactions has also been suggested (Miller, 1996; Gill *et al.,* 1993b). Examples are the ability of IgA to induce eosinophil degranulation (Abu-Ghazaleh *et al*., 1989), the binding of IgA/antigen immune complexes to inflammatory cells in the mucosa and the subsequent release of cytokines and inflammatory mediators (Dubucquoi *et al.,* 1994), and the cytophilic properties of IgG1 for

mast cells (Askenase, 1977) resulting in mast cell activation and degranulation. Eosinophils and mast cells have Fc receptors for various immunoglobulin isotypes (Table 3 for eosinophils).

The majority of studies on IgE responses to helminth infections have been in humans and rodents. In human subjects, high levels of serum IgE are thought to be associated with protection against gastrointestinal nematodes (Pritchard *et al.,* 1995). Studies on the role of IgE in helminth-infected sheep were aided by the development of a specific monoclonal antibody against ovine IgE by Kooyman *et al.* (1997). It was found that infection with *H. contortus* resulted in increased total serum IgE levels at 2-4 weeks after infection and values were negatively correlated to worm count at necropsy in repeatedly infected sheep. This is in line with the observations in *O. ostertagi* infected calves (Baker and Gershwin, 1993; Thatcher *et al*., 1989).

A number of parasite antigenic molecules can be recognised by the different immunoglobulin isotypes. Those antigens that can provoke immunological responses vary from surface antigens to excretory/secretory (ES) molecules of different developmental stages. These molecules are termed 'natural antigens' or 'conventional antigens' as opposed to 'hidden' (concealed) antigens. They are recognised by the host during an infection and are targets of the naturally acquired immune response. A wide range of ES and somatic molecules including crude extracts have been tried to induce a protective immunity against *Haemonchus, Ostertagia* and *Trichostrongylus* species. Cysteine proteinases extracted from adult *H.contortus* or its ES by anion-exchange chromatography or thiolsepharose column conferred significant levels of protection against homologous challenge in sheep (Bakker *et al.,* 2004; Redmond and Knox, 2004). Fourth stage larvae secreted/excreted several enzymes into culture medium including a metalloprotease, an acid phosphohydrolase, a cathepsin C-like enzyme, a phospholipase C-like enzyme etc (Gamble and Mansfield, 1996). A 15 and 24 kDa ES proteins with respectively 148 and 222 amino acids were identified as potential antigenic products for vaccine production. The expression of both ES products appears to be developmentally regulated (Schallig *et al.,* 1997) where mRNA encoding occurs only in the parasitic life stages. Furthermore, analysis of a P26/23 antigen, a purified somatic fraction of *H. contortus*, with Western blotting showed an extensive reactivity of sera from P26/23 vaccinated lambs (Domínguez-Toraño *et al*., 2003). Similarly, a 70-90 kDa region in L3

extract but less commonly in L4 stage have been identified by antibody secreting cell probes (Raleigh and Meeusen, 1996) suggesting that different stages of the parasites have developmentally regulated antigenic expression.

3. EOSINOPHILS

3.1. Biology

Most studies related to eosinophil biology and functions are mainly concerned with human and rodent sources with the objectives of elucidating their participation in the pathogenesis of allergy/asthma and in the protective responses to parasitic helminth infections. Eosinophils have been considered end-stage cells involved in host protection against parasites. However, numerous lines of evidence have now changed this perspective by showing that eosinophils are pleiotropic multifunctional leucocytes involved in initiation and propagation of diverse inflammatory responses (Figure 6), as well as modulators of innate and adaptive immunity (Rothenberg and Hogan, 2006).

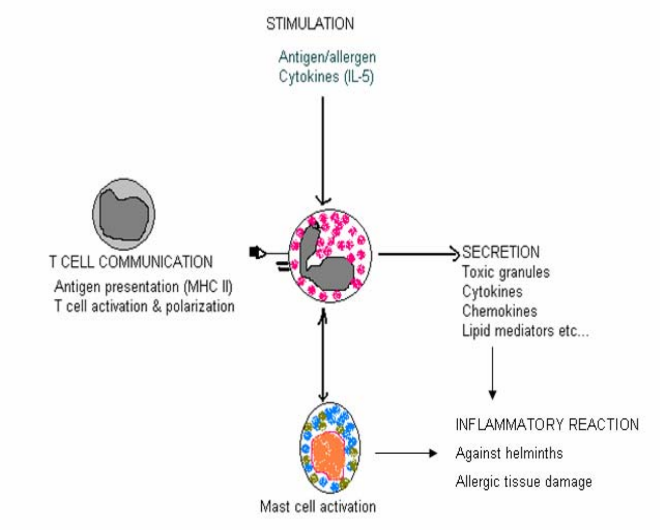


Figure 6. Putative role of eosinophils in the Th2 polarised immune system (Adapted from Rothenberg and Hogan, 2006).

3.1.1. Morphology, surface molecules and granules

Eosinophils are granulocytes that were first described to stain with acid aniline dyes, such as eosin. Because there is no eosinophil-specific cell-surface marker, their morphologic and tinctorial properties visible by light microscopy and their granule ultrastructure visible by electron microscopy remain the most common identifying features. Dyes such as eosin bind to cationic proteins in eosinophil specific granules. Eosinophils typically exhibit a bilobar

nucleus with highly condensed chromatin and cytoplasm containing two major types of granules: primary and specific (secondary) granules. Primary granules are small granules similar to those found in other granulocyte lineages and are found early in eosinophil development. Secondary granules have a distinctive ultrastructural appearance, consisting of an electron-dense crystalloid core. These granules contain the many cationic proteins that give eosinophils their unique staining properties. Eosinophils express a wide array of cell-surface molecules (receptors), including receptors for cytokines, chemokines, immunoglobulins, complement and adhesion molecules (Rothenberg and Hogan, 2006). Table 3 depicts the details of selected receptors and associated ligands for human eosinophils.

Table 3. Human eosinophil receptors for selected immunologic ligands

RECEPTORS	LIGANDS	RECEPTORS	LIGANDS
Complement		**Cytokine**	
C3a receptor	C3a	IL-1R (CD121a)	IL-1
C5a receptor (CD88)	C5a	IL-2Rα chain (CD25)	IL-2
CR1 (CD35)	C3b	IL-3Rα chain (CD123)	IL-3
CR3(Mac-1, CD11b/CD18	C3bi, ICAM-1	IL-4R (CD124)	IL-4
p150, 95 (CD11c/CD18)	C3bi	IL-5Rα chain	IL-5
Immunoglobulin		GM-CSF Rα chain (CD116)	GM-CSF
FcαR (CD89)	IgA	Common β chain	IL-3/IL-5/GM-CSF
FcγRI (CD64)	IgG	CD4	IL-16
FcγRII (CD32)	IgG	IFN-α receptor	INF-α
FcγRIII (CD16)	IgG	IFN-γ receptor	INF-γ
FcεRI (high-affinity R)	IgE	TNF receptor	TNF-α
Chemokine		**Selected others**	
IL-8 receptor (CDw 128)	IL-8	CD9	
CCR-1	MIP-1α, RANTES, MCP-2	CD40	
CCR-3	Eotaxin, RANTES, MCP-3, 4, 5	CD69	
		MHC class I & II	
		FAS (CD95)	

ICAM, Intracellular adhesion molecule-1; TNF, tumor necrosis factor, MIP, macrophage inflammatory protein; MCP, monocytes chemotactic protein; MHC, major histocompatibility complex; FAS, fatty acid synthetase. Adapted from Weller (1997)

3.1.2. Eosinopoiesis

Pluripotent stem cells of the myeloid lineage expressing $CD34^+$ first differentiate into a hybrid precursor with shared properties of eosinophils and basophils (eosinophil/basophile colony forming unit). Eosinophil lineage specification is dictated by the interplay of at least three classes of transcription factors (GATA-1, PU-1 and C/EBP) (Rothenberg and Hogan, 2006). Eosinophils develop and mature in the bone marrow from these $CD34^+$ progenitors under the influence of IL-3, IL-5 and GM-CSF; IL-5 being the most specific to eosinophil lineage (Rothenberg, 2004; Ishihara *et al*., 2000; Tomaki *et al*. 2000; Costa *et al*., 1997; Sanderson, 1992; Enokihara *et al*., 1988). These cytokines (eosinophilopoietins) likely provide permissive proliferative and differentiation signals following the instructive signals specified by the transcription factors (Rothenberg and Hogan, 2006).

3.1.3. Eosinophil homing in tissues

Eosinophils are released constitutively to the peripheral blood as terminally differentiated cells (Hitoshi *et al*., 1991; Shalit *et al*., 1995). Eotaxin and IL-5 cause bone marrow release of mature eosinophils as well as eosinophil precursors (Palframan *et al*., 1998; Mould *et al*., 1997; Collins *et al*., 1995). Eosinophils circulate in the peripheral blood and then traffic to tissues with a peripheral blood half-life (human) of 8-18 hours (Teixeira *et al*., 1995). Although, eosinophils are best known as peripheral blood leukocytes, the vast majority of them are located in the gut, lungs and skin (Behm and Ovington, 2000; Costa *et al*., 1997). The steps that lead to eosinophil recruitment into certain sites of inflammation are multiple and require a combinatorial interplay of enhanced expression and/or function of integrins on intravascular eosinophils and their counterligands such as the vascular cell adhesion molecule-1 (VCAM-1) on the vascular endothelium, as well as the action of eosinophil chemoatractant factors (Weller, 1997; Weller *et al*., 1991). To be effective, blood leucocytes must be non-adherent to the vascular endothelium while they are in the circulation and adherent when they should enter the tissues. IL-4, IL-5 and IL13 play central roles in promoting eosinophil trafficking to tissues by increasing endothelial cell VCAM-1 expression and up-regulating eotaxin expression (Foster *et al*., 2001; Mould *et al*., 1997; Walsh *et al*.,

1990; Sano *et al*., 1995). Eotaxin is also required for the baseline level of tissue eosinophils in mice (Matthews *et al*., 1998). In addition to eotaxin, platelet-activation factor (PAF) and leukotriene B4 (LTB4) are also potential eosinophil chemotactic and transendothelial migration factors (Noble *et al*., 1997; Casale *et al*., 1993). Eosinophil chemotactic factors have also been found in a wide range of parasites including *H. contortus* and *O. ostertagi* (Wildblood *et al.,* 2005; Klesius *et al.,* 1989). Recent advances have helped delineate the adhesive pathways used by eosinophils in their interactions with the vascular endothelium. Eosinophils express two α4 integrins (α4β1, α4β7), not shared by neutrophils, yet found on basophils and some lymphocytes. These integrins bind to VCAM-1 and to domains within tissue fibronectin (Weller, 1997; Bochner and Schleimer, 1994). During tissue localization, circulating eosinophils lightly tether to and roll on endothelial cells by binding to selectins (on the platelets and endothelial cells) followed by migration between endothelial cells by traversing the basement membrane (Gleich, 2000; Sriramarao *et al*., 2000; Broide *et al*., 1998; Patel, 1998). Intuitively, these processes must result in stepwise cell activation sufficient to permit completion of vessel transmigration but not to cause degranulation and release of oxidants, otherwise frank vasculitis would occur.

3.1.4. Eosinophil activation

Once they have trafficked to the tissue, leukocytes need an action signal to exhibit their effector function. Eosinophils are said to be activated by binding of immunoglobulins such as IgG, IgE, IgA or secretory IgA, the last being the most potent (Weller, 1997; Capron *et al*., 1982, 1981) to their specific Fc receptors. Eosinophils also show the capacity to be primed by a number of mediators, including IL-5, GM-CSF, CC chemokines, and PAF (Takafuji *et al*. 1998; Yamaguchi *et al*., 1988). Eosinophils in the blood and tissues of helminth-infected patients and experimental animals exhibit morphologic and functional changes associated with their activation *in vitro* (Bochner, 2000). These include decreased density (Sugaya *et al*., 2001; Hua *et al*., 1990), up-regulation of surface activation molecules such as CD69, CD25, CD44 (Matsumoto *et al*., 1998; Mawhorter *et al*., 1996), enhanced cellular cytotoxicity and release of granule proteins and other mediators of inflammation. In peripheral blood, only 10% of available eosinophils are hypodense (density between 1.075 and 1.077) in normal

human subjects while patients having pathological conditions associated to hypereosinophilia have much higher percentage of circulating hypodense eosinophils (Frick *et al*., 1989; Fukuda *et al*., 1985). Whether the morphologic unity or the diverse density of eosinophils belies a greater diversity of functions analogous to what has been revealed for the lymphocytes (Swain, 2003) remains to be elucidated. Hypodense eosinophils might be activated eosinophils with higher metabolic activity characterized by increased oxygen consumption and elevated superoxide anion production (Owen *et al*., 1991; Yukawa *et al*., 1989). The normal life span of eosinophils is not known, but they are believed to survive for several days (Iversen *et al*., 1997) in healthy tissues and even longer during helminth infections (Gon *et al*., 1997; Simon *et al*., 1997; Walsh *et al*., 1995). IL-5 and GM-CSF have an antiapoptotic effect on eosinophils and promote eosinophil survival in tissues (Prussin and Metcalfe, 2003; Yamaguchi *et al*., 1988).

3.1.5. Granule proteins and inflammatory mediators

Eosinophils synthesize, store, and release a wide range of pro-inflammatory mediators, including at least four cationic proteins and a number of cytokines and chemokines. Crystalloid secretory granules are the major sites for storage of cytotoxic cationic proteins, cytokines and chemokines. The membrane-bound crystalloid granule (Figure 7) comprises an electron-dense crystalline core where major basic protein (MBP) is stored and an electron-lucent matrix where three other cationic proteins namely, eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN) are stored (Gleich and Adolphson, 1986; Ackerman *et al*., 1983).

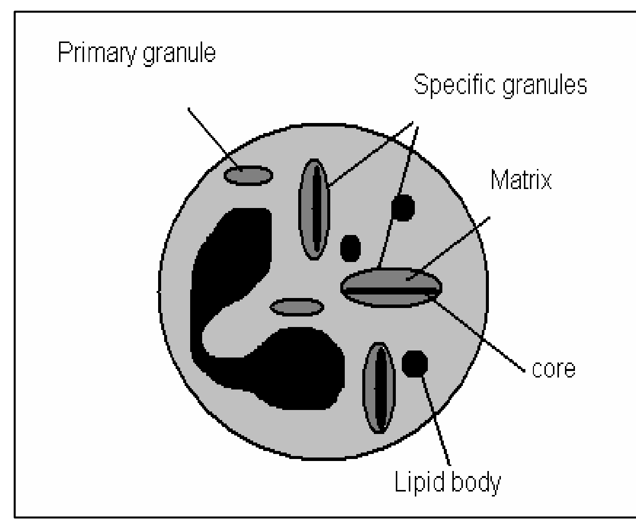


Figure 7. Schematic representation of eosinophil granules (G. Terefe)

Furthermore, a growing list of cytokines has been found within human eosinophil granules, including: prototypical Th1 cytokines (such as IL-12 and IFN-γ), prototypical Th2 cytokines (such as IL-4, IL-5, IL-13) and various growth factors etc. (Bandeira-melo and Waller, 2004; Chen *et al.*, 2004; Kita, 1996; Bjerke *et al.*, 1996; Moqbel *et al.*, 1994). Similarly, Bao *et al.* (1996) have demonstrated that intestinal lamina propria eosinophils in sheep infected with *T. colubriformis* expressed IL-5 mRNA. Upon stimulation, eosinophils also elaborate several bioactive lipids such as PAF and leukotriene C_4. Lipid bodies, intracellular lipid-rich domains, are induced to synthesise lipoxygenase- and cycloxygenase-derived eicosanoids. Collectively, these molecules have pro-inflammatory effects, including up-regulation of adhesion systems, modulation of cellular trafficking, and activation and regulation of vascular permeability, mucous secretion and smooth muscle contraction (Rothenberg and Hogan, 2006; Weller, 1997).

Various mediators released by activated mast cells (histamine, LTB4, PAF, IL-5, GM-CSF, TNF-α etc.) can also trigger eosinophil differentiation, tissue recruitment, activation and degranulation (Shakoory *et al.*, 2004; Takafuji *et al.*, 1998; Okayama *et al.*, 1997; Oliveira *et al.*, 1997). It has also been indicated in the preceding paragraphs that eosinophil granule products can influence the biological activity of mast cells, which altogether strongly suggest the existence of synergism between these two cell types during inflammatory processes.

Table 4. Some properties of specific granule proteins of mature human eosinophils

Protein	***Physical characteristics***	**Biological activity**
MBP	Location: granule core Mw: 14 KDa	• Strong helminthotoxic and cytotoxic activity • Histamine release from mast cells and basophils • Neutralizes heparin • Bactericide • Causes bronchial hyperreactivity
ECP	Location: granule matrix Mw: 18-21 KDa	• Strong helminthotoxic activity • Strong neurotoxic activity • Histamine release from mast cells
EDN	Location: granule matrix Mw: 18-19 KDa	• Strong neurotoxic activity • Strong RNase activity • Weak helminthotoxic activity
EPO	Location: granule matrix Mw: 66 KDa	• Activity in the presence of H_2O_2 and halogens (Br^-, I^-, etc) • Kills micro-organisms and tumour cells • Histamine release from mast cells • Helminthotoxic activity • Provokes bronchospasm in primates

Mw: Relative molecular weight

Adapted from Holgate and church (1993) and Gleich (2000)

Eosinophil peroxidase, MBP and ECP have toxicity (Table 4) to schistosomulae of *S. mansoni* and microfilaria of *Brugia pahangi* and *Brugia malayi* (Specht *et al*., 2006; Abdallahi *et al*., 1999; Hamann *et al*., 1990; Ackerman *et al*., 1985). EDN is a weak helminthotoxic protein (Ackerman *et al*., 1985) but has potent toxicity to myelinated neurons. EPO, which constitutes about 25% of the total protein mass of specific granules, catalyses the oxidation of pseudohalides [thyiocyanate (SCN^-)] and halides (Cl^-, Br^- and I^-) in the presence of H_2O_2 to form highly reactive oxygen species [hypothiocyanous acid (HOSCN) and hypohalous acids such as hypochlorous acid (HOCl) and hypobromous acid (HOBr)] (Wang and Slungaard, 2006). Although EPO shares about 70% amino acid homology to myeloperoxidase (MPO) of neutrophils, SCN^- and bromide anions are preferentially oxidised by EPO while MPO preferentially oxidises chloride anions (Wang and Slungaard, 2006).

EPO

Example: $H_2O_2 + Br^- + H+$ ---------------- $HOBr + H_2O$

Hypohalous compounds are potentially toxic to both micro-organisms and different species of helminths (Hamann *et al*., 1990; Buys *et al*., 1981; Jong *et al*., 1981).

3.1.6. Eosinophil mediator release

In view of such diverse functional potentiality, eosinophil degranulation is a critical process by which these cells may damage, activate or down-regulate the neighbouring cells, as is the case in allergic inflammation. Hence, a regulated mechanism is necessary to specifically select and release proteins from eosinophil granules. Three types of mediator release mechanisms have been described: cytolysis or necrotic release, compound exocytosis, and piecemeal degranulation (PMD). In cytolysis, the cell membrane loses its integrity and crystalloid granules are released to extracellular space. In compound exocytosis, a number of granules fuse intracellularly to form a large degranulation chamber or cavity, which in turn fuses with the cell membrane before discharging its contents to extracellular space (e.g. against parasites) (Logan *et al*., 2003; Scepek *et al.,* 1994). This secretory pathway allows, for instance the release of granule-derived toxic proteins onto helminth surfaces by eosinophils but does not enable them to differentially release their granule-stored cytokines (Bandeira Melo and Waller, 2004). Exocytic degranulation is an acute event that does not depend on protein synthesis or vascular transport. In physiologic conditions, a more commonly seen mode of mediator release in eosinophils that does not involve the wholesale secretion of granule content like in exocytosis is PMD, whereby stored mediators are selectively released from an intragranular pool, leaving portions or all of the granules empty in the intact cell (Erjefalt *et al.,* 1998; Dvorak *et al.,* 1991,). Various stimuli, including cross-linking of different sub-classes of immunoglobulin receptors, are known to induce selective mediator release from eosinophils.

3.2. Eosinophils in allergy

The development of experimental models of allergy has provided important insights into the immunological mechanisms regulating systemic (e.g., anaphylaxis) and pulmonary (e.g., asthma) allergic diseases. Collectively, these studies have identified a central role for Th2 cytokines (e.g., IL-4, IL-5, and IL-13), $CD4^+$ T cells, mast cells, and, in particular, eosinophils, in the induction and maintenance of allergic inflammatory responses (Drazen *et al*., 1996). It is abundantly believed that eosinophils are major participants in the immunopathogenesis of allergic inflammation and eosinophilic gastrointestinal disorders. They are characteristically recruited to inflammatory sites, and their cationic proteins are released, as are their lipid mediators. Antiallergic treatments commonly prescribed (corticosteroids, nedocromil sodium and cromolyn sodium or sodium cromoglycate) are known to reduce the recruitment and functional activity of such inflammatory cells as mast cells and eosinophils in human patients (Rothenberg, 2004; Thomas and Warner, 1996).

3.2.1. Asthma

Asthma is a complex respiratory allergic disease influenced by genetic factors, immunological response, and environmental conditions. The early event in the development of this disease is the immediate hypersensitivity reaction mediated by IgE-allergen interaction. This reaction is followed by an inflammatory response leading to changes in the bronchial structure and airway hyper-responsiveness involving inflammatory cells such as eosinophils. Studies of the activities of the cationic proteins of eosinophils provide a wealth of evidence, albeit circumstantial, that they contribute to damage and dysfunction of other cell types in sites of allergic inflammation (Gleich, 2000; Bochner, 2004). Eosinophils may produce acute and chronic effects on the lungs in asthma, through their capacity: (1) to secrete leukotriene C4, which can cause bronchial smooth muscle contraction, (2) to secrete PAF, which produces bronchial hypersensitivity and vascular permeability, (3) to secrete toxic granule basic proteins, which can induce epithelial cell desquamation and mucus production by goblet cells, franc damage to epithelial cells, and alteration in ciliary beating, and (4) to release reactive oxygen products, which can cause direct membrane damage to epithelial cells (Gleich, 2000; Thomas and Warner, 1996; Spry, 1988).

3.2.2. Eosinophilic gastrointestinal disorders (EGIDs)

Although substantial progress has been made in elucidating the inflammatory mechanisms involved in allergic responses in the lung, there has been limited progress in understanding the pathogenesis of allergic disorders of the GI tract. Eosinophil accumulation in the GI tract is suggested as a common feature of numerous digestive disorders, including classic IgE-mediated food allergy, eosinophilic gastroenteritis, allergic colitis, eosinophilic oesophagitis, inflammatory bowel disease and gastrointestinal reflux disease (Rothenberg, 2004; Sampson, 1999). EGIDs are defined as disorders that primarily affect the GI tract with eosinophil-rich inflammation in the absence of known causes for eosinophilia (eg. drug reactions, parasitic infections and malignancy) (Rothenberg, 2004). Significant progress has been made in elucidating that eosinophils are integral members of the GI mucosal immune system. Interestingly, despite the common finding of food-specific IgE in patients with EGIDs, food-induced anaphylactic responses only occur in a minority of patients (Sampson, 1999). Thus, EGIDs are primarily polygenic disorders that involve mechanisms that fall between pure IgE-mediated food allergy and cellular-mediated hypersensitivity disorders (Rothenberg, 2004). Preclinical studies in human subjects have identified a contributory role for the cytokine IL-5 and the eotaxin chemokine. EGIDs typically occur independent of peripheral blood eosinophilia, indicating the potential significance of GI-specific mechanisms such as those mentioned above for regulating eosinophil levels (Rothenberg, 2004). However, some patients with EGIDs (typically those with eosinophilic gastritis) can have substantially increased levels of peripheral blood eosinophils (in the absence of known cause) and meet the diagnostic criteria for the idiopathic hypereosinophilic syndrome (Waller, 1994). However, it remains to be determined why some disease states are characterized by peripheral blood eosinophilia, whereas others are associated with a tissue eosinophilia in the presence or absence of peripheral blood eosinophilia.

From what has been explained in the preceding sections, a clear similarity between helminth infections and allergic disorders can be appreciated since both are mediated by a Th2-type response. However, there is one important difference between these polarized type 2 responses. In allergy, there is no appropriate modulation of the immunological response whereas in infection with helminths several host mechanisms (such as those involving

regulatory T cells and IL-10) down-regulate the host immune response whenever appropriate. As a result, patients with chronic helminth infections have moderate (minimized) Th2-type responses (Carvalho *et al*., 2006; Capron *et al*., 2004). From this, it may be possible to suggest a regulatory effect of helminth infections over allergic problems when the two occur at the same time. A large number of studies have shown that infections with helminths such as *Schistosoma mansoni, Ascaris lumbricoides, Ancylostoma duodenale* etc. can protect against allergy (Carvalho *et al*., 2006; Capron *et al*., 2004). While acute helminth infections may aggravate manifestations of allergy, chronic infections decrease atopy probably through the initiation of those down-regulatory mechanisms (Negrao-Correa and Teixeira, 2006).

3.3. Role of eosinophils in GI parasitism

Concomitant with the study of Th2-polarized immune responses, the role of eosinophils in the resistance to helminth infections is also extensively examined in laboratory animals. Yet the precise functions of these cells are poorly understood. Increase in peripheral blood eosinophil numbers and the infiltration of target tissues by eosinophils are characteristic outcomes of helminth infection in mammalian hosts (Winter *et al*., 1996). The hypothesis that the primary function of eosinophils is to defend hosts against parasitic helminth infections in rodent species is based on the accumulation of observations *in vitro* and *in vivo*. These include: (a) the ability of eosinophils to mediate antibody-and/or complement-dependent cellular toxicity against helminths *in vitro* (Shin *et al*., 2001; Butterworth, 1984); (b) the increase in the levels of eosinophils during helminth infections and their aggregation and degranulation in the local vicinity of damaged parasites *in vivo* and (c) the results in the experimental parasite infected mice that have been depleted of eosinophils by IL-5 neutralisation and/or gene targeting (Behm and Ovington 2000).

Despite the consistent *in vitro* observations, a protective role for eosinophils has rarely been clearly identified *in vivo*. With the discovery of IL-5 as a key mediator of eosinopoiesis, a number of murine models of IL-5-dependent eosinophil depletion or overproduction have been used to examine the *in vivo* role of eosinophils in helminth infections. Treatment of mice with monoclonal antibodies that neutralise IL-5 generally reduced the development of eosinophilia upon infection with parasitic helminths, but had little effect on the survival or

reproduction of a number of nematodes such as *N. brasiliensis* (Coffman *et al*., 1989) and *T. spiralis* (Herndon and Kayes, 1992) strongly suggesting that eosinophils are not a major effector cells against helminths that reside in the gut (Finkelman *et al*., 1997). In contrast, there is compelling evidence for the presence of eosinophil-mediated protective immune response operating against the migratory tissue dwelling larval stages of two other nematodes, *Angiostrongylus cantonensis* (Sasaki *et al*., 1993) and *Strongyloides venezuelensis* (Korenaga *et al*., 1991) signifying the importance of these cells in the tissue stage immune response. One important problem associated to mice models is that mice are not natural hosts to many of the parasite species studied. More recent murine parasite models have demonstrated a requirement for IL-5 in protective immunity to challenge infection with *T. spiralis* (Vallance *et al*., 2000). Early studies demonstrating the close association of eosinophils with helminths in histological sections and significant correlations between susceptibility/resistance to infection and the magnitude of peripheral eosinophil response also supported a role for eosinophils in resistance to helminth infection (Behm and Ovington, 2000). This lack of consensus on the role of eosinophils in the helminth-induced immune response may be appreciated from the following examples, although incomplete presented in Tables 5 and 6.

Table 5. Effect of anti-IL-5 antibody treatment to mice on eosinophil levels and the development of different species of parasites

Parasite	**Response to treatment**	**Parasite development**	**Reference**
N. brasiliensis	↓eosinophilia	No change	Coffman *et al.* (1989)
T. muris	↓eosinophilia	No change	Betts and Else (1999)
H. polygyrus	↓eosinophilia	No change	Urban *et al.* (1991)
Strongyloides stercoralis	↓eosinophilia	enhanced	Rotman *et al.* (1996)
S. venezuelensis	↓eosinophilia	enhanced	Korenaga *et al.* (1994)
A. cantonensis	↓eosinophilia	enhanced	Sasaki *et al.* (1993)
Onchocerca lienalis	↓eosinophilia	enhanced	Folkard *et al.* (1996)

More recently, transgenic mouse strains constitutively over-expressing the gene encoding IL-5 have been developed. These mice display high blood and tissue eosinophilia (up to 60-90% of total leucocytes in blood) in uninfected state (Behm and Ovington, 2000; Martin *et*

al., 2000). These mice have now been infected experimentally with a variety of parasites, with quite variable outcomes.

Table 6. Parasite development in IL-5 transgenic mice

Parasite	**Response to infection**	**Parasite development**	**Reference**
N. brasiliensis	↑ eosinophilia	Reduced	Shin *et al*. (1997)
A. cantonensis	↑ eosinophilia	Reduced	Sugaya *et al.*(1997)
T. spiralis	↑ eosinophilia	No change	Hokibara *et al*. (1997)
S. mansoni	↑ eosinophilia	No change	Freeman *et al*. (1995)

A number of possible explanations can be suggested for the lack of response to IL-5-dependent manipulations.

1. Tissue recruitment of eosinophils can occur independent of IL-5. Indeed, maximal depletion of airway eosinophils has been reported to occur only in IL-5 and eotaxin double knockout mice in an allergic immunization model (Foster *et al*., 2001). Although Th2-type immune responses (IL-5 as the major mediator) are the dominant mechanism for the induction of eosinophilia, innate immunity is also capable of inducing tissue eosinophilia in the absence of a Th2 response (Alam and Busse, 2004).
2. Anti-IL-5 mAb may not completely abolish eosinophil production (Sutton *et al*., 2005) and hence, the few available eosinophils may be recruited to the site of infection, which is sufficient to induce effective protective responses.
3. Eosinophils from asthmatic subjects have been reported to reduce affinity for IL-5 (reduced IL-5Rα) and become more dependent on GM-CSF for survival and function (Liu *et al*., 2002).

Eosinophils are also found to be involved in immune responses to parasites in sheep and implicated in parasite rejection. An *in vitro* study realised in sheep on cells derived from antigen-induced mammary glands (Rainbird *et al*, 1998) has shown that ovine eosinophils in the presence of immune serum resulted in significant *H. contortus* larvae immobilisation after 24hrs. The effectiveness of larval immobilisation/killing was dependent on the activation status of eosinophils, which was enhanced by repeated *in vivo* stimulation with L3 or *in vitro* incubation with IL-5. In both *H. contortus* and *T. circumcincta* infections, eosinophil infiltration into the abomasal tissue seems to be closely associated with the presence of tissue larvae (Balic *et al.,* 2006; 2003, 2002). Indirect

support for the role of eosinophils in parasite resistance is provided by the significant correlations that exist between hosts' blood and tissue eosinophilic responses and various parasitological parameters. In the comparison of the resistance status of Santa Ines, Suffolk and Ile de France breeds of sheep infected by *H. contortus*, Amarante *et al.* (2005) have shown that all breeds expressed similar values of tissue eosinophilia. In this study, abomasal inflammatory cells including eosinophils and parasite-specific IgA responses were negatively correlated to worm burden and FEC. Similarly, tissue eosinophilia was negatively associated to FEC, eggs *in utero* and female worm length in sheep experimentally infected with *H. contortus* (Lacroux *et al.*, 2006). Furthermore, after trickle infection of naturally immunized sheep with this parasite, peripheral eosinophil counts were found significantly higher in responders than non-responder sheep lines (Hooda *et al.*, 1999). From these circumstantial evidences, one can suggest that eosinophils have an influential role on the regulation of parasite development *in vivo*.

On the contrary, no apparent correlation between eosinophilia and resistance to infection was reported by Gill (1991) and Beriajaya and Copeman (2006) in sheep infected with *H. contortus*. This may imply that eosinophils are not essential component of resistance to helminth parasites or there is a redundant mechanism involved against these large, multicellular pathogens. The above and more other studies were realised with different experimental protocols, used different species of parasites and animals of varied genetic backgrounds, which make comparisons practically doubtful. For example, Merino lambs with lower FEC (Resistant line) showed similar levels of eosinophilia to their contemporary lambs having high FEC (susceptible line) (Woolaston *et al.*, 1996) while in another study in the same breed, resistant lines have expressed higher eosinophilia after intramammary infusion with *H. contortus* antigen (Thamsborg *et al.*, 1999). On the other hand, Merino and Romney resistant line lambs experimentally infected with *T. colubriformis* were reported to have greater eosinophilic responses than their susceptible counterparts (Bisset *et al.*, 1996; Buddle *et al.*, 1992).

In general, eosinophils do not appear to be clearly defined cells. On the one hand, their biology is potentially well suited to cause different degrees of inflammation and tissue damage, on the other hand, they are constitutively mobilised during parasitic infections. In the latter case, their association to host resistance is not consistently revealed, and when such data exist, they are often subject to variations related to a number of factors such as parasite or animal species (tissue migratory/non-migratory parasites, laboratory/large animals) and

experimental protocols. With respect to experimental protocols, while the use of genetically modified mice (transgenic and gene knockout etc) has resulted in great advancement in the study of parasitism, similar modification does not exist for sheep.

DEUXIEME PARTIE

MATERIELS ET METHODES.

Cette seconde partie donne une description des animaux et de la souche du parasite utilisés dans les expérimentations. Les mesures quantitatives ou qualitatives (tests in vitro, études en microscopie électronique, tests biochimiques…) sont décrites brièvement. Le lecteur pourra se référer aux parties « Materials and méthods » des différents articles pour avoir une description plus détaillée.

PART II. MATERIALS AND METHODS

In this section, brief descriptions of experimental animals and infection protocols are given. Quantitative and qualitative measurements including *in vitro* tests, ultrastructural studies and biochemical assays conducted to achieve the goals of this thesis are described.

1. EXPERIMENTAL ANIMALS

Three breeds of sheep namely, INRA 401, Barbados Black Belly and Tarasconnais, reared and maintained helminth free, were used for our experimental studies. In most cases, lambs between the age of 4 and 6 months were used, but older animals have also been occasionally employed. Except in one study in which both sexes were included (Tarasconnais breed), all the other works have been realised on male (INRA 401 and Barbados Black Belly) animals generously provided by the INRA experimental station. This was due to shortage of female counterparts as they had to be maintained for breeding purposes. Based on the requirement of specific experimental protocol, animals were divided into infected and uninfected (control) groups. For infected groups, larvae were administered either once or twice. In the latter case, infections were separated by anthelmintic treatment. At the end of each study, sheep were killed by intravenous administration of 10 mg/kg pentobarbital sodium followed by exsanguinations. During each experimental study, animals were handled following European Union recommendations for animal welfare, under the supervision of the local INRA ethics committee.

1.1. The INRA 401 breed

The INRA 401 breed of sheep (Figure 8) is a product of a research program initiated by INRA experimental station (Bourges-La Sapinière), to enhance the productivity of sheep flocks of France by a reciprocal cross mating of the Romanov and Berrichon du Cher breeds. The INRA 401 is distributed particularly in the southern part of the country (François *et al.*, 1998). This breed is relatively susceptible to GI nematode (such as *H. contortus*) infections (Gruner *et al.*, 2003)

Figure 8. The INRA 401 breed of sheep (photo: G. Terefe)

1.2. The Barbados Black Belly breed

The Barbados Black Belly is a hair-breed of sheep characterised by black abdomen and extremities (Figure 9). Although there can be little doubt that the Black Belly has African ancestry, historical evidences show that this sheep, as a breed, originated and evolved on the island of Barbados, hence the name Barbados Black Belly, from crosses of African hair sheep and European wooled breeds of unknown origin (http://www.barbadosblackbelly.com/.

Embryos of this sheep were imported from Guadeloupe (French West Indies) to France by the INRA research centre (domaine INRA de la Sapinière (Cher)) where they are maintained by successive breeding programs. The Black Belly is known for its resistance to GI nematode infections such as *H. contortus* (Gruner *et al*. 2003; Yazwinski *et al*., 1980)

Figure 9. The Barbados Black Belly breed of sheep (Photo: G. Terefe)

1.3. The Tarasconnais breed

The Tarasconnais (Figure 10) is a local meat breed of sheep found in the Pyrenees region of France. It is susceptible to many species of GI nematode parasites such as *T. colubriformis* and *H. contortus*.

Figure 10. The Tarasconnais breed of sheep (photo: Traore I.)

2. THE PARASITE AND EXPERIMENTAL INFECTIONS

The larvae of *Haemonchus contortus,* « Humeau » strain were first isolated from a goat flock in the region of Quercy (France) and maintained in the Parasitology unit of INRA (Tours-Nouzilly). The larvae obtained from this station were maintained through passages in susceptible sheep. Larvae were prepared for each experiment by faecal egg culture in our laboratory and stored at 4°C for 6-7 weeks before each experimental manipulation. In most cases, animals were infected by oral route, with 10000 *H. contortus* L3 (all at once) per infection. This number was chosen to provoke the desired immune response without threatening the lifes of animals throughout the experimental period. Experiences in our laboratory by Lacroux (2006) in the preparation of her Doctoral thesis show that such a worm load can be well tolerated for at least one month by the INRA breed with no apparent physical weakness. In one experimental study, infective larvae incubated *in vitro* with blood cells were administered intra-abomasally to see their establishment potential. In both cases the duration of infection was limited to a maximum of one month.

3. METHODS

To meet our objective of studying the relationship between parasite development and host response, we have employed parasitological and immunopathological methods followed by *in vitro* tests, electron microscopic examinations and biochemical assays. Quantitative and qualitative measurements were performed on *ante-mortem* and *post-mortem* samples.

3.1. Parasitological parameters

Parasitological parameters such as faecal egg count, worm count, female worm size and *in utero* egg count can be good indicators for the comparison of the resistance status of groups, lines or breeds of sheep to *H. contortus* infection. Reduction in faecal egg count could be attributed to reduced ability of adult worms to produce eggs. It may also be related to reduced

worm establishment, reduced growth and development to adult stage, which altogether are in one way or another associated to or influenced by the host immune response.

3.1.1. Qualitative and quantitative assessment of parasite population

At the end of each experiment requiring necropsy, all sheep were killed humanely by intravenous injection of 10 mg/kg pentobarbital sodium. The abomasum was opened and its contents and washings were collected and sieved (40 µm), and the coarse material containing worms was retained. The abomasum was then digested in pepsin-hydrochloric acid solution (37°C, 6h) (Herlich, 1956) to collect the tissue-dwelling worms. The solution was prepared by mixing 20 gm of pepsin, 20 ml of HCl and distilled water to make a final volume of 1 litre. The contents have been preserved in absolute alcohol. The volume of the material was adjusted to 1 litre and worms were counted in a 10% aliquot. Worms were classified as adult male and female, immature male and female or L4 stages. The fecundity of female worms can be evaluated by worm length measurement and *in utero* egg count. Hence, whenever applicable, the lengths of 20 randomly selected adult female worms were measured. This was followed by digestion of each worm in a 200 µl of 20% mild bleaching agent (Milton Sterilizing fluid containing 2% w/v sodium hypochlorite and 16% w/v sodium chloride Milton Pharmaceutical LTD) diluted in distilled water (Kloosterman *et al*., 1978), and all eggs liberated from the uterus were counted. The advantage of this fluid is that it does not damage eggs for reasonably prolonged period and hence facilitates counting with minimum difficulty. The level of *in utero* egg count could either be a function of female worm size or worm's ability to produce eggs or both.

3.1.2. Faecal egg count

Egg counts were performed on faecal samples collected directly from the rectum at regular intervals. Three grams of the material was homogenised in 42 ml of salt solution (200 gm NaCl in 600 ml H_2O: density =1.8) which makes a final volume of approximately 45 ml (1gm/15ml). Eggs were counted according to the modified McMaster technique (Raynaud, 1970). The two McMaster egg-counting chambers contain 0.3 ml (0.15 ml x 2) of the

homogenate. Hence, counts were multiplied by 50 to calculate the number of eggs per gram (EPG) of the faecal material. This method provides information about the prepatent period of the infection and grossly allows estimating the developmental evolution of the parasite throughout the experimental period. Its limitation is that it does not provide full information about the parasite burden and extent of worm development, measurements that require in most cases killing of the animals.

3.2. Immunological parameters

Resistance to GI parasitism can also be explained in terms of immunological parameters. The levels of $CD4^+$ cells, Th2 cytokines and effector cell responses is often suggested to be associated to the hosts' ability to control worm population and development. The extent of the immune response varies with breed and age of animals, duration and frequency of exposure to infection as well as presence of other concurrent parasitic or non-parasitic problems. An increase in blood and tissue eosinophil number is a hallmark of gastrointestinal parasitism in general and *H. contortus* infections in particular. Furthermore, mast cells/globule leucocytes are also known to localise at the site in question at various stages of the infection. Therefore, measuring the levels of these and other related parameters could contribute to the understanding of the immune mechanism underlying resistance of sheep to *H. contortus* infection.

3.2.1. Measurement of cytokine gene expressions

3.2.1.1. mRNA extraction and reverse transcription- polymerase chain reaction (RT-PCR)

About 300 mg of abomasal lymph node or fundic mucosa were snap frozen in 1 ml of Trizol Reagent (Invitrogen, Ref. 15596-018), and stored at –70°C until used. Samples were homogenized using a Hybaid RiboLyser™ (two cycles of 30s each at a speed of 4.5). Fifty µl of the obtained homogenate was mixed with 950 µl of Trizol before adding 200 µl of chloroform at room temperature for 10 min. Samples were then centrifuged for 15 min at 20000g and 4°C. Supernatants were placed on an RNeasy Column (RNeasy Mini Kit, Qiagen) and then processed using the Qiagen protocol for RNA clean-up. This protocol allows the

recovery of highly pure RNA. The RNA concentration was measured by spectrophotometry at 260 nm and RNA quality was assessed by electrophoresis on 1.2% agarose gel stained with ethidium bromide. Two µg of total RNA were digested with 1U of RNase-free DNAse (Promega) for 1h at 37°C to remove any trace of genomic DNA, and then incubated for 10 min at 65°C with DNase Stop Solution. Treated RNAs were used as templates for single-stranded cDNA synthesis. They were incubated (10 min, 65°C) with Random Primers oligonucleotides (Invitrogen) and chilled on ice. A mix containing M-MLV transcriptase (400 IU/sample), 5x first-strand buffer (Life Technologies), dithiothreitol (DTT) 0.1 M (Life Technologies), 40 U of RNase Out Ribonuclease Inhibitor (Invitrogen), 10 mM of each four dNTPs (Promega) was then added and incubated (1h, 42°C). Reaction was heat-inactivated at 95°C (5 min) and kept at –20°C until used. For quantitative PCR, cDNA were used at dilutions of 1:100.

3.2.1.2. PCR primers

Specific primers and amplicon sizes are listed in Table 7. For each target cDNA, a primer pair was determined using Primer Express Software (Applied Biosystems) for SYBR Green real-time PCR assay on a GeneAmp 5700 Sequence Detection System (Applied Biosystems) and based on known ovine gene sequences (β-actin, IFN-γ, TNF-α, IL-3, IL-4, IL-5, IL-10, IL-12p40). Oligonucleotides were designed to amplify a product with a size of 51 bp, with a melting temperature (Tm) of 58-60°C. When the ovine gene sequence was not known (Eotaxin, IL-13), a consensus sequence was created, based on a minimum of three known sequences in other mammalian species. A first primer pair was then designed using Primer 3 Software in order to amplify the largest possible mRNA in all aligned sequence. PCR amplification on reverse transcript RNA obtained from ovine peripheral blood mononuclear cells (PBMC) stimulated with concanavalin A (ConA, 10 µg/ml, 24 hours in a 5% CO2 and 37°C atmosphere) was then performed. Amplicons were purified before cloning in TopoCloning system (Invitrogen) and sequenced (using M13 universal primers). The sequences thus obtained were then compared with Gene Bank database using blast to validate the identity of the amplified product. After validation, a new set of primers suitable for

quantitative PCR was designed using the obtained sequence. For IL-13, primers used were those published by Hein *et al*. (2004).

Table 7. Forward and reverse primer sequences for quantitative RT-PCR.

Ovine Cytokine	***Forward sequence***	***Reverse sequence***	***amplicon Tmp. (•C)***
Beta-actin	ACCAGTTCGCCATGGATGAT	AGCCGTTGTCAACCACGAG	78
IFN-γ	ATCTCTTTCGAGGCCGGAGA	ATTGCAGGCAGGAGAACCAT	77
IL-4	GGAGCTGCCTGTAGCAGACG	TTCTCAGTTGCGTTCTTTGGG	79
IL-10	CTGAGAACCATGGGCCTGAC	TCTCCCCCAGCGAGTTCAC	80
TNF-α	CCCGTCTGGACTTGGATCCT	TGCTTTTGGTGCTCATGGTG	75
IL-12p40	GAATTCTCGGCAGGTGGAAG	GTGCTCCACGTGTCAGGGTA	80
IL-13	AGAACCAGAAGGTGCCGCT	GGTTGAGGCTCCACACCATG	80
Eotaxin	ACAAGAAAATCTGTGTTGATCCCC	CCATGGCATTCTGGACCC	75
IL-5	CTGCTGATAGGTGATGGGAACTT	GGTGATTTGTATGCTGAGGAGTAGG	74
IL-3	AAGAATATCCTGGCGAATAAGAGC	GAACGCTTTCAGGTTTGCCT	75

3.2.1.3. Quantitative PCR

Quantitative PCR was performed on a GeneAmp 5700 (Applied Biosystems), using the double-stranded DNA binding dye SYBR Green I. PCR products corresponding to the different amplicons of target cytokines were cloned in PBR 2.1 plasmid (TopoClonA, Invitrogen) and sequenced (M13 universal primers). The plasmids harbouring the cloned sequences were then quantified by spectrometry to establish the number of target copy/µl. Ten-fold dilution series were prepared from the stock plasmid solution corresponding to 10^7 to 10^1 copies of the target sequence. Serial dilution of the plasmid was then used as an external standard, allowing the determination of the number of copies of the target cDNA in each assay. Each sample for each investigated cytokine was performed in duplicate. Ovine Beta-actin was used as a housekeeping gene to normalize expression between different samples. The results obtained for each cytokine are expressed as a ratio of the number of cytokine mRNA copies/Beta-actin mRNA copies. A non-template control reaction (NTC) was

included in each run. Finally, specificity of amplification reaction was assessed by acquisition of a dissociation curve. Data was obtained by slowly increasing the temperature of PCR reaction from 60 to 95°C. Denaturation of non-specific PCR products is followed by a faster decrease of fluorescence than for specific products. The melting profile of distinct PCR product was obtained by plotting the first derivative (-dF/dT) of fluorescence (F) versus temperature (T).

3.2.2. Blood parameters

3.2.2.1. Blood eosinophil count

Blood samples were collected in EDTA coated vacutainer tubes. 100 µl of each sample was mixed in 900 µl of Shandon eosin Y 0.5% (Ref. 6766009: Thermo electron corporation, U.S.A.) and allowed to stain for 5 minutes. The solution was then diluted with a similar volume of PBS (1 ml) to facilitate cell visualisation, a modification that allowed us to improve eosinophil counting by 10-15%. The resulting mixture was filled into a FAST READ 102 cell-counting chamber (ISL UK, Figure 11). Eosinophils were counted from 2 chambers in the grids containing 1 µl of the mixture (Dawkins, *et al.*, 1989) and values were multiplied by 10^4 to calculate the number of eosinophils per millilitre of blood.

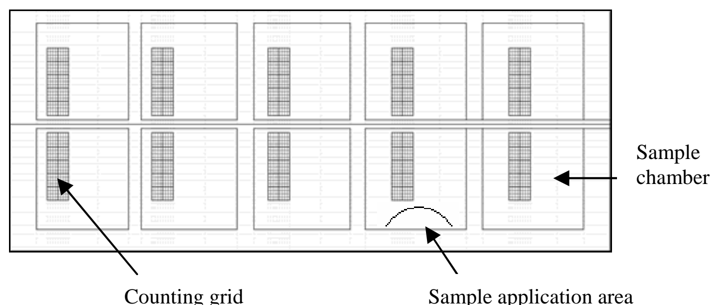


Figure 11. FAST READ 102 counting chamber. Each counting grid contains ten 4x4 grids. The sample chamber depth is 0.1 mm with a capacity of 7 µl, and the total counting grid volume is 1 µl.

3.2.2.2. Bone marrow eosinophil count

Bone marrow smears were made from the iliac crest after necropsy, and the slides were immediately fixed with absolute alcohol for three minutes. The fixed slides were stained by May Grünwald-Giemsa. The percentage of eosinophils was determined from 400 leucocytes counted at x1000 magnification.

3.2.2.3. Measurement of anaemia

Blood samples obtained for eosinophil counts have also served for packed cell volume (PCV) evaluation as a measure of anaemia. Samples were filled into capillary tubes and centrifuged at 12000 rpm for 10 minutes (Biofuge haemo). The PCV was registered as a percentage of whole blood volume.

3.2.3. Abomasal histopathology

3.2.3.1. Standard histology

Large volume of information about the local immune response can be obtained by examining histological samples taken from the abomasum. Representative samples have been taken in such a way as to include parasite specific predilection sites such as the fundic and pyloric regions. About 1 cm^2 tissue samples from both infected and control animals were collected and preserved in 10% buffered formalin for eosinophil and globule leucocyte or in Carnoy's fixative for mast cell staining. Because of its unstable nature, Carnoy's fixative was prepared few minutes before use by mixing 600 ml of absolute ethyl alcohol, 300 ml chloroform and 100 ml glacial acetic acid in 1 litre. Tissues in Carnoy's fixative were transferred to absolute alcohol after at least 6 hours of fixation. After routine histological processing, 5 µm-tick paraffin-embedded tissue sections were prepared. These were deparaffinised and stained with haematoxylin and then counterstained with either Carbol-chromotrope or eosin for counting eosinophils (Figure 12) and globule leucocytes. Similarly,

for counting mast cells, slides were stained with alcian blue and counterstained with safranine (Enerback 1966; Lendrum, 1944). The cells were counted at x400 magnification in 10 microscopic fields. Eosinophils and mast cells were counted throughout the mucosal layer while globule leucocytes were counted in the upper two-third of the mucosal layer.

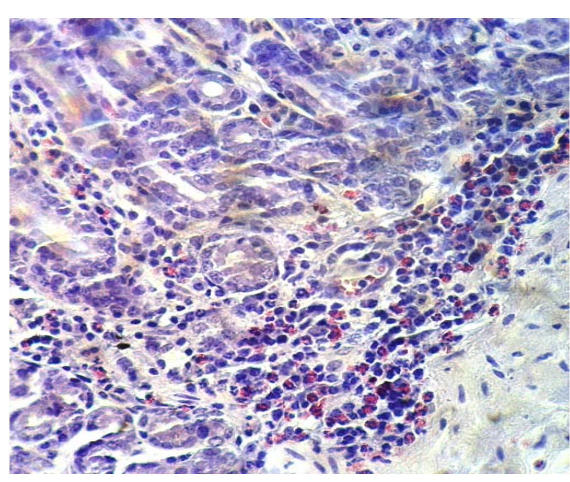


Figure 12. Eosinophils (pink) in the abomasal mucosa: Hematoxylin/carbol-chromotrope stain. (Photos: G. Terefe).

3.2.3.2. Immunohistochemistry (IHC)

Immunohistochemistry on paraffin embedded samples was performed using antibodies described for cell phenotyping on paraffin-embedded tissues in sheep (Lacroux *et al.*, 2006). This antibody set allows specific labelling of macrophages/monocytes (mouse anti-human CD68 antibody, clone Ki-M6, Serotec, 1:150 dilution), B lymphocytes (mouse anti-human CD20-like antibody, clone BLA-36, Novocastra, 1:50 dilution), or T lymphocytes (rabbit anti-human CD3 antibody, A 0542, DAKO, 1:100 dilution). Immunohistochemistry on frozen samples (non-fixed samples stored at –70°C) was performed on five µm sections of abomasal tissue obtained using a Leika cryomicrotome. Slides were fixed for 20 min in an –20°C acetone bath before drying and directly processed for IHC. Monoclonal antibodies directed against CD4 (mouse anti-ovine CD4, SEROTEC, Ref. MCA2213, 1:2000 dilution), or CD8 (mouse anti-bovine CD8, SEROTEC, Ref. MCA837G, 1:800 dilution) antigens were used. Cells were counted on five randomly selected fields at x 400 magnification.

3.2.4. Antibody responses

3.2.4.1. Collection of serum and mucus samples

Antibodies against *H. contortus* antigens can be detected in serum and mucus secretions of infected animals. Serum samples were prepared at regular intervals and stored at –20°C until analysis. Mucus samples were also collected after necropsy by PBS-impregnated filter paper strips deposited onto the abomasal mucosa for 5 min. The strips were then agitated in PBS for 2h at room temperature for eluting the mucus, and the liquid was centrifuged and stored at -70°C until analysed. Mucosal scrapings can be used as an alternative method of sampling but this must be made on approximately similar size of abomasal tissue to avoid sampling biases. Just after discarding the abomasal content, the mucosa was flushed with PBS before sampling. Excessive washing was avoided as this could wash away the mucous secretions from the surface.

3.2.4.2. Preparation of *H. contortus* antigens

Parasite antigens that can easily be recognised by the host's immune response are either somatic (crude extracts) or excretion-secretion products. Adult *H. contortus* worms were incubated at 37°C, 5% CO_2 overnight in PBS (pH 7.4) containing penicillin (100 IU/ml) and streptomycin (1 mg/ml). The liquid containing excretion-secretion products (ESP) from these worms were collected and centrifuged (10000g, 30min, 4°C), and the supernatant was stored at –70°C until used. A crude extract of *H. contortus* L_3 (L_3CE) was prepared after three cycles of freezing and thawing (-70°C / +25°C), homogenisation (Potter tissue homogeniser at 4°C) and centrifugation (30,000g, 30 min and 4°C). The supernatant was stored as above. The protein concentration of these two antigenic preparations was determined with the method of Lowry *et al*. (1951).

3.2.4.3. ELISA for antibody measurement

An indirect ELISA was applied as described by Jacquiet *et al*. (2005) on serum and mucus samples to determine *H. contortus* ESP- and L_3CE-specific IgG and IgA responses. Briefly, each well of a flat-bottomed microtitre plate (Nunclon Distr. VWR International, France) was coated overnight with 100 µl of antigen diluted in sodium carbonate buffer at 4°C. The plates were washed twice in PBS pH 7.2 containing 0.1% Tween 20 (PBS-T). To minimize non-specific binding of the antibody, the plates were then incubated for one hour at 37°C with 200 µl of 5% skimmed milk-PBS-T (PBS-TSM). Subsequently the PBS-TSM was discarded and the plates dried, before adding 100 µl of serum diluted in PBS-TSM or non-diluted mucus to each well for 1 hour at 37°C. After three washes with PBS-T (the third being for 5 min), the plates were subsequently incubated for 1.5 hours with 100 µl of Donkey anti-sheep IgG HRP (Ref. A3415, Sigma) for IgG determination, or for two periods of 1 hour for IgA determination with the first (Mouse IgG1 anti-IgA bovine/ovine, Serotec, Ref. MCA628) and the second (Goat anti-mouse IgG1 HRP, Ref. STAR81P: Serotec) conjugates separated by three washings in PBS-T. Finally, the plates were washed three times with PBS-T and 100 µl of 2-2'-azino-bis (3-ethylbenylthiazoline-6-sulphonic acid, ABTS) in 100 mM citrate buffer (Ref. 104.4: Sigma) containing 0.01% H_2O_2 was added to each well. The colour was allowed to develop for 1 hour at 37°C and the reaction was stopped (4°C, 15 min). The optical density was measured at 405 nm using a Microplate Reader (System Dias, Dynatech).

3.3. Blood eosinophil isolation and *in vitro* tests

3.3.1. Choosing eosinophil isolation method

The availability of a relatively pure preparation of viable eosinophils is an important prerequisite for studying their functions *in vitro*. The first problem in this respect is the difficulty in the separation of these cells from other leucocytes. Density gradient centrifugation and immunomagnetic methods or combinations of the two are the commonly employed methods for isolating eosinophils from mice and human blood. After eliminating lymphocytes and monocytes by percoll density gradient centrifugation, taking the advantage

of CD16 expressed on neutrophils, the immunomagnetic method uses anti-CD16-antibody coated magnetic beads to separate eosinophils from neutrophils by negative selection (Hansel *et al*., 1991). Our attempts to isolate sheep eosinophils by using magnetic beads coated with antibody against human CD16 failed probably due to lack of recognition of sheep neutrophils by human anti-CD16 antibody.

Density gradient centrifugation uses a dense solution or density gradient to separate particles based on their individual densities or mass/size ratio. In this regard, a method was described by Woldehiwet *et al.* (2003) for isolation of eosinophils from sheep blood. After initial removal of most mononuclear cells by a low speed centrifugation and lysis of erythrocytes by hypotonic shocks, the resulting pellet is further separated into mononuclear cells, eosinophils and neutrophils by percoll density gradient centrifugation (density: 1.10). Sheep eosinophils were found to form a distinct band under the mononuclear cell layer at the percoll-PBS interface. By this method, it was reported that 65.5-97% pure and viable eosinophils could be obtained. This technique, unlike the first one does not require the use of surface markers and solely depends on the density of the cells and the percoll. Although, we could not arrive at the level of purity indicated by the authors, we were able to generate a reasonable percentage of viable eosinophils by using this method. Thus, all *in vitro* works undertaken in our studies were based on eosinophil-enriched blood leucocytes prepared by the method adapted from Woldehiwet *et al*. (2003). As this fraction also contains variable proportions of contaminating cells, the major being lymphocytes and neutrophils, parallel studies were also carried out to see the possible interference of these contaminates during the *in vitro* manipulations.

3.3.2. Preparation of eosinophil-enriched blood leucocytes

Briefly, 30 ml of blood sample was taken in EDTA-coated tubes and centrifuged (4K15 centrifuge, Sigma) at 400g, for 20 minutes at 20°C. After discarding the plasma, the underlying buffy coat layer was carefully removed and kept in sterile tube. The material was then adjusted to the original volume by PBS containing EDTA (PBS-EDTA). Erythrolysis was made for 20 seconds by sterile distilled water (36 ml /4 ml of the diluted blood) followed by addition of 4 ml of 9% sterile NaCl solution to restore isotonicity. The tubes were then

centrifuged for 5 minutes (250g, 20°C). The supernatant was discarded and the cell pellet was washed with 10 ml of PBS-EDTA (250g, 5 min and 20°C). The pellets were re-suspended in 5 ml of complete cell culture medium (RPMI 1640, supplemented with 1% penicillin-streptomycin and 10% FCS) and gently deposited on a percoll (P1644-Sigma) solution (density: 1.090 g/ml) for density gradient centrifugation (400g, 20 min and 20°C). As our cell culture medium contained phenol red, which may interfere with spectrophotometry, sterile PBS was used instead of RPMI 1640 for eosinophil peroxidase assay. As we were not able to see the distinct band expected to represent a layer of eosinophils, all cells at the percoll-RPMI/-PBS interface (EOS), and the pellet (PMN) at the bottom of the tube were separately aspirated and washed twice with 10 ml of cell culture medium or PBS by centrifugation (250g, 5 min and 20°C). Total cell count and May Grünwald-Giemsa stained differential counts were performed (Figure 13). Cells from the RPMI-/PBS-percoll interface containing more than 40% eosinophils were considered for all *in vitro* cultures and EPO assays. Whenever applicable, Pellets containing greater than 92% neutrophils or buffy coats containing more than 92% mononuclear cells were conserved and used for *in vitro* comparisons. Cell viability was assessed by trypan blue exclusion stain (> 95% viable). The proportion of eosinophils isolated by this method was found to increase with increasing percentage of blood eosinophils (higher in infected animals than in non-infected sheep, or at around peak blood eosinophilia than before or after the peak).

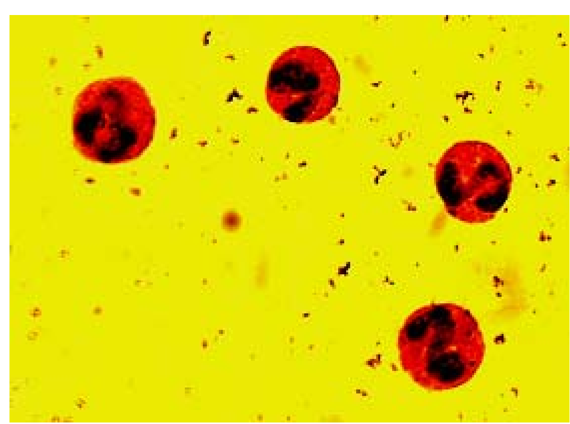


Figure 13. Sheep eosinophils isolated by percoll (1.090 g/ml) density gradient centrifugation (Photo: G. Terefe)

3.3.3. *In vitro* L3-cell co-culture and larval mobility

Cellular suspensions were classified as eosinophil-enriched (EOS: from RPMI-percoll interface), neutrophils-enriched (PMN: from the pellet at the bottom) and lymphocyte-enriched (LYP: from the buffy coat layer) and adjusted to 1.5×10^6 cells/ml and 200 µl of the suspension (3×10^5 cells/well) was deposited in a 24 well micro-plate (TPP-92024). This was done with or without 100 µl of immune and non-immune and whole and decomplemented (heat-inactivated) sera in triplicates. Decomplemented serum was prepared by heating the serum in a water bath at 56°C for 1hr. In one of the experiments in which the activities of eosinophils from two breeds were compared, decomplemented serum was replaced by foetal lamb serum (without complement inactivation). In the latter case, each well of the culture plate was filled with leucocyte fraction adjusted to a fixed number of eosinophils (i.e. 1×10^5 eosinophils). Exsheathed *H. contortus* L3 (200 µl) in RPMI tissue culture medium was added and the plates were incubated overnight (18-24 hrs) at 37°C and 5% CO_2 atmosphere. The culture plates were taken out of the incubator and kept for 30 minutes at room temperature. The motility of larvae was examined directly in the culture plates using an inverted microscope. Larvae were considered motile if any part of the body was seen moving. The addition of similar volume of eosin Y to the culture (minimum 3 hours) allowed the demonstration of cells (mainly eosinophils) attached to the larval surface (Figure 14). After 18 hours of L3-cell co-culturing, the suspension was collected and processed either for intra-abomasal infection or for ultrastructural studies according to the experimental protocol in question.

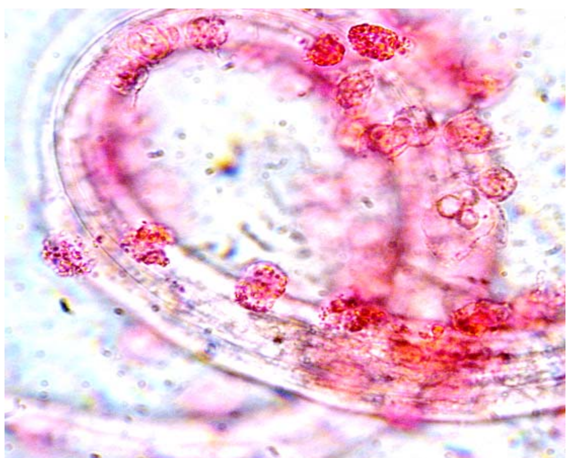


Figure 14. Larva cultured with eosinophil-enriched fraction in the presence of immune serum. Intact eosinophils and their granules are visible adherent to the larva (original magnification: x1000. photo: G. Terefe).

3.4. Intra-abomasal infection

For intra-abomasal inoculation, larvae co-cultured with different leucocyte-preparations, (eosinophil-enriched, lymphocyte-enriched or neutrophil-enriched) were put in distilled water overnight to remove adherent and non-adherent cells from the suspension. The material was then centrifuged at low speed (250g , 5 min and 20°C) and the number of larvae was adjusted to 800/animal in 2 ml of distilled water. A 5 cm incision was made on the right flank under general anaesthesia and the abomasum was exposed. Larvae were injected into the abomasal lumen by using a hypodermic needle.

3.5. Ultrastructural studies

Ultrastructural studies were performed to demonstrate structural changes and adherence of eosinophils co-incubated with *H. contortus* L3 *in vitro*.

3.5.1. Scanning electron microscopy

Larvae co-cultured with eosinophil-enriched leucocytes were collected on the second day of culturing and fixed in 2% glutaraldehyde in 0.1 M phosphate buffer at 4°C for 4 hrs. Following two washes with the same buffer, the larvae were dehydrated in a graded ethanol series, dried by critical point drying with EMSCOPE CPD 750 and coated with gold-palladium for 5 min at 100 Å/min. The material was then observed with a S450 scanning electron microscope (Hitachi) at an accelerating voltage of 15kV.

3.5.2. Transmission electron microscopy

Co-cultured larvae were fixed in a 2% glutaraldehyde in 0.1M Sorensen phosphate buffer (pH 7.4) for 4 hrs at 4°C, washed overnight in 0.2M phosphate buffer and then post fixed for 1 hr at room temperature with 0.1% osmium tetroxide in 250 mM saccharose and 0.1 M phosphate buffer. The samples were then dehydrated in a series of graded ethanol solutions and embedded in an epon-araldite resin (Embed 812-Araldite 502, Electron Microscopy Sciences). Finally, 70 nm thick sections were made (Ultracut Reichert Jung) and mounted on 100-mesh collodion-coated copper grids prior to staining with 3% uranyle acetate in 50% ethanol and Reynold's lead citrate. Examinations were carried out on a transmission Hitachi HU12A electron microscope at an accelerating voltage of 75 kV (Figure 15).

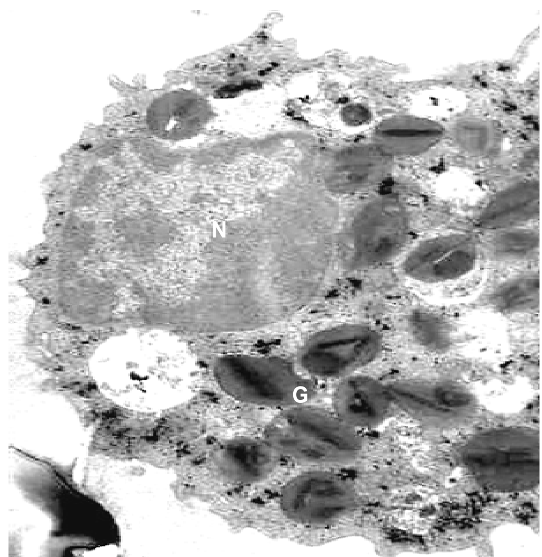


Figure 15. Transmission electron micrograph of an eosinophil. Note numerous granules with electron-dense central cores (Photo: C. Grisez)

3.6. Biochemical tests

3.6.1. Eosinophil peroxidase assay

Demonstration of the levels of most eosinophil granular products is a complex process, which often requires the use of radioimmunoassay. Here, a biochemical method was chosen

for eosinophil peroxidise (EPO) assay for its relative simplicity. EPO is a highly cationic protein that readily adheres to cell surfaces, which results in oxidant formation in close proximity to a pathogen or host cell (McCormick *et al.* 1994). Physiologically, eosinophils consume oxygen in a respiratory burst that produces superoxide and hydrogen peroxide. EPO catalyses the oxidation of halides (such as Br, I^- and the pseudohalide thiocyanate (SCN^-)) to their respective hypohalous acids in the presence of H_2O_2. However, a very closely related peroxidase, the myeloperoxidase (MPO) produced and stored by neutrophils frequently interferes with the EPO assay. Resolving such interference depends on the choice of halides such as Br^-, substrate pH (> 6) and use of specific inhibitors such as resorcinol (Varga *et al.* 2002; Schneider and Issekutz, 1996).

Accordingly, eosinophil-enriched leucocytes were prepared as described above. $5x10^6$ cells in 500 µl were lysed with 0.2% triton X-100 and stored at -80°C. Eosinophil peroxidase assay was performed according to the method described by (Adamko *et al.*, 2004) with slight modifications. Briefly, a substrate solution containing 3 mM o-phenylenediamine dihydrochloride (OPD), 6 mM KBr and 8 µM H_2O_2 was prepared in a 50 mM Hepes (EPO diluting buffer, pH 7) with (R^+) or without (R^-) 120 µM of the EPO-inhibitor, resorcinol. A 1:1 volume of the substrate solution and the cellular suspension was mixed and incubated in the dark at 23°C for 30 minutes. The reaction was stopped by adding 500 µl of 4 M H_2SO_4. 150 µl of the coloured solution was distributed in triplicate in a flat-bottomed 96-well micro-plate and the colour absorbance was read at 490 nm by a spectrophotometer. The absorbance for EPO was calculated as the difference between absorbance without resorcinol and absorbance with resorcinol (R^--R^+).

3.6.2. Serum pepsinogen assay

Following infections with abomasal parasites, a rise in the concentration of serum pepsinogen is a common phenomenon (Fox, 1997, Simpson 2000). Hence, serum pepsinogen concentrations were evaluated using the method described by Dorny and Vercruysse (1998). Briefly, the serum sample was acidified with HCl and incubated overnight at 37°C with bovine serum albumin (BSA). The reaction was stopped with 4% trichloro-acetic acid (TCA) and the mixture was centrifuged at 14000 rpm for 5 minutes. An aliquot from the supernatant

was added to 0.25 M NaOH and the plates were incubated at room temperature with folin reagent for 30 minutes. The liberated tyrosine was estimated by reading the absorbance at 680 nm and the values were expressed as unit (U) or milliunit (mU) tyrosine/litre of serum.

TROISIEME PARTIE

RESULTATS

Les travaux expérimentaux de ce travail de thèse ont été réalisés au sein de l'équipe « Exploration fonctionnelle du résistome des ruminants » de l'UMR INRA/ENVT 1225 (Interactions Hôtes Agents pathogènes). Ils ont pour but d'étudier l'implication des éosinophiles dans la régulation des populations d'*Haemonchus contortus* chez le mouton.

Ces résultats sont présentés sous forme d'articles regroupés en trois sections. Chaque section est précédée par une brève introduction.

PART III. RESULTS

The experimental works realised in the laboratory of Parasitology (UMR 1225, IHAP, INRA/Ecole National Vétérinaire de Toulouse: France) in the preparation of this thesis were destined to study the role of eosinophils in the regulation of *Haemonchus contortus* population in sheep.

Although, much remains to be done to elaborate the exact role of eosinophils in the immune response of sheep to helminth infections, the findings presented in this thesis are believed to provide additional input in the journey towards this goal. Parts of this work have already been published or accepted for publication in different journals, and others are either submitted for publication or under preparation. The manuscripts illustrating our findings are presented in three sections, each of them preceded by a brief summary.

1. DEMONTRER LA CAPACITE DES EOSINOPHILES SANGUINS DE MOUTONS A TUER LES LARVES INFESTANTES D'*H. CONTORTUS IN VITRO*

Le rôle protecteur des éosinophiles dans les infestations par des helminthes a été démontré à la suite d'études *in vitro* chez la souris. Chez le mouton, une seule étude (Rainbird *et al*. 1998) a montré, *in vitro*, un probable effet létal des éosinophiles (recrutés dans la mamelle de brebis) mis au contact de larves infestantes d'*H. contortus*. Dans cette étude, la mort des larves était suggérée par l'observation au microscope de leur immobilité. Toutefois, nous avons pu constater qu'il est très difficile de classer certaines larves dans l'une ou l'autre de ces deux catégories, mobile ou immobile. De plus, rien ne permet d'affirmer qu'une larve jugée mobile pourra effectivement s'implanter et se développer chez l'hôte. De même, une larve considérée comme immobile pourrait très bien poursuivre son développement une fois transplantée chez l'hôte. C'est pourquoi, dans cette étude, l'observation de la mobilité/immobilité larvaire a été associée, pour la première fois chez le mouton, à des études *in vivo* après transplantation chirurgicale dans l'abomasum d'agneaux receveurs naïfs.

Les animaux ayant reçu par voie chirurgicale des larves incubées préalablement avec des préparations cellulaires enrichies en éosinophiles ont montré une réduction significative de l'excrétion d'œufs dans leur matières fécales et une réduction drastique du nombre de vers installés en comparaison avec des animaux témoins (inoculés avec le même nombre de larves *per os* ou ayant reçu des larves préalablement incubées avec le milieu de culture, des neutrophiles ou des lymphocytes uniquement). L'étude *in vivo* a non seulement confirmé que l'immobilisation larvaire observée *in vitro* conduisait à une réduction de la capacité d'implantation chez l'hôte mais également que de nombreuses larves considérées comme « mobiles » *in vitro* avaient en fait perdu de leur virulence.

Nos résultats ont permis enfin de confirmer que les éosinophiles sanguins de moutons sont capables d'immobiliser (et de tuer) des larves infestantes *d'H. contortus* uniquement en présence du sérum de l'animal. Lorsque ce sérum est chauffé à 56°C pendant une heure, cet effet est presque totalement aboli suggérant un rôle important du complément dans les interactions entre éosinophiles et larve infestantes (Publication (N° 1) : **Getachew Terefe *et al*., 2007. *Veterinary Research*, 38: 647-654**).

2. DEMONSTRATION OF THE LARVAL-KILLING ABILITY OF SHEEP EOSINOPHILS *IN VITRO*

Based mostly on *in vitro* evidences of parasite killing in laboratory mice, eosinophils have been widely regarded as host-protective effector cells. The single *in vitro* study conducted by Rainbird *et al*. (1998) using eosinophils derived from sheep mammary glands infused with *H. contortus* antigens reveal that these cells are potentially lethal to the infective larvae of the parasite. In this study, parasite killing was judged only from the observation that certain proportion of larvae co-cultured with eosinophil-enriched suspensions remained immobile for three days. However, besides the difficulty in classifying larvae as mobile or immobile, the measurement of larval mobility in a cell-larvae culture medium may not always confirm the death of those that are 'immobile', or the ability of those larvae that are considered 'mobile' to develop after inoculation into their appropriate hosts.

In this study, *in vitro* tests were performed in which the mobility of larvae was assessed after incubation with blood eosinophils. As a confirmatory procedure, we were also interested to see the developmental potential of incubated larvae after surgical deposition into the abomasum in sheep.

Our result shows that sheep eosinophils are capable of immobilizing *H. contortus* infective larvae *in vitro*. Interestingly, compared to control animals, which were orally infected with non-incubated intact larvae, sheep receiving incubated larvae intra-abomasally (eosinophil + larvae) excreted significantly lower faecal eggs and harboured drastically reduced number of worms at necropsy. Hence, the *in vivo* experiment not only confirmed our *in vitro* observations but also demonstrated that the majority of those larvae which were considered mobile (live) *in vitro* were incapable of surviving further in the host. *In vitro*, eosinophils were more efficient in the presence of immune serum. Heating the serum significantly reduced their larval immobilizing potential suggesting a possible role of complement in the eosinophil-mediated larval killing (Publication (No.1) : **Getachew Terefe *et al*., 2007. *Veterinary Research*, 38: 647-654**).

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Short note

In vitro pre-exposure of *Haemonchus contortus* L3 to blood eosinophils reduces their establishment potential in sheep

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Abstract – Different authors have reported that eosinophils are capable of immobilising infective larvae of different species of nematodes in vitro. However, classifying larvae as mobile or immobile is so subjective that it does not always mean all apparently immobile larvae are dead or those that are mobile are capable of surviving further immune responses if administered to their natural hosts. The objective of this experimental study was therefore to substantiate the role of eosinophils in the killing of *Haemonchus contortus* infective larvae by comparing the infectivity in sheep of larvae that had been incubated with eosinophil-enriched cell suspensions with control larvae. Since it was not possible to isolate pure eosinophils from sheep blood, we were compelled to evaluate the effects of other blood cells contaminating our eosinophil-enriched suspensions. Although eosinophils and neutrophils were the only cells found adherent to *H. contortus* infective larvae in vitro, induced eosinophils in the presence of immune serum were primarily responsible for the drastic reduction in larval motility compared to the minor effects of neutrophils and mononuclear cells. Corresponding reductions in faecal egg count and worm numbers were observed when the incubated larvae were transferred intra-abomasally to sheep. Interestingly, the proportion of larvae that failed to establish was much higher following incubation with induced eosinophils compared with other cells or with immune serum alone. Although this study did not address the in vivo role of eosinophils in sheep, the results strongly indicate that sheep blood eosinophils have a larval killing potential in vitro, and a larval mobility test alone may not fully explain the level of damage inflicted on the larvae.

***Haemonchus contortus* / eosinophils / in vitro / intra-abomasal / sheep**

1. INTRODUCTION

Haemonchus contortus infection in sheep is known to elicit Th2 type immune responses characterised by the recruitment of a large number of eosinophils, mast cells and globule leucocytes and the production of local and circulating antibodies [1, 4, 8, 12]. It has been reported that eosinophils in the presence of immune

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serum were able to immobilise *H. contortus* infective larvae (L3) in vitro [9], but larval killing was suggested only from the observation that the larvae remained immobile for three days. Furthermore, in vivo studies have also described the close association between eosinophils and death of *H. contortus* larvae in the abomasal mucosa of sheep [2]. However, besides the difficulty in classifying larvae as mobile or immobile in an in vitro cell-larvae culture medium, larval motility tests may not always reflect the mortality or inability of those larvae to develop into adult stages if inoculated into their appropriate hosts. Therefore, the present study gives special attention to the capacity of *H. contortus* infective larvae, incubated with blood derived eosinophil-enriched cellular suspensions, to establish in a susceptible sheep. Since eosinophils cannot be completely purified from sheep blood, parallel examinations of the roles of neutrophils and mononuclear cells were also performed.

2. MATERIALS AND METHODS

Four 12 month-old Black belly sheep, raised helminth free indoors, were orally infected with 10 000 *H. contortus* (Humeau strain) infective larvae to become donors of induced eosinophils and immune serum. Blood samples were collected regularly and eosinophils were counted according to the method described previously [3] to judge when a reasonable proportion of eosinophils could be isolated.

The different types of blood leucocytes were isolated using the method adapted from techniques developed by Woldehiwet et al. [15]. Briefly, 30 mL of blood was taken from each Black Belly sheep, on day 15 post infection, in EDTA-coated tubes. After initial centrifugation, the plasma was discarded and the underlying buffy coat layer was carefully removed and washed. Only buffy coats with more than 92% mononuclear cells (LYP) were considered for the experiment. Residual red blood cells (RBC) and granulocytes (majority) were processed by haemolysis and subsequent centrifugation to obtain pellets free of RBC. Cells were resuspended in RPMI 1640 (Ref. 21875 GIBCO, Cergy-Pontoise, France) complete medium (supplemented with 1% penicillin-streptomycin and 10% FCS). After centrifugation of the pellets on a percoll density gradient (P1644-Sigma Diagnostics, St. Louis, USA, density: 1.090), cells at the percoll-RPMI interface (EOS) and the bottom-pellet (PMN) were aspirated separately and washed twice with 10 mL cell culture medium. Total and differential cell counts were performed using May Grünwald-Giemsa stain. Pellets containing less than 92% neutrophils were discarded while cells from the RPMI-percoll interface containing more than 40% eosinophils were considered for the in vitro culture. The viability of cells was verified using trypan blue staining. Serum samples were also collected for use in all in vitro tests.

H. contortus L3 were exsheathed using 10 µL of Milton Sterilising fluid containing 2% w/v sodium hypochlorite and 16% w/v sodium chloride (Milton Pharmaceutical LTD, Thouars, France). After the motility and exsheathement of the larvae were verified, three successive washes with physiological saline solution were performed at low speed centrifugation (250 *g*, 5 min and 20 °C) and the number was adjusted to 1500/mL in a sterile RPMI 1640 complete medium. Cell suspensions were classified as eosinophil-enriched (EOS: from RPMI-percoll interface), neutrophil-enriched (PMN: from the cell pellet) and lymphocyte-enriched (LYP: from the buffy coat layer), and 200 µL (i.e. 3×10^5 cells/well) of the suspension in RPMI 1640 complete medium were deposited in triplicate in a 24 well test plate

(Ref. 92024, TPP, Geneva, Switzerland) with or without 100 μL of immune (homologous) whole/decomplemented serum (heat-inactivated at 56 °C, 1 h). In addition, larvae were also incubated with immune serum (SER), and in culture medium only as a control. Finally, 300 exsheathed *H. contortus* L3 in 200 μL of RPMI 1640 were added and the plates were incubated overnight (18 h) at 37 °C, 5% CO_2.

After 18 h of culture, the motility of 100 larvae was examined directly in the culture plates using an inverted microscope. Larvae were considered mobile if any part of the body was seen moving. An aliquot was taken in a different culture plate from each representative well and stained for three hours using equal volume of eosin Y (Ref. 6766009, Thermo Electron Co., USA) to identify the types of cells (especially eosinophils) adhering to the larvae. Co-cultured larvae were also fixed in glutaraldehyde, dehydrated in ethanol and coated with gold palladium (scanning electron microscopy) or embedded in resin, sectioned and stained (transmission electron microscopy) according to standard procedures for electron microscopic imaging.

After being evaluated in vitro, the contents of the culture plates were pooled according to cell types and left overnight at 37 °C mixed with the same volume of distilled water to remove cells. The tubes were then centrifuged at low speed and the number of larvae collected was adjusted to 400 L3/mL in distilled water. Twenty-five four-month old INRA 401 lambs, reared parasite free, were allocated to five groups of five animals. Control lambs (group POS) received 800 non-exsheathed L3 while the remaining four groups received the same number of exsheathed larvae incubated with the following: cellular suspension enriched for induced eosinophils (group EOS), neutrophils (group PMN) or lymphocytes (group LYP) in the presence of immune serum or with serum in a culture medium without cells (SER). Except for the POS group which was orally infected, direct intra-abomasal injections in lambs of the other four groups were performed under general anaesthesia. Faecal egg counts were performed at regular intervals between days 15 and 28 post infection by the modified McMaster technique [10]. The lambs were killed on day 28 by intravenous barbiturate. Abomasal contents and washings were collected and the worms from a 10% aliquot were counted and classified according to sex and development. The lengths of 20 adult female worms randomly picked from each lamb were measured and the number of eggs in utero was determined as previously described [14]. Faecal egg count kinetics were compared between the five groups by analysis of variance with repeated values (SYSTAT software). Comparisons between total worm counts, worm lengths and eggs in utero were performed with the non-parametric Kruskall-Wallis test (SYSTAT software) while the proportions of mobile larvae in different culture media were compared using a chi square test.

3. RESULTS

Following *H. contortus* infection of the Black Belly sheep, peak blood eosinophilias were attained between days 9 and 16 post infection (PI). Based on previous observations, day 15 PI was chosen as an appropriate time to isolate induced eosinophils (43–63%). Percentage ranges of neutrophil- and mononuclear cell-enriched suspensions were 92–96% and 92–99% respectively, and viability was between 94 and 96% for all cell types.

Control wells (larvae + culture medium) had 93% actively motile larvae after 18 h of incubation. Eosinophil-enriched cellular suspensions in the presence of immune serum were the most powerful in immobilising the larvae of *H. contortus* ($P < 0.01$)

Table I. Percentages of motile larvae after culture with immune serum and cell suspensions enriched for eosinophils (EOS), neutrophils (PMN) or mononuclear cells (LYP), or with immune serum alone (SER). Values are presented as means and standard deviations for four blood donor animals. Cells from each donor sheep were treated separately for the in vitro tests.

	EOS	PMN	LYP	SER	POS
% Motile larvae (estimated on 100 larvae)	49 ± 4[a]	74 ± 7[b]	82 ± 4[b]	89 ± 4[b]	–

Values in different letters are statistically different ($P < 0.05$).

followed by neutrophils to some extent and mononuclear cells to a lesser degree (Tab. I). Staining with eosin Y revealed that larvae in the eosinophil- and neutrophil-enriched suspensions were coated with large numbers of cells (Fig. 1A) while those in the mononuclear cell suspensions had very few or no adherent cells (data not shown). Indeed, maximal cell adherence was observed in the first few hours of culture while enhanced larval immobility was seen only after overnight incubation. Lack of further cell adhesion after overnight incubation may indicate cell degeneration or apoptosis. In all cases, heat inactivation of immune serum abolished cell adherence as well as their larval immobilising ability but larval motility was not significantly affected by the presence of serum alone (SER: data not shown). Scanning electron microscopy revealed the close apposition of eosinophils to the larval surface (Fig. 1B) while in transmission electron microscopy, eosinophils had their pseudopodia-like cytoplasmic extensions (Fig. 1C) filled with an electron dense material (Fig. 1D) penetrating into the normally sealed striae of the larval cuticle.

Lambs that received larvae incubated with eosinophil-enriched cells in the presence of immune serum (group EOS) excreted significantly fewer eggs than all the other groups ($P < 0.01$), none of which differed significantly from each other (Fig. 2). Fewer worms (only 12%) were recovered from the EOS lambs compared to the other groups ($P < 0.05$, Tab. II). All recovered worms were adults

Table II. Individual worm counts after intra-abomasal inoculation or oral administration of larvae. Groups EOS, PMN, LYP and SER received exsheathed L3 (800/animal) intra-abomasally while the control group (POS) was infected orally with the same number of larvae. Co-cultured larvae were pooled according to cell type for the intra-abomasal infection.

	EOS	PMN	LYP	SER	POS
Worm counts for each animal	7	760	62	120	475
	136	476	400	633	329
	69	69	405	787	543
	227	640	624	580	513
	45	674	396	378	587
Mean	97[a]	524[b]	377[b]	500[b]	489[b]
SD	87	274	132	258	98

Values in different letters are statistically different ($P < 0.05$).

and there was no difference in either female length or number of eggs in utero between groups (data not shown).

4. DISCUSSION

In this study, we were able to obtain 43 to 63% of eosinophils with the major contaminants being lymphocytes. In spite of repeated attempts to optimise the isolation method, we did not attain previously reported levels of purity [15] probably because of the difference in the sheep breed used. Our efforts to isolate eosinophils by the immunomagnetic method [5] have also failed probably due to lack of recognition of sheep neutrophils by human anti-CD16 antibody. However, the 92–99%

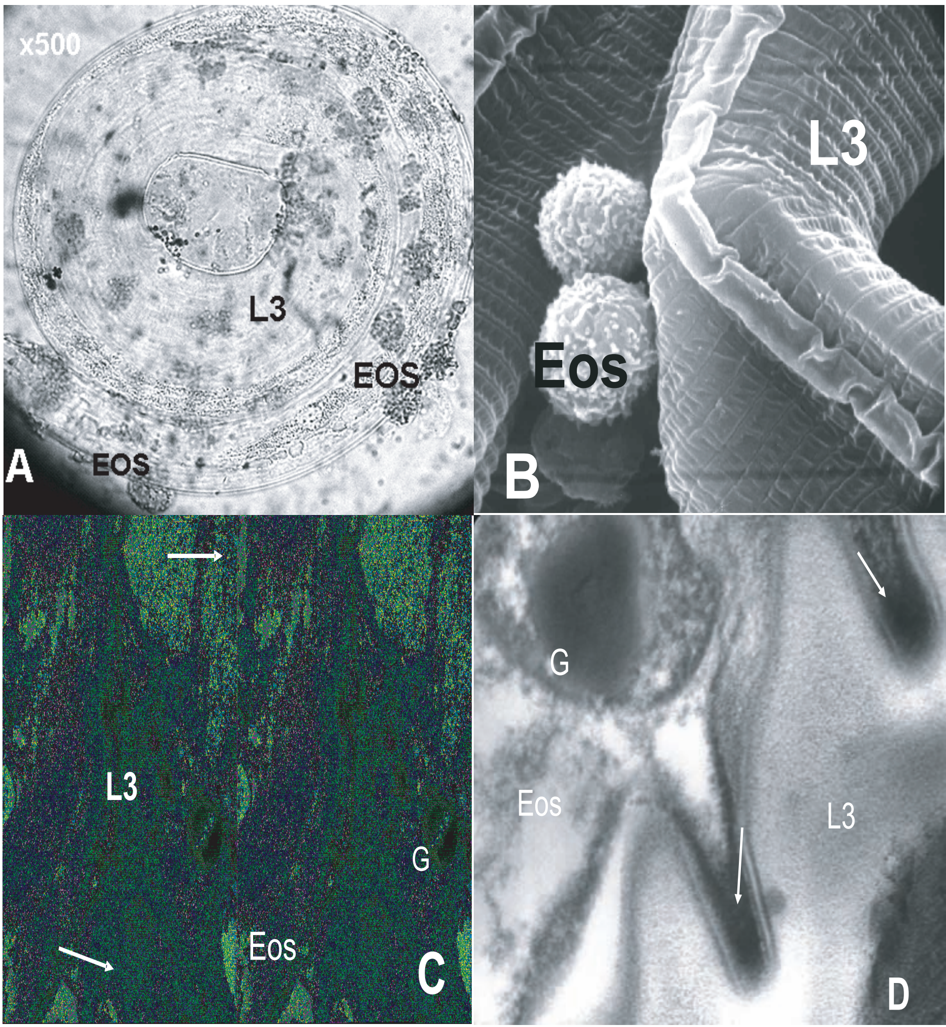

Figure 1. (**A**) Eosinophils adherent to larvae in eosinophil-enriched culture medium (eosin Y stain). (**B**) Scanning electron micrograph showing cells apparently eosinophils (Eos) attached to the striated larval surfaces. (**C**) Transmission electron micrograph showing one eosinophil with partially degranulated granule (G) and cytoplasmic processes penetrating (arrows) into the cuticular striae of the larva. (**D**) An electron dense material fills the ends of the processes (arrows) in contact with the larval surface.

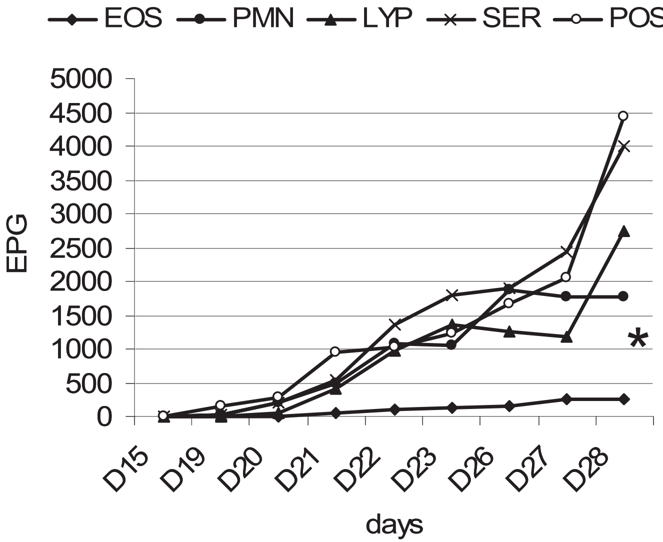

Figure 2. Kinetics of *H. contortus* faecal egg count in four groups of lambs infected by intra-abomasal inoculation of 800 L3 incubated with the following: eosinophil-enriched (EOS), neutrophil enriched (PMN) or mononuclear cell-enriched (LYP) suspensions in the presence of immune serum or with serum alone (SER). A fifth or control group (POS) received the same number of larvae per os. * $P < 0.01$ for group EOS versus other groups.

neutrophils isolated with this technique was close to those found by other authors [15] and the mononuclear cell suspensions were almost free of granulocytes. The motility of larvae incubated for 18 h with eosinophil-enriched suspensions was drastically reduced while the effects of neutrophils and mononuclear cells were much lower. Similar findings were reported using *Strongylus vulgaris* incubated in blood from eosinophilic ponies [7]. The effect of eosinophils on *H. contortus* larval motility was only obvious when immune serum was added and heat inactivation of the serum abolished this effect. Furthermore, as revealed by light and electron microscopy, the eosinophils showed a very intimate contact with these larvae in the presence of immune serum. Although the role of antibody and other serum components cannot be ruled out, it seems very likely that the presence of complement in the culture was essential for maximum adherence and immobilisation of larvae by eosinophils supporting the findings of Rainbird et al. [9]. Similar in vitro findings were also reported in the study with *Nippostrongylus brasiliensis* in mice [13]. Moreover, by using *Strongyloides stercoralis* L3 in diffusion chambers implanted in immunised mice, Rotman et al. [11] have demonstrated that the majority of the cells recruited towards the larvae are eosinophils and maximal cell number coincides with parasite killing. In this study, it was suggested that direct contact between the cells and the L3 is required for larval killing. Whether this holds true in a classical in vivo condition is not yet clearly demonstrated. Indeed, on the contrary to what has been observed here, there are reports where mice treated with anti-IL-5 mAb exhibit highly reduced numbers of eosinophils but normally reject infections

of helminth parasites [6]. In our study, we also indicate that neutrophils are capable of adhering to the larval surface and hence one can not undermine the contribution of these and other contaminating cells although larval motility was only minimally reduced.

In agreement with our in vitro observations, group EOS lambs excreted significantly fewer *H. contortus* eggs and harboured fewer worms than the remaining four groups where any inter-group differences were not significant. Obviously, since female length and number of eggs in utero were identical in the five groups, the difference in egg excretion was explained by the difference in total worm burden between groups. Interestingly, most (75%) of the apparently motile eosinophil treated larvae did not establish indicating that eosinophils are far more potent than can be shown by in vitro larval motility tests. The larvae were so weakened by the action of eosinophils that they might have been easily expelled before they established.

To our knowledge, this is the first study that combines in vitro larval immobilisation tests with the subsequent in vivo development of the same larvae in sheep. Although this study provides no data on the in vivo role of eosinophils, the in vivo experiment not only confirmed our in vitro observations but also demonstrated that the majority of those larvae that were considered mobile (alive) in vitro were incapable of surviving further in the host.

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3. EFFETS D'UNE AUGMENTATION OU D'UNE REDUCTION ARTIFICIELLE DE LA REPONSE EOSINOPHILIQUE SANGUINE SUR LA RESISTANCE DU MOUTON A *H. CONTORTUS*

2.1. Augmentation de la réponse éosinophilique sanguine à *H. contortus* lors de co-infection avec *Oestrus ovis*.

Les larves d'*Oestrus ovis* (Diptera : Oestridae) causent une réaction inflammatoire intense dans les voies respiratoires supérieures. Les moutons infestés naturellement ou expérimentalement avec ce parasite développent une importante éosinophilie sanguine et tissulaire (Khanh *et al.* 1996 ; Yacob *et al.* 2002 ; Tabouret *et al.* 2003). Une infestation expérimentale par des larves d'*Oestrus ovis* a donc été utilisée pour induire une hyperéosinophilie sanguine pendant cinq semaines avant l'administration de larves infestantes d'*H. contortus*. Un autre groupe d'animaux n'a reçu que l'infestation par le nématode. Comme attendu, l'éosinophilie sanguine a considérablement augmentée dans le groupe infesté par les larves du Diptère. L'intensité d'excrétion d'œufs, le développement des vers et la fécondité des femelles ont été considérablement diminués dans le groupe co-infesté en comparaison avec le groupe infesté par le nématode seulement. De plus, les paramètres parasitologiques étaient corrélés à la réponse éosinophilique sanguine et tissulaire ainsi qu'à la mastocytose de la muqueuse abomasale. Nous suggérons que la présence d'une forte éosinophilie sanguine au moment de l'infestation par le nématode a contribué à une résistance accrue chez les animaux co-infestés même si le rôle complémentaire des mastocytes ou d'autres facteurs ne peut pas être écarté (Publication (N° 2) : **Terefe *et al.,* 2005. *Veterinary Parasitology*, 128 : 271-283**).

2. EFFECT OF ARTIFICIALLY ENHANCING OR REDUCING BLOOD EOSINOPHILIC RESPONSES ON THE LIFE TRAITS OF *H. CONTORTUS* IN SHEEP

2.1. Enhancing host's eosinophilic response to *H. contortus* by co-infection with *O. ovis* larvae

Oestrus ovis larvae cause a serious inflammatory reaction in the upper respiratory tract. Sheep naturally or artificially infected with these larvae are known to mount high level of tissue and blood eosinophilia (Khanh *et al*., 1996; Yacob *et al*. 2002; Tabouret *et al*., 2003). Based on this information, *O. ovis* larvae were used to induce elevated blood eosinophilia for five consequetive weeks before *H. contortus* administration. This was compared mainly with sheep infected with *H. contortus* only. As expected, blood eosinophilia was tremendously increased after nasal-larvae administration. FEC, worm development, and fecundity were significantly reduced in the presence of *O. ovis* and this was correlated to blood and tissue eosinophilia and mastocytosis. We suggest that the presence of high blood eosinophilia at the time of nematode infection has contributed to the resistance of the animals although the supportive role of mast cells and other factors could not be ruled out (Publication (No. 2): **Terefe *et al*., 2005. *Veterinary Parasitology*, 128, 271-283).**

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Haemonchus contortus egg excretion and female length reduction in sheep previously infected with *Oestrus ovis* (Diptera: Oestridae) larvae

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Abstract

Mixed parasitic infection of animals is a common phenomenon in nature. The existence of one species often positively or negatively influences the survival of the other. Our experimental study was started with the objectives to demonstrate the interaction of *Haemonchus contortus* and *Oestrus ovis* in relation to cellular and humoral immune responses in sheep. Twenty-two sheep of Tarasconnais breed (France) were divided into four groups (O, OH, H and C) of five or six animals. Group O and OH received 5 weekly consecutive inoculations with *O. ovis* L1 larvae (total = 82 L1) in the first phase of the experiment between days 0 and 28. On the second phase, groups OH and H received 5000 L3 of *H. contortus* on day 48 while group C served as our control throughout the experimental period. Parasitological, haematological, serological and histopathological examinations were made according to standard procedures and all animals were slaughtered at day 95. There was no significant variation in the number and degree of development of *O. ovis* larvae between the two infected groups. Furthermore, in tissues examined in the upper respiratory tract (nasal septum, turbinate, ethmoide and sinus), group O and OH has responded similarly on the basis of cellular inflammatory responses (blood and tissue eosinophils, mast cells and globule leucocytes (GL)) and serum antibody responses against the nasal bots. This may indicate that the presence of *H. contortus* in the abomasa of group OH had no marked influence over the development of *O. ovis* larvae in the upper respiratory tract. On the other hand, we have observed a significantly lower *H. contortus* female worm length, fecal egg count (FEC) and in utero egg count in animals harbouring the nasal bot (OH) than in the mono-infected group (H). This was significantly associated with higher blood eosinophilia, higher packed cell volume (PCV) and increased number of tissue eosinophils and globule leucocytes. We conclude that, the establishment of *O. ovis* larvae in the upper respiratory tract has initiated higher inflammatory cellular activity in group OH there by influencing the development and fecundity of *H. contortus* in the abomasum.
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Keywords: *Oestrus ovis*; *Haemonchus contortus*; Mixed infection; Inflammatory cells; Serology; Immune response

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1. Introduction

In the field, domestic animals are exposed to a multiplicity of parasites among which are gastrointestinal nematodes that cause considerable animal health problem in many parts of the world. *Haemonchus contortus*, a blood feeding trichostrongyle of the abomasum, is a widely spread nematode infecting ruminants (Miller et al., 1998; Tembely et al., 1997; Waller et al., 2004).

It is known that in many countries, helminths and myiasis agents are sympatric. One of the myiasis agents affecting sheep and goats in both tropical and Mediterranean regions is the dipteran fly, *Oestrus ovis* (Dorchies et al., 2000; Murguia et al., 2000; Papadopoulos et al., 2001; Yilma and Dorchies, 1991). *O. ovis* larvae, commonly known as nasal bots, cause variable degree of inflammatory reactions in the upper respiratory tract. Sheep naturally or artificially infected with these larvae were known to mount high level of tissue and blood eosinophilia (Khanh et al., 1996; Tabouret et al., 2003).

In most cases of natural infections, animals harbour more than one parasite species of the same or different order or genera (Clark, 2001; Cox, 2001). There are evidences that demonstrate the existence of synergistic or antagonistic effects between two or more parasites in a given host (Dobson et al., 1992; Onah and Wakelin, 1999). Immunity against gastrointestinal nematodes can be manifested as expulsion of adult parasite, reduction of worm length, decreased female worm fecundity, failure of infective larvae to establish and arrested development of larvae (Onah and Nawa, 2000; Douch et al., 1996b).

Recently, it has been demonstrated that mixed infection with *O. ovis* larvae and some strongyle species in sheep, in spite of the site of infection and their very distant biology, has favourably enhanced inflammatory responses of the animals to the nematodes. In this regard, Yacob et al. (2002) have reported that sheep harbouring *O. ovis* larvae shed lower number of *Trichostrongylus colubriformis* eggs per gram. Furthermore, the number and length of adult worms collected after autopsy were also significantly lower in the former than in the later category. This suggests an enhanced rejection and stunted development of the parasites in previously *O. ovis*-infected sheep. Moreover, Dorchies et al. (1997) noted a significant difference in *H. contortus* egg output between sheep harbouring *O. ovis* larvae and those without it. However, in the latter case, data regarding the involvement of local inflammatory cells and serum *H. contortus*-specific antibody are lacking. Furthermore, the evaluation of important parasitological parameters like adult worm measurement and in utero egg counts was not done.

The objectives of this experimental study were therefore to confirm the negative interaction between *O. ovis* and *H. contortus* and to generate new information regarding the local cellular reactions, serum *H. contortus*-specific antibody responses and degree of worm development and fecundity.

2. Materials and methods

2.1. Experimental animals

Twenty-two 3–4 months old sheep of Tarasconnais breed with body weights between 25 and 30 kg were used in this experiment. The animals were raised indoors under worm free conditions. They were divided into four groups of five/six animals namely, groups O (six lambs), OH (six lambs), H and C (five lambs each) (Table 1). Each group was housed separately and lambs were provided with free access to roughage feed with sufficient quantity of concentrate mix. For 8 days before the start of the experiment, blood and faecal samples were collected and the animals were treated with oxfendazole (Synanthic, MERIAL SAS) at a dose of 5 mg/kg body weight.

2.2. Parasites and experimental infections

L1 larvae of *O. ovis* have been collected from heads of freshly slaughtered sheep at the days of experimental infections. The larvae were carefully picked up, washed

Table 1
Experimental design

Group	Days of infection with *O. ovis* L1 and *H. contortus* L3						
	0	7	14	21	28	48	95
O	22 L1	20 L1	14 L1	20 L1	6 L1		Necropsy
OH	22 L1	20 L1	14 L1	20 L1	6 L1	5000 L3	
H						5000 L3	

with physiological saline solution, and examined for their viability. Sheep in Groups O and OH received equal number of L1 *O. ovis* larvae (82 L1) on a weekly basis for 5 consecutive weeks. The larvae were deposited with 1 ml of saline water in both right and left nostrils (Yilma, 1992). Following the last infection with *O. ovis*, infective larvae (L3) of *H. contortus* (Humeau strain) obtained from Cabaret J., INRA (Tours-Nouzilly) were orally inoculated to sheep in group OH and H at a dose of 5000 L3/animal at D48. Animals in group C were left uninfected until the end of the experiment and served as negative controls.

2.3. Parasitological examinations

2.3.1. Faecal egg count

Egg counts were performed on fecal samples collected twice a week from D60 to D95, according to the modified McMaster technique (Raynaud, 1970).

2.3.2. Worm counts and female fecundity

At the end of the experiment (D95), all sheep were killed humanely. Each head was split into two on the midline. *O. ovis* larvae were recovered after careful dissection of the septum, turbinate, ethmoide and frontal sinus. The larvae were counted and the different larval stages were identified as L1, L2 or L3.

The contents and washings of the abomasum were collected and passed through a sieve (40 μm) to conserve the coarse materials. The abomasum was digested in pepsin–hydrochloric acid solution (37 °C, 6 h) to collect the tissue dwelling nematode stages. The contents have been preserved in absolute alcohol. The volume of the material was then adjusted to 1 l and worms were counted in a 10% aliquot and classified as adult male and female, immature male and female or L4 stages. Furthermore, 20 adult female worms were randomly picked up from each sample for total length measurement and egg counts eggs in utero. For this purpose, individual female worms were allowed to disintegrate using sodium hypochloride 4% solution (Kloosterman et al., 1978) and all eggs liberated from the uterus were counted for each worm.

2.4. Blood and serum evaluation

Blood samples were collected twice a week for eosinophil counts and packed cell volume (PCV) determination, and once a week for serum antibody and pepsinogen quantification.

2.4.1. Blood eosinophils count

Hundred microliters of heparinised blood was mixed in 900 μl of eosin Y (Carpenter's solution) and 10 μl of the mixture was filled into a FAST READ 102 counting chamber (ISL, UK) and eosinophils were counted in the grids containing 1 μl of the mixture (Dawkins et al., 1989).

2.4.2. Evaluation of PCV

Heparinised blood was filled into capillary tubes and centrifuged at 2000 rpm for 2 min. The packed cell volume was registered as the percentage of whole blood volume.

2.4.3. Serum antibody (IgG) measurement (ELISA)

2.4.3.1. Antigen preparation. *O. ovis* L2 larval crude antigen extract was prepared according to the method described by Yilma (1992). Briefly, freshly collected larvae were washed, homogenised with phosphate buffered saline (PBS) (pH 7.2). The extract was centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatant was taken and its protein concentration was determined (Lowry et al., 1951).

Adult *H. contortus* worms were incubated at 37 °C, 5% CO_2 overnight in PBS and antibiotic solution. Excretion–secretion products from the worms were collected and centrifuged (10,000 × *g*, 30 min, 4 °C), their protein concentrations was determined as above and the supernatant was stored at −70 °C until use.

2.4.3.2. ELISA procedures. Enzyme-linked immunosorbent assay (ELISA) against *O. ovis* L2 antigen was made, as described previously (Papadopoulos et al., 2001). Each well of the ELISA microplates (Nunc, Denmark) were coated with 100 μl of the antigen at 2 μg/ml dilution in carbonated buffer (pH 9.6). The plates were incubated at 37 °C for 1 h followed by overnight incubation at 4 °C. They were washed three times with PBS–Tween (pH 7.2) and filled with 200 μl of 10% skimmed milk and incubated for 30 min at 37 °C. After emptying the plates, 100 μl of serum sample diluted at 1:200 was filled into the wells in triplicates and incubated at 37 °C for 1 h followed by washing with PBS–Tween. The microplates were incubated with 100 μl of peroxidase-conjugated anti-

sheep immunoglobulin G (Sigma, Ref. A3415) at a dilution of 1:1000 for 1 h. After washing three times, the reaction was revealed by incubating the plates with 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid, Sigma, USA, Ref. A9941) for 1 h at 37 °C. Optical densities (OD) of the test plates were read at 405 nm using an ELISA reader (Dynatech) and results were expressed as percentage of the OD of a positive reference serum.

Serum *H. contortus*-specific antibody level was determined according to the method previously described by Schallig et al. (1995a). ES antigen was used at 2 μg/μl while serum samples were diluted at the rate of 1:100 in 5% skimmed milk. ELISA procedures were similar as above. Except for the following minor details: the plates were directly incubated at 4 °C overnight, 5% skimmed milk was incubated for 1 h and anti-sheep IgG conjugate was diluted in PBS–Tween instead of carbonated buffer.

2.4.3.3. Serum pepsinogen. Serum pepsinogen concentrations were evaluated using the method described by Dorny and Vercruysse (1998). Briefly, the serum sample was acidified with HCl and incubated overnight at 37 °C with bovine serum albumin (BSA). The reaction was stopped with trichloro-acetic acid (TCA) 4% and the mixture was centrifuged at 14,000 rpm for 5 min. An aliquot from the supernatant was added to 0.25 M NaOH and the plates were incubated at room temperature with folin reagent for 30 min. The liberated tyrosine was estimated by reading the absorbance at 680 nm and the values were expressed as mU tyrosine/l of serum.

2.5. Histopathological examinations

Tissue samples were collected from four different parts of the upper respiratory tract (septum, turbinate, ethmoide and sinus) and two areas of the abomasum (fundus and pylorus). They were preserved in 10% buffered formalin or Carnoy's fixative. The samples were paraffin embedded and 5 μm sections were stained with hemalun and eosin for the examination of globule leucocytes (GL) and eosinophils in the abomasal mucosa. Eosinophils and globule leucocytes in the respiratory tissues where selectively stained by carbol chromotrope (Lendrum, 1944), while mast cells were stained with alcian blue for all the tissues (Enerback, 1966). Eosinophils, mast cells and globule leucocytes were counted at 400× magnification in 10 different optical fields.

3. Statistical analysis

Kinetics of egg excretions, blood eosinophils counts, packed cell volumes, serum pepsinogen values and *O. ovis* or *H. contortus*-specific IgG in serum were compared between the four groups of lambs using analysis of variance with repeated values (SYSTAT software). Comparisons of the numbers of *O. ovis* larvae, *H. contortus* worm populations, and eosinophils, mast cells and globule leucocytes in mucosae were performed with a non-parametric test, Kruskall–Wallis (SYSTAT software).

4. Results

4.1. Parasitological data

Group O and group C lambs were negative for *H. contortus* throughout the experimental period. A great individual variation in the *O. ovis* establishment rate was observed in both infected groups (O and OH). Among the larvae recovered, the proportion of L1 was 88 and 88.6% for groups O and OH, respectively, while L2 and L3 accounted only for 4.9–6.5% of the larval population in both groups. Therefore no significant difference in the total number and development of the larvae could be found (Table 2).

H. contortus eggs appeared in faeces on the third week (D67) of the experimental infection. From D74 to D95, the fecal egg count (FEC) was quite constant in group OH, but showed large time variations in group H (Fig. 1). Significant differences were observed between groups H and OH from day 67 to the end of the experiment ($P < 0.01$) with lower FEC in group OH. There was no significant variation in the total number of worms recovered from the abomasa of both groups (Table 2). Male and female proportions were also similar (group OH: 49 and 51%, group H: 48 and 52% male and female, respectively) while very few immature or L4 stages were observed in both groups (data not shown). By contrast, female worm length and in utero egg counts per female in group OH

Table 2
Establishment rate of *O. ovis* and *H. contortus* larvae and length and fecundity of female *H. contortus*

	Group (O)	Group (OH)		Group (H)
	O. ovis 82 L1	*O. ovis* 82 L1	*H. contortus* 5000 L3	*H. contortus* 5000 L3
% Establishment	19.1 ± 17	35.4 ± 24	58 ± 17	60 ± 9
Fecundity (egg/female)			535 ± 95	803 ± 143
Female worm length (mm)			20.79 ± 1.2	23.05 ± 0.5

were significantly lower than that of group H ($P < 0.05$).

4.2. Blood parameters

4.2.1. Blood eosinophils count

The number of blood eosinophils in the control group was low throughout the experiment (Fig. 2). Eosinophilia was a very prominent phenomenon in animals infected with *O. ovis* larvae. The number of blood eosinophils attained a peak value at the third week of *O. ovis* infection period (D22) then decreased until day 42 despite further infection with L1 larvae. Blood eosinophilia was also characteristic of *H. contortus* infection and peak values were seen on D60 for group OH and D64 for group H. Towards the end of the experiment, a gradual fall in eosinophilia approximately to the control level was seen in all three groups. Between group differences (both before and after infection with the nematode) were highly significant ($P < 0.001$). During infection with the nematode, higher number of blood eosinophils were observed in group OH than in group H ($P < 0.001$).

4.2.2. Packed cell volume

The development of anaemia was compared to the PCV value (100%) registered 2 days before the day of infection with *H. contortus* (D46). The PCV declined from D64 until the end of the experiment (Fig. 3) in *H. contortus*-infected groups. PCV values of group OH remained significantly higher than that of group H ($P < 0.01$).

4.2.3. Serology

The kinetics of serum antibody levels against antigens of *O. ovis* and *H. contortus* are shown in Fig. 4a and b, respectively. The mean serum *O. ovis*-specific IgG for group OH reached the positive threshold (% OD = 20) at day 39, followed by a peak on day 81 after the first inoculation. Although the difference was not statistically significant, development of antibody in group O was very slow and remained below that of group OH. Due to low values for group H and C, between group differences were found highly significant ($P < 0.01$). On the other hand, the level of serum *H. contortus*-specific IgG increased 2 weeks after inoculation and gradually

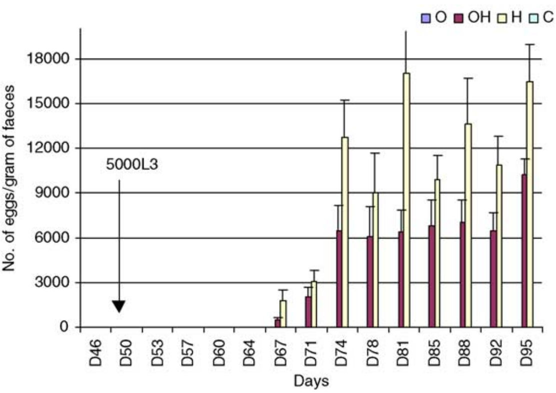

Fig. 1. Kinetics of fecal egg counts in sheep concurrently infected with both *O. ovis* and *H. contortus* and those infected with *H. contortus* alone.

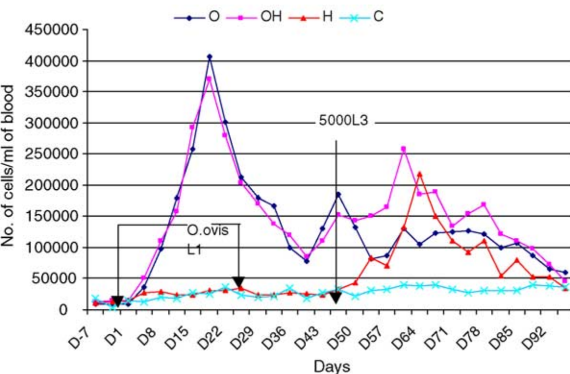

Fig. 2. Development of blood eosinophilia in response to infections with *O. ovis* and/or *H. contortus*. Five weekly consecutive inoculations with *O. ovis* L1 have been made from D0 to D28.

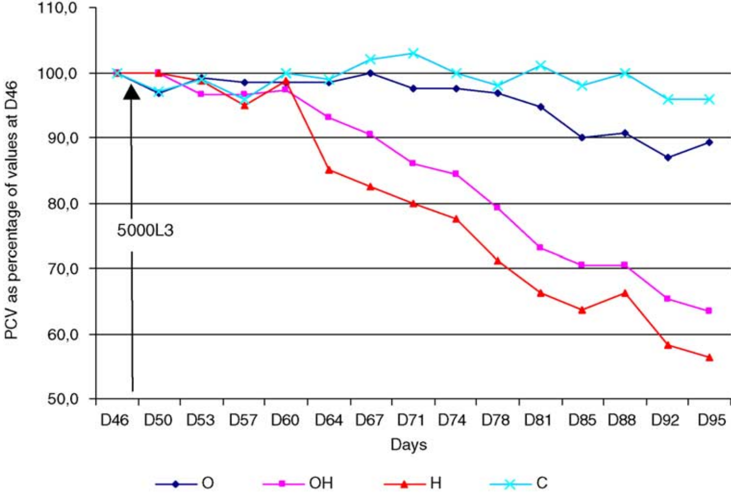

Fig. 3. Blood packed cell volumes in response to infections with *O. ovis* and/or *H. contortus*. Values are presented as percentage of the mean PCV 2 days before the date of infection (D46) with *H. contortus* L3.

reached a peak at the end of the experiment for both infected groups. Control animals had significantly lower values ($P < 0.01$) while there was no significant difference in serum-specific IgG between groups OH and H.

4.2.4. Serum pepsinogen

There was a gradual but progressive increase in serum pepsinogen level of group OH until the end of the experiment (Fig. 5). In group H, a sharp rise in serum pepsinogen level was seen from D53 to D67 followed by a rapid drop to low values (D74–95). No significant difference was shown between the two *H. contortus*-infected groups.

4.2.5. Histopathology

O. ovis infections were followed by a large cellular recruitment (eosinophils, mast cells and globule leucocytes) in the mucosa of the upper respiratory tract, but no differences were recorded between the two groups, O and OH. Interestingly, the presence of *O. ovis* larvae led to a slight, but significantly different, mucosal eosinophil and mast cell reaction in the fundus and pylorus compared to controls (Fig. 6).

H. contortus-infected lambs showed high numbers of eosinophils (mucosal and intraepithelial), mast cells and globule leucocytes in the fundic and pyloric mucosae. Significant differences ($P < 0.05$) were observed for mucosal eosinophils in the fundus and globule leucocytes in pylorus between groups OH and H. By contrast, *H. contortus* infection alone was not associated with an inflammatory process in mucosae of the upper respiratory tract.

4.2.6. Correlations between the measured variables

Coefficients of correlation in groups OH and H were calculated for the values of different parameters examined at D95 (Table 3). Blood eosinophilia and tissue cellular reactions have shown strong correlations with the various parasitological parameters. In utero egg, count was negatively associated with globule leucocytes in the pylorus ($r = -0.88$) and blood eosinophils ($r = -0.57$). Similarly, number of worms in both groups was negatively correlated with GL in the fundus and number of mast cells in the fundus and pylorus. Female worm length was strongly associated with in utero egg count ($r = 0.79$) and moderately with FEC ($r = 0.53$).

5. Discussion

This experiment was performed with the objectives to confirm the negative effects of *O. ovis* infection on the subsequent development of *H. contortus* in sheep and to investigate the factors associated with this

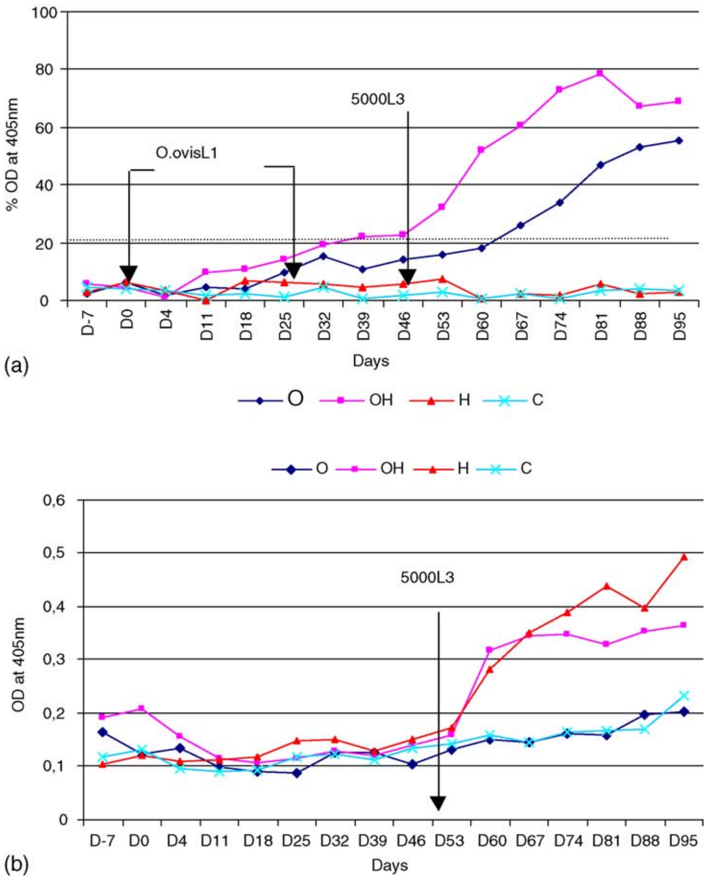

Fig. 4. Evolution of serum-specific antibody (IgG) levels against *O. ovis* (a) and *H. contortus* (b) in sheep experimentally infected with one or both parasites.

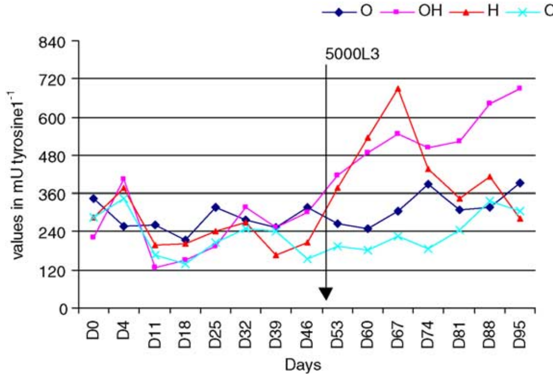

Fig. 5. Weekly serum pepsinogen values in mU tyrosine/l of serum. Groups OH and H were inoculated with 5000 L3 of *H. contortus* on D48 of the experimental period.

concurrent infection. There was no marked difference in the number and development of *O. ovis* larvae collected in the two groups O and OH. This was further supported by the presence of similar values of inflammatory cells and serum-specific IgG levels against the nasal bot. Hence, it is assumed that the presence of *H. contortus* in the abomasa of animals in group OH had no notable influence on the development of *O. ovis* in the upper respiratory tract. This was in agreement with other similar works carried out with *O. ovis* and *T. colubriformis* (Yacob et al., 2002, 2004). On the other hand, it was noticed that higher numbers of eosinophils and globule leucocytes were recruited into the abomasa of animals in group O compared to control animals. Similarly, the number of these cells

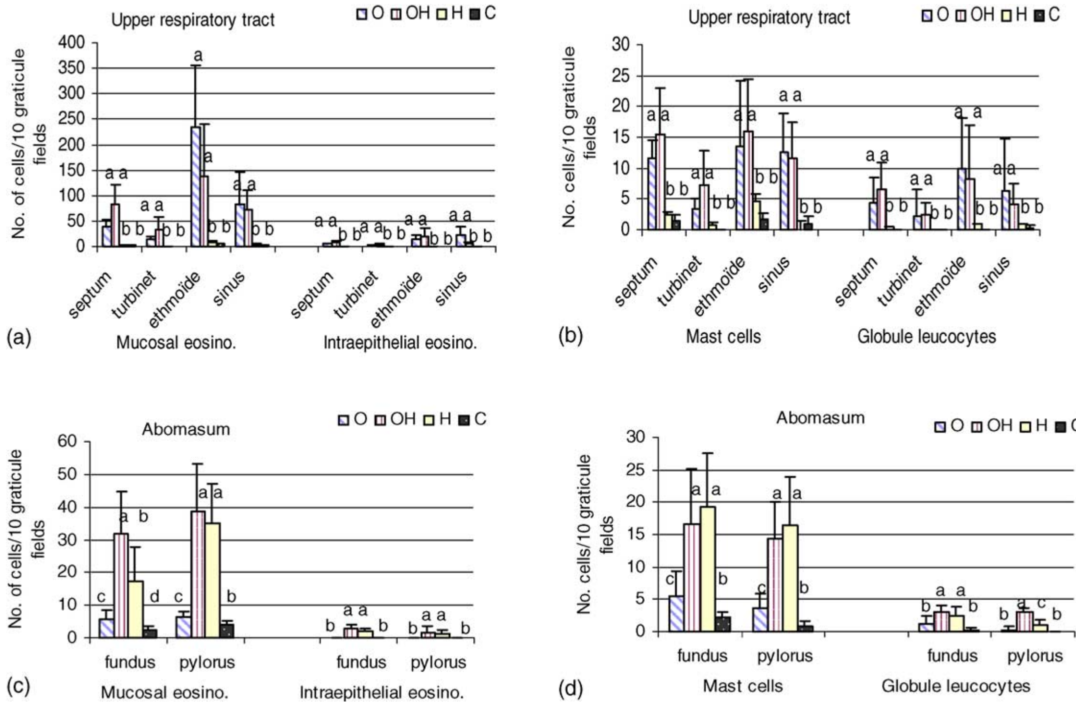

Fig. 6. Comparison of cellular responses: (a and b) in the upper respiratory tract and (c and d) in the abomasal tissues. Mucosal eosinophils, intraepithelial eosinophils, mast cells and globule leucocytes were counted in samples from the nasal septum, turbinate, ethmoide and sinus as well as two parts of the abomasum (fundus and pylorus).

was higher in the abomasa of group OH animals than that of group H. The existence of a high number of inflammatory cells (mainly eosinophils and globule leucocytes) in the abomasa in association with other relevant factors could explain the various differences noticed between the mono-infected (H) and dual infected (OH) lambs.

Resistance of sheep to nematodes has been associated with lower fecal egg count which may in turn be related to lower larval establishment rate

Table 3
Correlation coefficients between parasitological, haematological, serological and histopathological parameters established at the necropsy time

	Pepsinogen	GL pylorus	GL fundus	MC pylorus	MC fundus	Eos pylorus	Eos fundus	No. of worms	PCV	Eos blood	FEC	Egg in utero	Female length
Ab-Hc	−0.37	−0.39	0.43	0.27	0.37	−0.25	−0.21	0.37	−0.63	−0.11	−0.01	0.20	0.080
Female length	−0.52	−0.66	0.1	0.1	0.11	−0.05	−0.36	−0.36	−0.57	−0.67	0.53	0.79	1
Egg in utero	−0.3	−0.88	0.01	0.2	0.05	−0.45	−0.17	−0.15	−0.32	−0.57	0.51	1	
FEC	−0.39	−0.79	−0.77	−0.47	0.40	−0.20	−0.7	0.33	−0.47	−0.61	1		
Eos-blood	0.43	0.71	0.21	0.21	0.19	0.51	0.09	−0.28	0.28	1			
PCV	0.58	0.66	0.19	0.14	0.1	0.13	0.58	−0.4	1				
No. of worms	−0.14	−0.36	−0.67	−0.64	−0.53	−0.29	−0.67	1					
Eos-fundus	0.08	0.56	0.66	0.50	0.34	−0.04	1						
Eos-pylorus	−0.19	0.56	0.10	0.36	0.38	1							
MC-fundus	−0.33	0.14	0.77	0.77	1								
MC-pylorus	−0.18	0.24	0.70	1									
GL-fundus	0.02	0.35	1										
GL-pylorus	0.36	1											

Ab-Hc: *H. contortus*-specific antibodies; MC: mast cells; GL: globule leucocytes; Eos: eosinophils.

(worm burden) and development and lower female length and fecundity (Douch et al., 1996b; Onah and Nawa, 2000; Stear and Bishop, 1999a). Significant difference in FEC was reported between the relatively *H. contortus* resistant Florida Native and the susceptible Rambouillet breeds of sheep (Amarante et al., 1999), and between Corriedale and Crioula Lanada breeds (Bricarello et al., 2004). This shows that lower FEC can be a potential indicator for the development of some degree of resistance against the parasites.

In this study, the significantly lower FEC and in utero egg counts registered in animals of group OH as compared to group H indicates a previous *O. ovis* infection has led to limited fecundity of *H. contortus*. There was no significant difference in the total worm burden as well as in the number of female and male worms between the two groups. Hence, this difference in the number of eggs might be directly related to the reduced length of female *H. contortus* in group OH and/or retardation in worm development and suppression in egg production.

The strong positive correlation found between female nematode worm length and FEC or between worm length and in utero egg count was in agreement with Claerebout et al. (1998) and Gruner et al. (2003). Our results also supports (except for worm number) the findings of Yacob et al. (2002) who noted significant differences in these parasitological parameters between animals concurrently infected with the nasal bot and *T. colubriformis* and those infected with *T. colubriformis* alone. Similarly, Dorchies et al. (1997) reported differences in FEC while studying the effect of the presence of *O. ovis* larvae on the pathogenicity of *H. contortus* in sheep. However, they did not find notable difference in worm burden.

Mast cells and globule leucocytes are often associated with worm rejection. However, the findings so far accumulated concerning the role of mucosal mast cells and associated globule leucocytes are not consistent enough to establish a defined relationship between worm rejection and the number of these cells (Bricarello et al., 2004; Douch et al., 1996b; Gamble and Zajac, 1992; Huntley et al., 1992). The negative correlation established between mast cells and worm burden in our study was in agreement with the results of Emery et al. (1993) and Huntley et al. (1995). Similarly, the occurrence of negative correlation between GL and parasite burdens, total length and egg counts indicates a possible functional activity of these cells in accord with the findings of Bricarello et al. (2004) and Douch et al. (1996a). These data suggest an enhanced negative pressure on the development of the nematodes, which may in turn explain the low level of FEC and the shorter size of female worm length in this group. However, in cases of primary infections the number of mast cells and the level of activation depend on the duration of infection and in most instances large numbers of worms are able to stay in the abomasum for months in susceptible animals (Bendixsen et al., 1995). This was also the case in our study where up to 60% of adult worms were recovered at the end of the seventh week of infection, suggesting that the lambs were not able to eliminate most of the worms during the relatively short experimental period in both groups. It has been indicated that lambs preferentially control worm length before acting on worm number (Stear et al., 1999b). Moreover, young animals being unable to control worm burden are known to be more susceptible to parasitic infections (Colditz et al., 1996; Manton et al., 1962).

Eosinophils are considered to be important elements in the response against helminth infections and are frequently associated with the expression of resistance to the parasites (Balic et al., 2000; Dawkins et al., 1989; Pfeffer et al., 1996). In this study, there was an increased mobilisation of eosinophils against *O. ovis* larvae both at blood and tissue levels. This was in agreement with Yacob et al. (2004). Furthermore, animals inoculated with *H. contortus* mounted considerable degree of blood and tissue eosinophilia as compared to the non-infected animals. Moreover, the higher numbers of eosinophils registered both in blood and abomasal tissues (fundus) were correlated with lower FEC and reduced female length and fecundity in group OH.

Eosinophils mobilised against specific parasites were frequently found to cause immobility and death of larvae of homologous or heterologous parasites often in association with antibodies and/or other factors (Emery et al., 1993; Kazura and Grove, 1978; Rainbird et al., 1998; Rotman et al., 1996). Hence, in our study, activated circulating eosinophils produced against *O. ovis* larvae might have influenced the development of *H. contortus* in the abomasum. This

may be through migration into the abomasum and acting directly on the worms in association with antibodies, complements and other inflammatory cells or by releasing various toxic proteins/factors (Wardlaw, 1996) into the blood circulation, which could be in contact with the haematophagous stage of *H. contortus*.

The haematocrit is an essential parameter, which may be used beside FEC to describe resistance against nematode parasites in sheep in situations where the dominant nematode species sucks blood (Amarante et al., 2004; Gauly and Erhardt, 2002). In this regard, relatively *H. contortus* resistant animals were reported to have a higher packed cell volume as compared to the susceptible ones. Gulf Coast Native sheep had relatively stable PCV values and low FEC as compared to Suffolk breed (Miller et al., 1998). We have observed that PCV was negatively correlated with female worm length and faecal egg count in support of the findings of Gauly and Erhardt (2002). There was a positive correlation between PCV and numbers of mucosal eosinophils (fundus) and GL (pylorus), two candidates as phenotypic markers for resistance to parasitic infections (Douch et al., 1996b). Differences between groups as well as between the two nematode-infected groups were significant, group OH being less anaemic than group H. This might be attributed to the difference in the size of the parasites, assuming that longer parasites feed more blood than the shorter ones. Hence, animals concurrently harbouring larvae of *O. ovis* seem to tolerate the pathogenic effects of haemonchosis by reducing parasite length, thereby minimising the quantity of blood loss caused by the parasites.

The development of local and circulating antibody responses against one or both parasites has been described by different authors (Abebe et al., 1997; Schallig et al., 1995b; Yacob et al., 2004). However, a direct relationship between serum antibody and the immune status of sheep is questioned. There are several reports denouncing the role of serum antibodies in the development of resistance against, particularly primary, parasitic infections which can be explained by lack of significant correlation between antibody levels and various parasitological parameters (Gamble and Zajac, 1992; Gomez-muñoz et al., 1999; Sréter et al., 1994). In this experimental study, a progressive rise in total serum IgG level was observed against both *O. ovis* and *H. contortus*. Values for infected groups were significantly higher than those of non-infected groups. However, notable difference has not been discovered between groups O and OH against *O. ovis* or between groups H and OH against *H. contortus*. In addition, we were not able to demonstrate high profile correlations between these values and worm/larval burdens, egg count, worm length or number of inflammatory cells. Antibodies produced against primary infections with *H. contortus* did not result in protective immunity when compared to secondary infection (Schallig et al., 1995b). Hence, in this primary infection, serum IgG levels alone had no obvious effect on the development and persistence of *H. contortus* although its contribution in association with potent inflammatory cells can not be discounted as were reported by various authors (Kazura and Grove, 1978; Rainbird et al., 1998).

It has been demonstrated that serum pepsinogen values correlate with infection levels of abomasal nematodes (Berghen et al., 1993; Ploeger et al., 1990; Stear et al., 1999c).

Following infections with abomasal parasites, there was a rise in the concentration of pepsinogen in the plasma (Fox, 1997). We have noticed a sharp increase in serum pepsinogen level from D53 to D67 in group H, which may indicate the occurrence of severe damage to the abomasal mucosa during this period. The increase in gastric secretions is mainly associated with the development of the larvae into adult worm stage (Scott et al., 2000), and hence, we assume that within the above mentioned time period, most larvae have emerged from the gastric glands and developed into adult stage. This was also supported by the first emergence of *Haemonchus* eggs in faeces on D67 in both infected groups. The sharp decline in pepsinogen concentration thereafter may indicate that most of the worms were already withdrawn from the gut mucosa to the lumen, and hence, major cellular functions, including functions of parietal cells, have been restored to the normal level (Lawton et al., 1996). In group OH, a gradual but progressive rise in the concentration of pepsinogen right from the date of infection with the nematode to the end of the experimental period was observed. This may suggest that there has been certain degree of retardation in the full development of the parasites into adult stage although this was not revealed at the end of our

experiment (49 days after infection) except for the reduced female length. A gradual and continuous withdrawal of the parasites into the abomasal lumen could cause a progressively increasing abomasal pH and level of pepsinogen.

In conclusion, the nematode *H. contortus* and the dipteran fly *O. ovis* are often sympatric. As for most parasites, these two species being in the same animal host interact in a variety of ways with the host defence mechanism directed against one or both of the parasites.

In this study, we have demonstrated that repeated infections of sheep with *O. ovis* larvae had a prominent influence on the growth and egg laying capacity of a subsequent *H. contortus* infection. This influence was probably through the enhanced recruitment of activated inflammatory cells (eosinophils, mast cells and globule leucocytes) and/or their products towards the gut mucosa that finally created an unfavourable environment to the nematodes, thereby reducing worm length and fecundity.

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2 .2. Diminution de la reponse inflammatoire à l'aide du cromoglycate de sodium lors d'infestation experimentale par *Haemonchus contortus* chez une race resistante (Barbados Black Belly).

Le cromoglycate de sodium (CDS) est un médicament anti-allergique non stéroïdien utilisé dans la thérapie et la chimioprophylaxie de l'asthme et des syndromes allergiques gastro-intestinaux chez l'homme. Il inhibe la dégranulation du mastocyte et donc la libération de cytokines et de médiateurs inflammatoires, en particulier de l'IL-5 produite par le mastocyte (Shin *et al.* 2004 ; Hoshino et Nakamura, 1997). Il a également été utilisé avec succès dans le traitement des syndromes hyperéosinophiliques du tube digestif (Moots *et al.* 1988). Des animaux de race résistante (Barbados Black Belly) ont été traités avec du CDS avant et après une infestation expérimentale et comparés avec un groupe d'animaux de la même race mais non traités avec du CDS. Les résultats indiquent que l'éosinophilie sanguine ainsi que la mastocytose tissulaire ont été significativement diminuées dans le groupe CDS mais pas l'éosinophilie tissulaire mesurée 30 jours après infestation. De plus, il y avait une tendance à une plus forte intensité d'excrétion d'œufs et à une meilleure installation des vers dans le groupe CDS. Ce travail (Publication (N°3) **Terefe *et al.*, 2007. *Revue de Médecine Vétérinaire* : sous presse**).

2.2. Suppressing the inflammatory responses in a resistant breed (Barbados Black Belly) using sodium cromoglycate during *H. contortus* infection

Sodium cromoglycate (SCG) is a non-steroid anti-allergic drug used in the prophylaxis and treatment of asthma and GI allergic disorders in human subjects. It inhibits mast cell degranulation and subsequent release of cytokines and inflammatory mediators among which is the eosinophil-active IL-5 (Shin *et al*., 2004; Hoshino and Nakamura, 1997). It has also been used to treat eosinophilic gastroenteritis (Moots *et al*., 1988). Hence, a resistant breed of sheep (Barbados Black Belly) known for its high basal blood eosinophilia was treated with SCG before and after infection with *H. contortus* and compared with a non-treated group. The results indicate that blood eosinophilia and tissue mastocytosis but not tissue eosinophilia were significantly reduced in the treated group. Furthermore, there was a trend for higher FEC and worm count in the CSG-treated group though not statistically different from those of untreated animals (Publication (No. 3) **Terefe *et al*., 2007. *Revue Médecine Vétérinaire* (in press).**

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Monsieur le Professeur Dorchies,

J'ai le plaisir de vous informer votre manuscrit :

Daily sodium cromoglycate treatment decreased mast cell and blood eosinophilic responses in *Haemonchus contortus* resistant Black Belly sheep.

est accepté pour publication dans la Revue de Médecine Vétérinaire.

En vous remerciant d'avoir choisi la RMV pour la publication de vos travaux, je vous prie de croire, cher Auteur, à l'expression de mes sentiments dévoués.

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SHORT COMMUNICATION

Daily sodium cromoglycate treatment decreased mast cell and blood eosinophilic responses in *Haemonchus contortus* resistant Black Belly sheep

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Abstract

Mast cells and eosinophils are known to participate in the processes of allergic inflammation and gastrointestinal parasitic diseases. Sodium cromoglycate (SCG), a non-steroid anti-allergic drug is used in the prophylaxis and treatment of asthma and GI allergic disorders in human. This experimental study has used SCG to reduce the population and degranulation of mast cells and the subsequent mobilisation of eosinophils during *Haemonchus contortus* infection, in an attempt to rendering the relatively nematode resistant Black Belly breed of sheep more prone to the infection. Animals were divided in to three groups of 5 sheep (groups SH, H and C: SCG treated and infected, non-treated and infected and negative control, respectively). Blood and bone marrow eosinophil counts, faecal egg count (FEC), tissue eosinophil, mast cell and globule leukocyte counts as well as measurement of other parasitological parameters were performed. Oral administration of SCG for 5 consecutive weeks successfully reduced the level of blood and bone marrow eosinophil number but not tissue eosinophil level. Furthermore, a significant reduction in mast cells, and a moderate reduction in globule leucocyte numbers were observed in the abomasal mucosa. Mean values for FEC and worm counts were higher in group SH. However, there was no significant difference between SCG-treated and non-treated group, which suggests the reduction in the inflammatory response alone, may not be sufficient to influence *H. contortus* development in sheep. As the dose and mode of administration of this drug is not fully established for sheep, further studies might be required to make SCG a useful tool for experimental studies during gastrointestinal parasitism.

Keywords: Haemonchus contortus; Sodium cromoglycate; Eosinophils; Mast cells; resistant breed.

Titre : Un traitement quotidien au cromoglycate de sodium diminue les réponses mastocytaire et éosinophilique sanguine chez des moutons de race Black Belly résistant à *Haemonchus contortus*.

Résumé :

Mastocytes et éosinophiles sont des cellules impliquées dans les processus allergiques et les maladies parasitaires du tube digestif. Le cromoglycate de sodium (CGS), une drogue non stéroïdienne, est utilisée dans la prévention et le traitement de l'asthme bronchique et des manifestations allergiques du tube digestif. Cette expérimentation a utilisé le CGS afin de réduire la population et la dégranulation des mastocytes muqueux mais également la mobilisation des éosinophiles durant une infestation par *Haemonchus contortus* chez des moutons de race Barbados Black Belly réputés résistants à ce parasite. 15 agneaux de cette race ont été répartis en trois groupes de 5 animaux : i) traités au CGS et infestés par *H. contortus* (SH), ii) non traités au CGS et infestés (H) et iii) témoins non traités et non infestés (C). Des comptages réguliers d'éosinophiles sanguins ont été réalisés de même que des comptages d'œufs dans les matières fécales. Après l'autopsie, 30 jours après l'infestation, une évaluation de la proportion d'éosinophiles dans la moelle osseuse, du nombre d'éosinophiles, de mastocytes et de globule leucocytes dans la muqueuse abomasale a été menée sur tous les animaux. L'administration quotidienne de CGS avant l'infestation puis durant toute la durée de l'infestation a réduit significativement l'éosinophilie sanguine et la proportion d'éosinophiles dans la moelle osseuse mais n'a pas eu d'effet sur le recrutement de ce type cellulaire dans la muqueuse abomasale. Une réduction significative du nombre de mastocytes et, dans une moindre mesure, de globule leucocytes est constatée chez les animaux du groupe SH. La diminution de réponse cellulaire dans ce dernier groupe ne s'est pourtant pas traduite par une installation significativement supérieure, un meilleur développement ou une plus grande fécondité des vers femelles même si des tendances apparaissent dans ce sens. Comme la posologie et le rythme d'administration du CGS ne sont pas complètement validés chez le mouton, d'autres expérimentations sont nécessaires pour faire du CGS un outil d'étude de la réponse cellulaire des ovins aux strongyloses gastro-intestinales.

Mots clés : *Haemonchus contortus*, cromoglycate de sodium, éosinophiles, mastocytes, race résistante.

Introduction

Mast cells and eosinophils are often incriminated for the pathogenesis of asthma and inflammatory gastrointestinal (GI) disorders in human (3, 20). Interestingly, helminth infections also are characterised by the induction of IgE synthesis and mast cell and eosinophil accumulation suggesting some parallels in the pathogenesis of helminthosis and allergy (9). However, despite their *in vitro* activity (18), *in vivo* studies often produced controversial results on the role of these cells in GI parasitism (15). The invasion of the GI mucosa with helminths causes the development of a Th2 type of response, where mast cells exert their biological effects by releasing preformed and de novo synthesized mediators (histamines, prostaglandins and cytokines) upon stimulation. IL-5, one of the cytokines produced by the mast cells, is critical for the mobilisation of eosinophils from the bone marrow while IL-4 and leucotrienes facilitate their recruitment in tissues (17). Mast cells and eosinophils increase in number following gastro-intestinal nematode infections and are supposed to be involved in mounting a certain degree of resistance, which greatly varies between individuals and breeds of animals (1). In this regard, the Black Belly breed of sheep is known to resist *Haemonchus contortus* infection as compared to other breeds (7). One of the mechanisms envisaged to induce such resistance is the development of a high blood eosinophilia following experimental parasitic infections (1). However, there is no detailed *in vitro* study to substantiate this role of eosinophils and /or mast cells in the resistance of the Black Belly breed to gastrointestinal parasites. Sodium cromoglycate (SCG), a non-steroidal anti-allergic drug is used in the prophylaxis and treatment of asthma and GI allergic disorders in human. It blocks the degranulation (14, 21) and reduces the population (8) of mast cells thereby reducing the severity of allergic diseases. This drug was also reported to act on eosinophilic gastroenteritis in humans (13). Different from the corticosteroid drugs (16), SCG is poorly absorbed through the digestive mucosa (11) and hence, its direct effect may be limited to the digestive tube after oral delivery. Assuming that a similar mechanism of action of sodium cromoglycate could take place during parasitic infection in ruminants, this experiment attempted to reduce mast cell populations and degranulation as well as to increase mobilisation and recruitment of eosinophils that would influence the development of *H. contortus* in the abomasum of Black Belly breed of sheep. The association of this drug and

the characteristics of parasite populations (establishment, egg count and female worm length) as well as local inflammatory responses in the abomasum has been described in this paper.

Materials and methods

Fifteen male lambs of Black-Belly breed (4 to 5 months old and 30-40 kg body weight) raised in worm free condition were generously provided by INRA (Bourges, Domaine de la Sapinière). Animals were divided in to three groups of 5 lambs (groups: H, SH and C). The *H. contortus* L3 (Humeau strain) were originally obtained from INRA (Tours-Nouzilly) and maintained by passages in donor sheep in our laboratory. A human preparation of oral Sodium Cromoglycate (Nalcron ND., Specia Laboratories, France) in 5ml (100mg) ampoules was purchased from a local pharmacy. The usual dose indicated for an adult person is 1-6 ampoules per day (corresponding to 2-10 mg/kg BW/day). Animals of group SH received three ampoules/day (10mg/kg) of Nalcron for 7 days before and for 30 consecutive days after *H. contortus* infection (D_{-7} to D_{30}) while groups H and C animals were left untreated. On $D_{0,}$ all animals in group SH and H were infected orally with 10000 L3 of *H. contortus* and group C served as negative control. Blood samples were collected in EDTA coated tubes once before the beginning of the treatment, and then daily until day 15 after infections followed by three times per week until the end of the experiment. 100µl of each blood sample was mixed in 900µl of eosin Y (Carpenter's solution) and allowed to stain for 5 minutes. The solution was then diluted with similar volume of PBS (1 ml) to facilitate the visualisation of cells, and the mixture was filled into a FAST READ 102 cell-counting chamber (ISL UK). Eosinophils were counted in the grids containing 1µl of the mixture (4). Egg counts were performed according to the modified McMaster technique (19) on faecal samples collected three times a week from D_{10} to D_{32}. At the end of the experiment (D_{32}), all sheep were killed by intravenous injection of 10 mg/kg pentobarbital sodium. Abomasal contents and washings were collected and sieved (40µm). The abomasum was digested in pepsin-hydrochloric acid solution (37°C, 6h) to collect the tissue dwelling worms. The contents were then preserved in absolute alcohol. The volume of the material was adjusted to 1 litre and worms were counted in a 10% aliquot and classified according to their developmental stages. Furthermore, 20 adult female worms were randomly picked up from each sample for total parasite length measurement and counting of eggs *in utero*. For this

purpose, individual female worms were allowed to disintegrate using 200 µl of Milton Sterilising fluid (contains: 2% w/v Sodium hypochlorite and 16.5% w/v Sodium chloride) diluted in a 1:4 ratio in distilled water (10) and all eggs liberated from the uterus were counted. Bone marrow smears were made from the iliac crest after autopsy and stained with May Grünwalds-Giemsa. 400 leucocytes were counted and the percentage of eosinophils was determined. Tissue samples were collected from fundic and pyloric regions of the abomasum for histopathological examinations. They were preserved in 10% buffered formalin or Carnoy's fixative. 5µm sections were stained with Hemalun and counterstained with Carbol-chromotrope for counting eosinophils and globule leucocytes while mast cells were stained with alcian blue-safranine (6). The cells were counted at X400 magnification in 10 microscopic fields. The evolution of faecal egg excretion and blood eosinophil counts was compared between the three groups of lambs using analysis of variance with repeated values (SYSTAT software). The number of worms recovered, female worm length, *in utero* egg counts, and abomasal eosinophils, mast cells and globule leucocytes were compared by the non-parametric, Kruskall-Wallis test (SYSTAT software) while the percentage of eosinophils in the bone marrow was compared using the Chi-square test between the three groups.

Results and discussion

In a separate trial before the start of this experiment, we have confirmed that SCG at various concentrations had no positive or negative effect on the mobility and viability of *H. contortus* infective larvae *in vitro*. In addition, no animal has shown overt symptoms of incompatibility or illness characteristic of the SCG treatment. The amount of SCG administered was based on the prescriptions designed for human beings (10mg/kg per day: maximum dose). Although this drug is said to be poorly absorbed through the digestive mucosa (11), there is no data concerning the non-glandular compartments of the stomach in sheep. In spite of such ambiguity on the use of this drug in sheep, the results of our experiment have shown some important points deserving discussions. While control animals expressed a constant and low blood eosinophilia, a rise in the number of blood eosinophils started in all infected groups approximately eleven days after infection (Fig. 1). Between D_{12} and D_{18}, the mean blood eosinophil number for group SH was significantly lower than that of group H ($P < 0.05$). Furthermore, the values have fallen close to the non-infected level within

few days of the appearance of the peak in the group treated with SCG as compared to the non-treated infected group ($P < 0.05$).

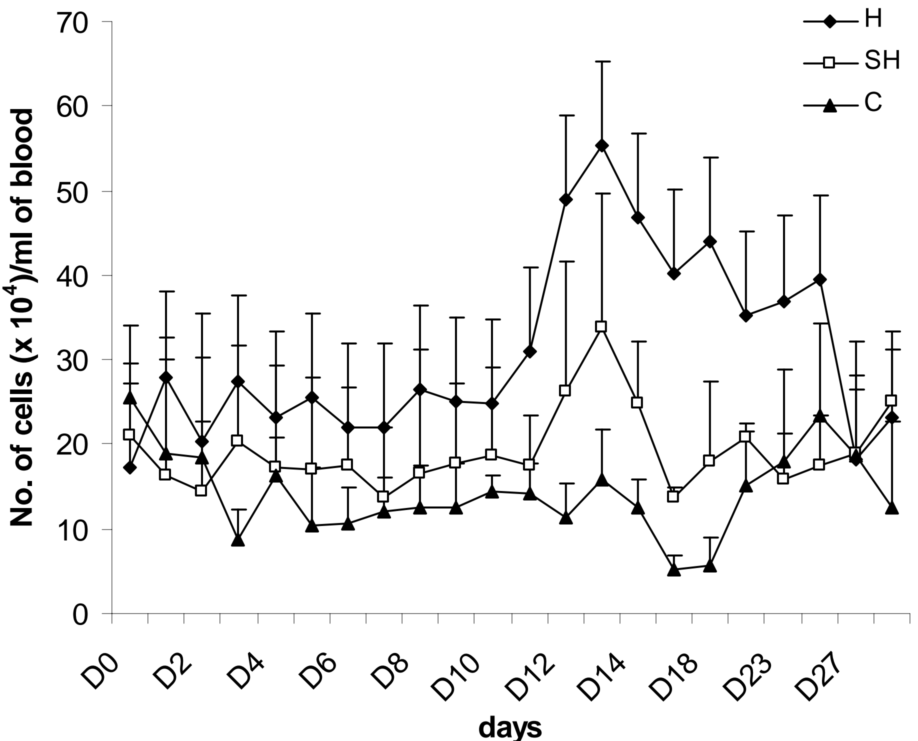

Fig.1. Evolution of blood eosinophilia in infected and control groups. Group SH has received a daily SCG treatment for 7 days before and 30 days after infection (D_{-7} to D_{30}). Both Groups H and SH received 10000L3 of *H. contortus* on D_0. Group C remained neither treated nor infected. Error bars indicate individual variations within a group.

Similarly bone marrow eosinophil values were lower in group SH than in group H ($P<0.05$). These may suggest the indirect effect of SCG exerted on the production and mobilisation of these cells to the blood circulation. Although, the nature of the signal mediating the activation of bone marrow after parasite exposure is not very clear, it could probably be through the involvement of essential cytokines (IL5 etc...) mediating these functions. Control animals had a very low level of tissue eosinophils, mast cells and globule leucocytes when compared to infected groups ($P<0.05$). Irrespective of the treatment with sodium cromoglycate, both infected groups (SH and H) have demonstrated a significant level

of inflammatory reactions, characterised by the recruitment of eosinophils and mast cells. The number of abomasal mast cells in treated and infected group (SH) was significantly lower than that of the non-treated infected group (H) but greater than that of control group ($P<0.05$). The number of globule leucocytes in both infected groups was significantly higher than that in the control group whereas SCG-treated animals demonstrated moderately lower values than group H animals ($P=0.06$) (Table. I).

Table I. Mean number of mast cells, globule leucocytes and eosinophils in the abomasum and percentage of eosinophils in the bone marrow.

	Abomasum						***Bone marrow***	
Groups	Mast cell	(SD)	Globule leucocytes	(SD)	Eosinophils	(SD)	% of eosinophils	(SD)
H	22^{a}	(8.4)	4.3^{a}	(2.4)	156^{a}	(33)	13.8^{a}	(4.4)
SH	11.6^{b}	(2.2)	2.6^{a}	(1.2)	188^{a}	(130)	8.6^{b}	(2.8)
C	3.4^{c}	(1.8)	0^{b}	(0)	4^{b}	(4)	9.2^{b}	(2.9)

Abomasal tissue cells were counted in 10 microscopic fields on a 5µm section of the abomasum. Bone marrow eosinophils were evaluated as a percentage of 400 leucocytes counted. Values marked by different letters are statistically different.

Reduction in the number of mast cells and globule leucocytes at the level of the abomasal mucosa supports the findings of Hoshino and Nakamura (8). Assays for mast cell products were not included in our experimental protocol, which could probably have served as a more direct method of demonstrating mast cell degranulation. On the other hand, similar numbers of eosinophils were observed in the abomasal mucosae of the two *H. contortus* infected groups suggesting the absence of effect of SCG on eosinophil tissue recruitment.

Control animals were negative for faecal egg count (FEC) and worm counts. Though the difference was not statistically significant, worm counts were relatively higher in group SH as compared to group H (Fig. 2). The mean number of worms recovered was 1226(±1062) and 1986(±1771) for groups H and SH respectively. No significant difference was observed between groups for the number of eggs *in utero* and female worm length and developmental stages of worms (data not shown).

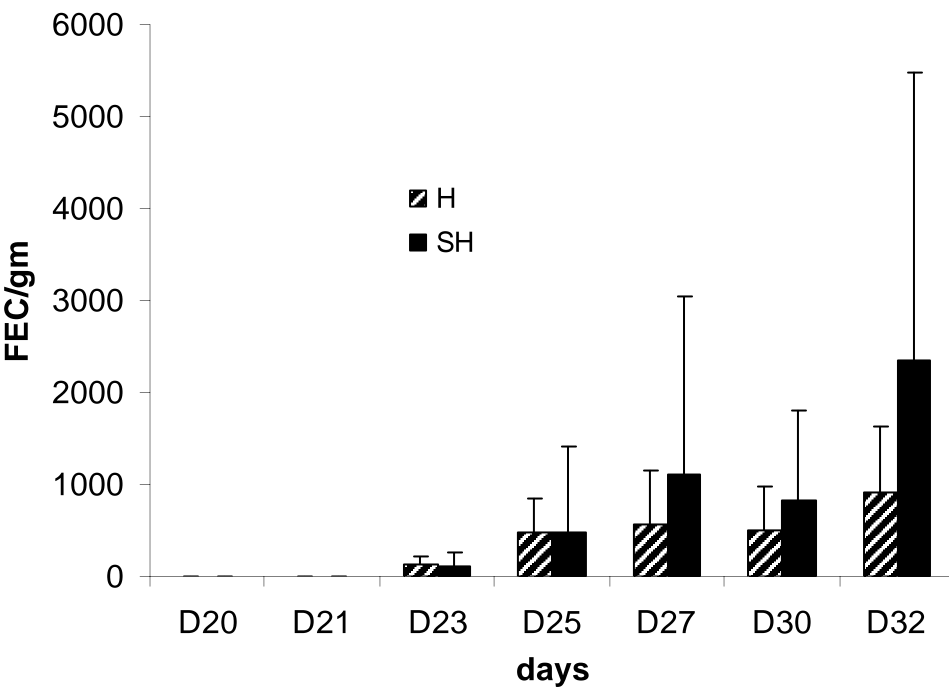

Fig.2. Mean faecal egg count in the two groups of sheep infected with 10,000 *Haemonchus contortus* L3. Error bars indicate individual variations within a group.

These findings are in agreement with Mckellar and Bogan, (12) who found no difference in the development of *Teladorsagia circumcincta* between SCG-treated and non-treated sheep. It appears that the reduction in the degree of blood eosinophilia and tissue mastocytosis was not to the level that could sufficiently influence *H. contortus* development in the abomasum. Possibly, sufficient numbers of cells have been recruited into the abomasal tissue before a pronounced effect of SCG could be observed. This may either be attributed to the dose administered (underdosage) or the duration of administration (prolonged duration required) both before and after infection with *H. contortus*. Furthermore, the role of other immunological factors that could equally contribute in the control of worm development cannot be excluded. Though some IL-5 transgenic mice showed enhanced resistance to *Nippostrongylus brasiliensis* (5), administration of anti-IL-5 monoclonal antibody to mice infected with *Trichuris muris* has greatly reduced the development of eosinophilia but had little effect on worm expulsion (2).

In conclusion, the changes in the number of mast cells and globule leucocytes in the abomasal mucosa and the number of blood and bone marrow eosinophils in the group treated

with SCG indicates some effect of this drug in the reduction of inflammatory reactions during *H. contortus* infection in the Black Belly sheep. Parallel to this, treatment with SCG showed a tendency of favouring parasite establishment and faecal egg excretion. However, this effect was not pronounced enough to result in a significant difference between treated and non-treated groups. Although, inadequate administration of SCG could be taken as one cause for the absence of statistical differences between the two groups, it could also be the case that suppression of the inflammatory response alone may not be sufficient enough to influence parasite establishment and development.

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3. FACTEURS ASSOCIES A LA RESISTANCE A UNE INFESTATION PAR *HAEMONCHUS CONTORTUS* CHEZ LE MOUTON, ROLE DES EOSINOPHILES EN PARTICULIER

3.1. La race de mouton comme facteur de variation

Expérience 1 : Les réponses immunitaires ont été comparées entre une race résistante, Barbados Black Belly (BBB) et une race sensible, INRA 401, lors d'infestations par *H. contortus*. Les animaux de race résistante ont présenté une expression des gènes de cytokines Th2 plus élevée que les animaux de race sensible. En particulier, l'expression du gène de l'IL-5 a été plus soutenue dans le temps chez les BBB que chez les INRA 401 chez qui elle diminuait rapidement après avoir augmenté dans un premier temps. L'éosinophilie sanguine était nettement plus importante dans la race résistante, cependant, les animaux des deux races ont montré des niveaux similaires de recrutement d'éosinophiles, de mastocytes ou de globule leucocytes dans la muqueuse abomasale à 4 et 30 jours post-infestation. Les BBB se sont montrés beaucoup plus résistants que les INRA 401 dès la première infestation. En revanche, lors de la seconde infestation, les INRA 401 ont développé une immunité acquise ce qui leur a permis de parvenir à un niveau de résistance comparable aux BBB chez qui la réinfestation a eu très peu d'effet (Publication (N°4) **Terefe *et al.,* 2007. *Parasite Immunology* : sous presse).**

Expérience 2 : comment expliquer que les recrutements tissulaires en éosinophiles à J4 et J30 post-infestation soient identiques dans les deux races alors qu'une différence d'éosinophilie sanguine et de résistance a été constatée dès la première infestation ? Deux explications sont proposées : a) le maximum de recrutement tissulaire des éosinophiles pourrait avoir lieu au moment, ou très peu de temps après, le pic d'éosinophiles sanguins, b) même si le nombre d'éosinophiles recrutés dans la muqueuse abomasale est similaire, il pourrait y avoir une différence fonctionnelle des éosinophiles entre les deux races. En conséquence, des études complémentaires ont été réalisées sur des animaux abattus 16 jours post primo-infestation. A nouveau, les BBB ont démontré un grand niveau de résistance (très petit nombre de vers retrouvés à l'autopsie et forte éosinophilie sanguine). Trois des quatre BBB infestés avaient beaucoup plus d'éosinophiles dans la muqueuse abomasale que les INRA 401. La capacité d'immobiliser des larves infestantes d'*H. contortus* ne diffère pas significativement entre les deux races. Les éosinophiles isolés des BBB 14 jours post-infestation ont montré une plus grande capacité à immobiliser les larves infestantes d'*H. contortus* que les éosinophiles isolés avant infestation et 7 jours après. Ceci n'a pas été retrouvé chez les INRA 401. Ces éléments nous ont encouragés à rechercher une éventuelle activation des éosinophiles de BBB au cours d'une infestation. Quatre autres agneaux BBB ont donc été infestés et des dosages de peroxydase de l'éosinophile (EPO) ont été réalisés de façon hebdomadaire de J0 à J30 post infestation. Il a été démontré que les niveaux d'EPO par éosinophile ont augmenté régulièrement de J0 à J22 puis ont décliné rapidement à J30 (**Publication (N°5) en préparation**).

3. FACTORS INFLUENCING RESISTANCE TO *H. CONTORTUS* INFECTION WITH SPECIAL REFERENCE TO EOSINOPHILIA IN SHEEP

3.1. Animal breed as a factor of variation

Experiment 1: The immunological responses of resistant (Barbados Black Belly) and susceptible (INRA 401) breeds of sheep infected with *H. contortus* were compared. The resistant breed showed higher Th2 cytokine mRNA gene expressions. The level of IL-5 was persistent in the BBB and downregulated over time in the INRA breed. Both breeds have shown equivalent fundic mucosal eosinophil, mast cell and globule leucocyte numbers at 4 and 30 days post challenge while blood eosinophilia was higher in the BBB throughout the experimental period. In both breeds, a challenge infection following anthelmintic treatment of the first immunizing infection did not cause significant changes in these parameters. In terms of parasitological parameters, the BBB was extremely more resistant than the INRA during the primary infection. However, after re-infection of both breeds, the INRA breed has developed an acquired immunity to the extent that it equalizes with the re-infected BBB breed in which further change was not observed. (Publication (No. 4) **Terefe *et al.,* 2007. *Parasite Immunology:* in press**)

Experiment 2: The question was, why similar tissue eosinophil levels were recorded in spite of the difference in blood eosinophilia and levels of resistance between the two experimental breeds in experiment 1. Two speculations were put forward though the involvement of other factors could not be excluded: (a) maximal tissue recruitment of these cells could have occurred at around the period of peak blood eosinophilia and (b) there might be a difference in the functional state of eosinophils between the two breeds. Hence, an additional comparative study was performed where animals were killed on day 16 after primary infection. Once again, the BBB breed has proven its resistance with very low worm counts and high levels of blood eosinophilia. In addition, three out of four BBB sheep had very high abomasal tissue eosinophil number compared with their INRA counterparts. No significant difference was observed in the *in vitro* larval immobilizing potential of eosinophils between the two breeds. The observation that larval immobilizing activity was higher at D_{14} compared to D_0 and D_7 in the BBB has encouraged us to further investigate if there is any change in the quantity of granule proteins over time. For this, another four BBB sheep were infected with *H. contortus* and serial eosinophil peroxidase assays were performed. It was found that absorbance for EPO increases from D_0 to D_{22} and then declines abruptly (Publication (No. 5) **in preparation).**

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Publication No. 4 (in press)

Immune response to *Haemonchus contortus* infection in susceptible (INRA 401) and resistant (Barbados Black Belly) breeds of lambs

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SUMMARY

The immune responses to Haemonchus contortus *were compared in studies in resistant Barbados Black Belly (BBB) and susceptible INRA 401 (INRA) breeds of lambs. The cytokine patterns indicated a Th2-biased response in both breeds. A more persistent and elevated Th2 cytokine mRNA transcription and blood eosinophilia were noted in the BBB lambs. However, at days 4 and 30 post-infection, abomasal recruitment of eosinophils and mast cells were similar between the two breeds. Following primary infections, the BBB demonstrated a substantially lower faecal egg count compared to the INRA lambs. Furthermore, worm counts at 4 and 30 days post-infection, and adult female worm size and* in utero *egg counts 30 days after the first infection were significantly lower in the BBB than in the INRA breed. In the INRA breed, re-infection caused a significant reduction in most parasitological parameters compared with those observed after the primary infection. A similar response was not observed in the BBB sheep. In conclusion, while the major driving force in the response to* H. contortus *infection is a Th2-biased immunity in which the BBB showed its maximal performance during the primary infection, the INRA breed performed better after re-infection compared to its response to first exposure.*

Keywords *Black Belly, cytokines, effector cells,* Haemonchus contortus, *INRA-401, sheep*

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INTRODUCTION

Rapid expulsions of primary nematode infections can occur in most murine models (1,2) while in ruminants, elimination of adult nematodes from the GI tract commonly occurs as a consequence of the development of adaptive immunity (3). In this respect, $CD4^+$ T lymphocytes are said to play a pivotal role in mediating resistance to *Haemonchus contortus* in sheep (4,5), and significantly higher lymphocyte blastogenic responses to parasite antigen have been reported in animals with genetic resistance to haemonchosis (6). Moreover, *H. contortus* infections in lambs has been shown to induce a Th2-polarized adaptive immune response with mobilization of characteristic effector cells (mast cells, eosinophils) and production of immunoglobulin isotypes such as IgA, IgG1 and IgE (7,8). The relative importance of the different effector mechanisms in influencing parasite development is still under discussion, but their involvement in the regulation of nematode populations is now generally accepted (9–11). While a reduction in larval establishment and their subsequent development into adult stages is considered as a 'final point' of the adaptive immune response to GI nematodes (12), diminished female worm size and reduced fecundity are major regulatory forces for gastrointestinal parasitic populations in lambs (7,13). However, there is evidence, which indicates that these responses can be influenced by factors including the genetic background of the animals. Inter-breed and within-breed variations in the response to infection with *H. contortus* in sheep have been demonstrated in a number of studies (14–17). Similarly, using different isolates of *H. contortus*, the Barbados Black Belly (BBB) breed has been shown to be more resistant than the INRA 401 (INRA) breed (18). In addition, Gruner *et al.* (19) found that a similar resistance of this breed was observed in infections with *Trichostrongylus colubriformis* and, to a lesser extent, with *Teladorsagia circumcincta*. While comparisons have been made to elucidate differences in parasitological parameters

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between these two breeds, little or no attempt has been made to reveal the potential immune mechanisms underlying such differences. The objective of this study was to compare immunological mechanisms such as cytokine gene expressions and effector cell (eosinophils, mast cells, etc.) and antibody responses between the resistant (BBB) and the susceptible (INRA 401) breed relative to the development of their *H. contortus* populations after experimental infection.

MATERIALS AND METHODS

Animals and experimental infections

Six-month-old male INRA 401 ($n = 28$) and BBB lambs ($n = 25$) which had been reared and were maintained under worm-free conditions were randomly allocated, for each breed, into three experimental groups: Group A (previously infected, dewormed and re-infected), Group B (primary-infected) and Group C (uninfected control) sheep. Lambs received concentrate feed appropriate for their age, and hay and water were available *ad libitum*. Animals were handled according to the European Union recommendations for animal welfare, and were under the supervision of the local INRA ethics committee.

In Phase I, lambs of Group A were infected on D_0 with 10^4 *H. contortus* infective larvae (L_3) (Humeau strain) followed by treatment of all lambs (Groups A–C) with oral ivermectin (Oramec® 0·2 mg/kg, Merial SAS) on D_{30}. In Phase II, lambs of Group A were re-infected on D_{45} with 10^4 *H. contortus* L_3, at the same time as those of Group B received their first infection with the same number of larvae. The animals were killed either on D_{49}, 4 days post-challenge (dpc) or on D_{75} (30 dpc) by administration of intravenous barbiturate followed by exsanguinations (Table 1).

Parasitological measurements

Egg counts were performed according to the modified McMaster technique (20) on rectal faecal samples collected twice a week from D_{16} to D_{30} (Phase I) and from D_{59} to D_{75} (Phase II) after infection. Immediately after necropsy, the contents and washings of the abomasums were collected and processed as described previously (7,21) for worm counting, and female worm size measurement and *in utero* egg counts.

Blood eosinophil counts

Blood samples were collected in EDTA coated tubes once a week from D_0 to D_{45} for Group A in the first phase and from D_{45} to D_{75} for all groups in the second phase. Eosinophil counts were performed after staining with eosin Y 0·5% (Ref. 6766009, Thermo Electron Co., USA) as described previously (22,23).

mRNA extraction and quantification of cytokine gene expression

After necropsy, about 300 mg of abomasal lymph node (ALN) or fundic mucosal samples were snap frozen in 1 mL of Trizol Reagent (Invitrogen reference 15596-018), and preserved at –70°C for mRNA extraction, and subsequent amplification and quantification of cytokine gene expressions by reverse transcription-polymerase chain reaction (RT-PCR) and quantitative-PCR as described previously (7).

Histology and immunohistochemistry (IHC)

Two samples from the abomasal fundic region were taken from necropsied animals; one was fixed in 10% formalin for conventional histology and IHC while the second was stored at –70°C for frozen IHC analysis.

Abomasal tissue was paraffin-embedded and 3 µm sections were mounted on glass slides. Eosinophils and globule leucocytes were counted on haematoxylin–eosin stained sections whereas mast cells were counted after staining with Giemsa. IHC on paraffin-embedded tissues was performed using antibodies previously described for cell phenotyping

Table 1 Experimental design

Breed	Days	Phase I infection 0	Anthelmintic treatment 30	Phase II infection 45	Number of sheep killed 49	75
INRA	Group A (10)	+	+	+	*n* = 5	*n* = 5
	Group B (10)	–	+	+	*n* = 5	*n* = 5
	Group C (8)	–	+	–	*n* = 4	*n* = 4
BBB	Group A (9)	+	+	+	*n* = 4	*n* = 5
	Group B (9)	–	+	+	*n* = 4	*n* = 5
	Group C (7)	–	+	–	*n* = 3	*n* = 4

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on paraffin-embedded tissues in sheep (7). This antibody set allows specific labelling of macrophages/monocytes, B lymphocytes or T lymphocytes.

For IHC on frozen samples, 5 µm sections of abomasal tissue were obtained using a Leika cryomicrotome. Slides were fixed for 20 min in an acetone bath at –20°C before drying and were immediately processed. Monoclonal antibodies directed against CD4 (mouse anti-ovine CD4, SEROTEC, reference MCA2213, 1 : 2000 dilution) or CD8 (mouse anti-bovine CD8, SEROTEC, reference MCA837G, 1 : 800 dilution) antigens were used. In all cases, cells were counted on five randomly selected microscopic fields at ×400 magnification.

Antibody responses

Preparation of serum and H. contortus *antigens*

Antibody measurements were performed on regularly collected sera and mucus samples, which were collected after necropsy using a 4 cm^2 PBS-impregnated filter paper strip placed on the abomasal mucosa. Mucus was eluted by gently agitating the strips in PBS for 2 h at room temperature. The liquid was then centrifuged and the supernatant was stored at –70°C until used. Excretion–secretion products (ESP) from adult *H. contortus* were prepared by incubating the worms in PBS (pH 7·4) containing penicillin (100 IU/mL) and streptomycin (1 mg/mL) at 37°C with 5% CO_2 overnight. The fluid was centrifuged (10 000 *g*, 30 min, 4°C), and the supernatant was stored at –70°C for use as an antigen. A crude extract of *H. contortus* L_3 (L_3CE) was prepared after three cycles of freezing and thawing (–70°C/+25°C), homogenization at 4°C and centrifugation at 30 000 *g* for 30 min at 4°C. The supernatant was stored as above. The protein concentration of these two antigenic preparations was determined using the method of Lowry *et al*. (24).

ELISA for IgG and IgA antibodies

An indirect ELISA was applied on serum and mucus samples as described by Jacquiet *et al*. (25) to determine *H. contortus* ESP-specific IgG, and L_3CE-specific IgG and IgA responses, respectively. Briefly, each well of a flat-bottomed microtitre plate (Nunclon Distr. VWR International, France) was coated overnight with 100 µL of antigen diluted in sodium carbonate buffer at 4°C. The plates were washed twice in PBS pH 7·2 containing 0·1% Tween 20 (PBS-T). To minimize nonspecific binding of the antibody, the plates were then incubated for 1 h at 37°C with 200 µL of 5% skimmed milk PBS-T (PBS-TSM). Subsequently the PBS-TSM was discarded and the plates dried, before adding 100 µL of serum diluted in PBS-TSM or nondiluted mucus to each well for 1 h at 37°C. After three washes with PBS-T (the third being for 5 min), the plates were subsequently incubated for 1·5 h with 100 µL of donkey anti-sheep IgG HRP (Sigma, reference A3415) for IgG determination, or for IgA determination, for two periods of 1 h with the first (mouse IgG1 anti-IgA bovine/ovine, Serotec, reference MCA628) and the second (goat anti-mouse IgG1 HRP, Serotec, reference STAR81P) conjugates separated by three washings in PBS-T. Finally, the plates were washed three times with PBS-T and 100 µL of 2-2′-azino-bis(3-ethylbenylthiazoline-6-sulphonic acid, ABTS) in 100 mM citrate buffer (Sigma, reference 104·4), containing 0·01% H_2O_2 was added to each well. The colour was allowed to develop for 1 h at 37°C before the reaction was stopped by maintaining the plates at 4°C for 15 min. The optical density was measured at 405 nm using a Microplate Reader (System Dias, Dynatech).

Serum pepsinogen

Serum pepsinogen concentrations were evaluated as previously described by Dorny and Vercruysse (26). Briefly, the serum sample was acidified with HCl and incubated overnight at 37°C with bovine serum albumin (BSA). The reaction was stopped with 4% trichloro-acetic acid (TCA) and the mixture was centrifuged at 14 000 r.p.m. for 5 min. An aliquot from the supernatant was added to 0·25 M NaOH and the plates were incubated at room temperature with folin reagent for 30 min. The liberated tyrosine was estimated by reading the absorbance at 680 nm and the values were expressed as mU tyrosine/L of serum.

Statistical analysis

Comparisons between Groups (A–C), between the two necropsy dates (D_{49} and D_{75}) within a group and between the two breeds (BBB, INRA 401) for a given group were performed using Kruskal–Wallis nonparametric tests (SYSTAT software). $P < 0·05$ was considered significant. The kinetics of faecal egg count (FEC), blood eosinophil counts, pepsinogen concentrations and serum antibody responses were compared between experimental groups and breeds using repeated measures ANOVA (SYSTAT software). Within-subject comparisons are not shown.

RESULTS

Parasitological values

Faecal egg excretion

No faecal egg excretion was recorded in lambs of Groups C throughout the experimental period. This was confirmed by the absence of worms in the abomasum at necropsy. During

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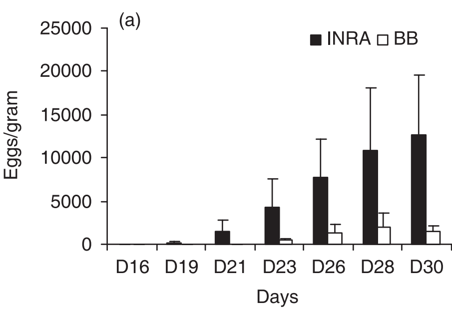

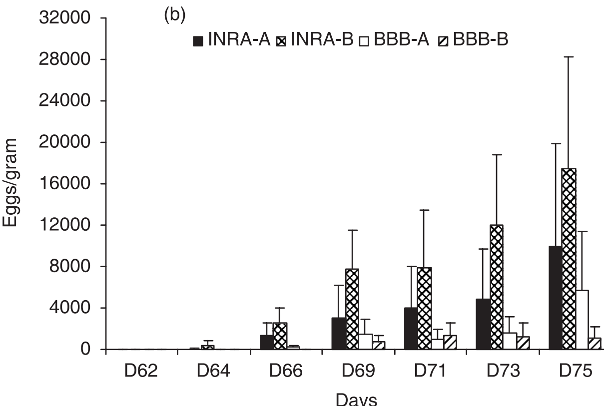

Figure 1 Faecal egg excretions in BBB and INRA breeds of lambs during: (a) Phase I (Group A) and (b) Phase II (Groups A and B). Animals were infected with 10 000 *H. contortus* L3. The primary-infection in Group A animals was terminated by oral ivermectin 2 weeks before re-infection with the same dose.

the immunizing infection (Group A: Phase I), faecal egg excretion started on D_{19} in the INRA lambs while it was delayed for about 4 days (D_{23}) in the BBB lambs (Figure 1a). Furthermore, FECs in the BBB animals were significantly lower than those recorded for the INRA breed ($P < 0{\cdot}001$). In Phase II, the first eggs were observed in the faeces 19 and 21 days after infection, respectively, for INRA and BBB lambs (Figure 1b). The difference between Groups A (re-infected) and B (primary-infected) within a breed was not significant. Similar to the observations during the first infection of Group A, the FEC's were lower in the BBB Group B than in INRA Group B lambs ($P = 0{\cdot}005$). However, this difference was absent between the two breeds after re-infection.

Worm burden and adult female worm size and fecundity

On D_{49} (4 dpc), both infected BBB Groups (A and B) exhibited significantly lower fourth stage larval counts than their INRA counterparts while there was no difference in the counts between Groups A and B within a breed (Table 2). On D_{75} (30 dpc), there was no statistical difference in worm counts between the INRA Group A and B lambs; at this time only the BBB Group B lambs had fewer worms than both INRA groups. Surprisingly, higher worm burdens were registered ($P = 0{\cdot}05$) in the re-infected BBB lambs of Group A compared with those in the primary-infected lambs of this breed (Group B). Worms of all developmental stages (L_4 to adults) were present in all infected groups. Re-infection in the INRA lambs resulted in higher percentages of L4 and immature stages and reductions in female worm length and *in utero* egg counts (Table 2) when compared with Group B animals of this breed. However, after the second exposure to infection in the BBB animals there were no significant changes in these parameters compared with those observed after the primary infection. Both infected BBB Groups had significantly lower numbers of eggs *in utero* and reduced female worm size than the primary-infected INRA lambs ($P < 0{\cdot}01$). However, values for these parameters in the BBB lambs were not statistically different from those of INRA Group A lambs.

Table 2 Parasitological parameters measured from samples collected at 4 (D_{49}) and 30 (D_{75}) days post-challenge. All infected groups have received 10 000 *H. contortus* L3 at a time. Animals were killed either at 4 or at 30 days post-infection

Days post-challenge	Breed	Group	Total worm burden (mean ± SD)	L_4 (%)	Immature male and female (%)	Adult males (%)	Adult females (%)	Adult female length (mean ± SD)	Number of eggs *in utero* (mm) (mean ± SD)
4 dpc (D49)	INRA	A	1650 ± 467[a]	100	–	–	–	–	–
		B	1892 ± 535[a]	100	–	–	–	–	–
	BBB	A	695 ± 458[b]	100	–	–	–	–	–
		B	1205 ± 246[b]	100	–	–	–	–	–
30 dpc (D75)	INRA	A	4370 ± 902[a]	15	13	34	38	16·8 ± 1·2[a]	334 ± 201[a]
		B	5558 ± 1763[a]	6	6	41	47	19·8 ± 1·4[b]	713 ± 129[b]
	BBB	A	4274 ± 1980[a]	8	9	40	43	14·8 ± 1·9[a]	234 ± 120[a]
		B	2465 ± 1623[b]	10	25	35	30	14·6 ± 1·2[a]	180 ± 70[a]

Means with different letters on the same column are significantly different for each autopsy date ($P < 0{\cdot}05$).

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Patterns of cytokine mRNA gene expression

For both breeds, *H. contortus* infections were followed by a significant increase in Th_2 cytokine (IL-4, IL-5 and IL-13) gene expression in the abomasal fundic mucosa (AFM) and ALN while these values remained low and constant in control animals (Figure 2). This was noticed earlier (4 dpc) in the re-infected groups than in the primary-infected groups especially in the ALN samples. There was no significant difference in cytokine mRNA transcriptions in the AFM between groups, breeds or between the two necropsy dates. In contrast, the IL-4, IL-5 and IL-13 mRNA transcription levels were considerably elevated 30 dpc in the ALN of infected BBB compared with those in the INRA lambs ($P < 0{\cdot}05$). Moreover, IL-5 gene transcription was found to be drastically down-regulated at 30 dpc in both infected INRA groups compared levels observed at 4 dpc. The IL-4, IL-5 and IL-13 gene expression values were highly correlated ($0{\cdot}85 < r < 0{\cdot}95$) in both breeds. No clear inter-breed or group differences in IFN-γ, IL-12, IL-10, TNF-α and eotaxin mRNA transcriptions were recorded (data not shown).

Cell recruitment in blood and abomasal mucosa

In the control groups, circulating eosinophil counts remained low and stable throughout the experimental period (Figure 3). In infected groups, peak values were observed 2–3 weeks after infection. During both experimental infections, blood eosinophil counts were higher in the BBB lambs than in the INRA lambs ($P < 0{\cdot}001$). After anthelmintic treatment, eosinophil counts returned to pre-infection levels within 2 weeks in the INRA lambs but remained higher than control values in the BBB lambs. However, re-infection in the BBB animals was followed by a further increase in blood eosinophil counts ($P < 0{\cdot}05$) compared with the primary infection; this was not observed in the INRA lambs. Blood eosinophilia was negatively associated with FEC and/or female worm length ($-0{\cdot}87 > r > -0{\cdot}99$) in the INRA breed while a similar relationship was only observed with worm burden in the BBB Group B lambs ($r = -0{\cdot}95$).

Numbers of mast cells, globule leucocytes and eosinophils in the AFM were very low in uninfected control animals (Figure 4). In both breeds, an increase in cell recruitment was obvious only 30 dpc in the primary-infected groups but

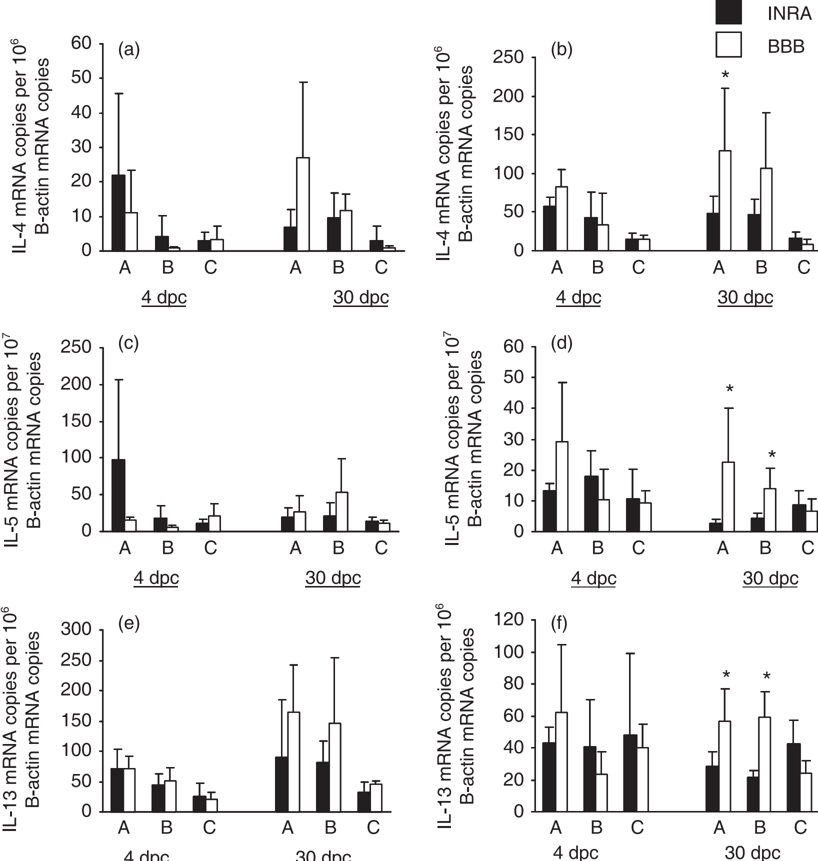

Figure 2 Cytokine mRNA transcriptions for IL-4, IL-5 and IL-13 in the abomasal fundic mucosa (a, c, e) and abomasal lymph node (b, d, f) in infected and control groups at 4 and 30 dpc. Assays were performed using an external standard and results were expressed after normalization (ratio) with a housekeeping gene (ovine β-actin). Asterisks indicate statistical differences between the two breeds ($P < 0{\cdot}05$).

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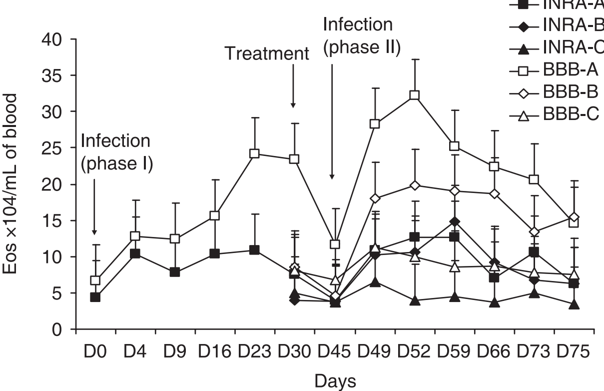
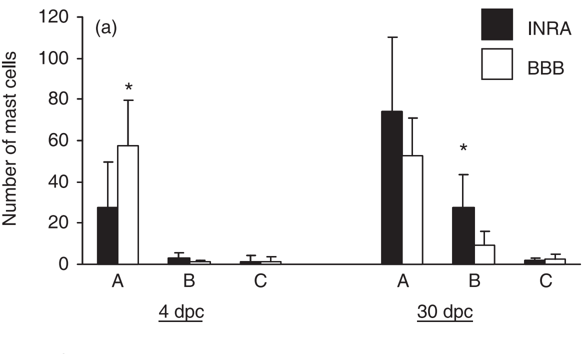
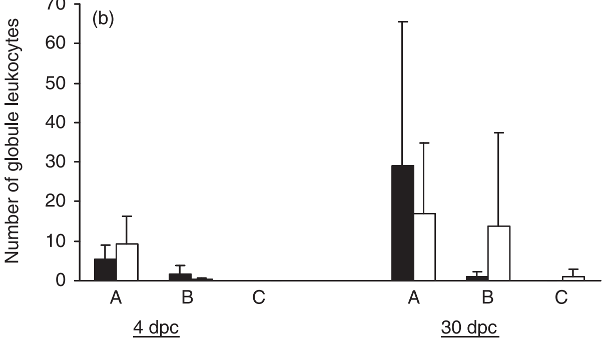


Figure 3 Mean blood eosinophilia in the BBB and INRA lambs following primary infection and re-infection with *H. contortus*. Eosinophilia in control groups was evaluated during the second phase.





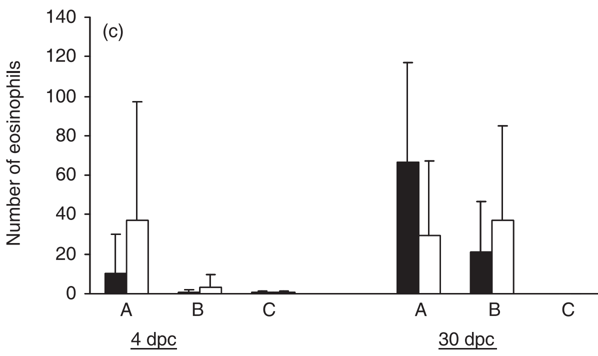


Figure 4 Cellular recruitment in the abomasal fundic mucosa: eosinophils (a), mast cells (b) and globule leucocytes (c) in infected and control animals. Values were expressed as sum of cell counts from five randomly selected microscopic fields (×400). Asterisks indicate statistical differences between the two breeds ($P < 0{\cdot}05$).

was already prominent 4 dpc in the re-infected groups. With the exception of mast cells, cellular recruitment into the AFM was similar between groups and between breeds at both 4 and 30 dpc. Higher counts of mast cells were noticed 4 days after re-infection in the BBB lambs and 30 days post-primary infection in the INRA lambs. Counts were comparable between breeds and between groups for $CD3^+$-T cells, $BLA36^+$-B cells, $CD68^+$-monocyte/macrophage cells and $CD4^+ : CD8^+$ ratio in the abomasal mucosa (data not shown). Abomasal eosinophils in both groups of BBB lambs and mast cells in the INRA Group A lambs were positively correlated to levels of IL-5 mRNA expressions ($0{\cdot}85 < r < 1$).

Serum antibody responses

In the BBB sheep, a significant rise in serum IgG level was observed 2 weeks post-exposure in the first phase (Group A) and after 3 weeks in the second phase (Groups A and B) of the experimental infections (Figure 5). On the other hand, this response was weak during the first phase but showed a significant rise after re-infection in the second phase ($P < 0{\cdot}05$) in the INRA breed. The BBB lambs of Group A had higher serum IgG levels during their first exposure than the corresponding INRA group. However, similar first-exposure responses did not show any difference between the Group B animals of the two breeds in Phase II. The serum IgG responses against L_3CE were very weak and comparable between breeds. In addition, serum IgA and mucus IgG and IgA levels were low and similar between infected groups (data not shown).

Serum pepsinogen concentration

Both infected BBB Groups A and B showed peak serum pepsinogen values between 14 and 16 days after primary-infection and re-infection while this occurred between 14 and 21 days in the INRA animals. In both breeds, values returned to pre-infection levels after treatment (Figure 6). Pepsinogen release into the systemic circulation was more intense in the BBB than in the INRA sheep ($P < 0{\cdot}01$) whereas no difference was noticed between Groups A and B of both breeds.

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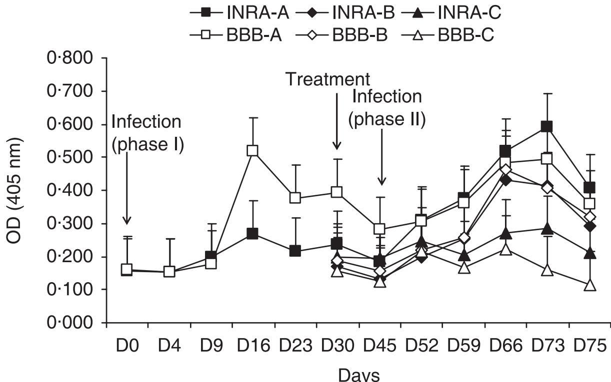

Figure 5 Serum IgG antibody responses to *H. contortus* adult ESP antigen in infected and control lambs. Optical densities were measured at 405 nm.

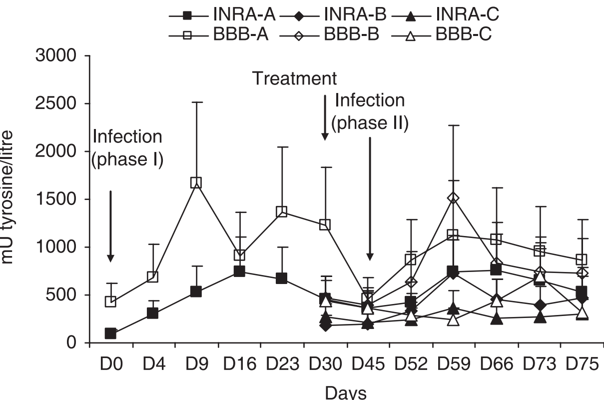

Figure 6 Kinetics of serum pepsinogen levels in infected and control animals. Values are given in mU/L of serum.

DISCUSSION

The adaptive immune response (cytokines and effector cells)

The resistance of the BBB sheep to *H. contortus* infections has been well documented (27–30). In the present study, the resistance of BBB lambs to *H. contortus* was compared with that of the INRA 401 (INRA) breed. It was designed to investigate whether the higher resistance of BBB lambs was due to a more rapid and/or intense Th_2 polarization and mobilization of associated effector cells and antibodies. The results show that a Th2-type immune response with characteristic cytokine gene expressions (IL-4, IL-5 and IL-13) was evident in the two breeds and that this was consistent with previous data reported for the INRA genotype (7). Moreover, 30 dpc, mRNA gene expressions for these cytokines in the ALN of both infected BBB groups were more pronounced than in the INRA breed, which indicated the development of a more pronounced Th2-biased immune response in the former compared with the latter. Moreno *et al*. (31) have recently detected a QTL for resistance to *H. contortus* on ovine chromosome 5 in the INRA × BBB backcross lines. The most likely location of this QTL corresponded to the IL-13/IL-4/IL-5 region suggesting that the elaboration of these cytokines is genetically controlled. Similar findings were reported for *T. colubriformis* in resistant and susceptible lines of Romney sheep (20,32). In these sheep, the resistant animals were found to develop better Th2-polarized cytokine IL-5 and IL-13 (but not IL-4) gene expressions than the susceptible lines. Moreover, the persistence of IL-5 mRNA expression in the BBB and its down-regulation in the INRA breed, together with its strong positive correlation to the numbers of blood and abomasal tissue eosinophils in the former, may suggest an enhanced contribution of IL-5 in the functional mobilization of these cells in the resistant compared with the susceptible breed. In rodent models, the production of some Th2 cytokines (particularly IL-5) can be down-regulated in chronic helminth infections irrespective of the continued production of IL-4 (33). It is possible therefore, that some inflammatory and protective immune responses, such as IL-5 dependent responses, could have been switched off in the INRA breed.

Irrespective of differences in cytokine expression in the ALN and eosinophil levels in blood, abomasal Th2 cytokine mRNA expression, tissue recruitment of characteristic effector cells, and local and systemic antibody responses remained similar between the BBB and INRA breeds. Similar findings were reported by Amarante *et al*. (34) where resistant Santa Ines, and susceptible Suffolk and Ile de France breeds of sheep had comparable numbers of abomasal eosinophils and mast cells after infection with *H. contortus*. On the other hand, Bricarello *et al*. (35) showed higher abomasal eosinophil and globule leucocyte counts in a resistant (Crioula Lanada) compared with the susceptible (Corriedale) breed after infection with *H. contortus*. Both before and after infection in this study, the resistant breed had a consistently higher blood eosinophilia but a similar density of tissue eosinophils compared with the susceptible breed. Eosinophil mobilization from the bone marrow into the systemic circulation could be mediated by IL-5 and the expression of this may have differed between the two breeds; however, the similar levels of eosinophil-active chemokine (eotaxin) observed and/or the possible release of parasite-derived eosinophil chemotactic factors (21,36) may explain the similar numbers of eosinophils observed in the AFM of the two breeds of sheep. Moreover, for animals of different genetic background, variations may also exist in the period during which maximal tissue recruitment of effector cells can be observed (36). In this study, day 4 and day 30 were

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the only two time points chosen to observe the early and late profiles of cytokines and effector cells, and this may not necessarily represent the dynamics of this aspect of the immune response between these two necropsy dates.

Interestingly, following primary infection and re-infection, the BBB lambs showed higher blood pepsinogen levels than the INRA lambs. As an increase in serum pepsinogen levels may signal abomasal tissue damage and disturbance in vascular permeability (37), minimizing the local inflammatory reactions by limiting cellular recruitment and other effector mechanisms may be an advantage for the animal although the effect of this response on parasite survival remains undefined.

Effect of breed and re-infection on the regulation of *H. contortus* populations

On the basis of the parasitological parameters, the BBB lambs appeared more resistant than the INRA lambs during their first exposure to *H. contortus*. The lower numbers of worms in the abomasum both at 4 and 30 days post-primary infection, and the reduced female worm size and number of eggs *in utero* observed in the BBB animals are without doubt responsible for the observed drastic reduction in FEC compared to the values recorded for the corresponding INRA groups. This was also associated with retardation in worm development, which was reflected by the higher number of immature worms and the longer prepatent period observed in the tropical breed. The lower FEC and worm establishment rate in the primary-infected BBB lambs compared with their INRA counterparts is in agreement with the earlier reports of Aumont *et al*. (18) and Gruner *et al*. (19). The negative correlation between FEC and worm length (in the INRA sheep) or worm burden (in the BBB animals) with the observed blood eosinophilia, suggests a possible role for these cells in the resistance of sheep to *H. contortus*. This agrees with the findings of Stear *et al*. (38) and Buddle *et al*. (39).

To study the effect of immunization on the immune response and parasite development, previously infected animals (Group A) were ivermectin treated and then re-infected with the same dose of *H. contortus* larvae. No significant differences in the immunological parameters measured were observed after the primary-infection and re-infection, with the exception of an elevation in the level of IL-5 four days after re-infection and a consistently higher blood eosinophilia throughout the rest of the experimental period in the BBB breed. Despite these observations, the INRA sheep were able to reduce worm development and fecundity in terms of female size, eggs *in utero* and number of immature worms after re-infection compared with after primary-infection. This is consistent with the findings of Lacroux *et al*. (7) in INRA 401 lambs. Contrary to the findings in the lambs of the INRA breed, the regulation of the *H. contortus* populations did not change after re-infection in the BBB lambs. This may suggest that the mechanism governing resistance to this parasite in the BBB lambs was efficient enough early in the first exposure and was not enhanced during the second infection. Bahirathan *et al*. (40) reported that suckling lambs of the Gulf Coast Native breed developed resistance to *H. contortus* infection after their first exposure to infection. Similarly, Florida Native lambs had lower FEC compared with lambs of the Rambouillet breed during their first exposure to natural infection while there was no difference between the two breeds of lambs after the second exposure (41). This is in contrast to the findings of Gauly *et al*. (42) for Rhön and Merinoland breeds, Aumont *et al*. (18) for the Black Belly and INRA 401 sheep, and Gamble and Zajac (16) for St. Croix and Dorset lambs; in all of these studies, increased resistance and breed differences were demonstrated in re-infected animals. While differences in experimental protocols, and the age and breed of animals could be responsible for variations in the findings of different studies, the precise origin of these discrepancies is not yet clear.

In conclusion, both the BBB and INRA breeds of animals used in this study showed a Th2-type immune response with characteristic cytokine gene expressions and a corresponding recruitment of effector cells. While elevated levels of Th2 cytokine mRNA expression and persistently higher blood eosinophilia characterized the response in the BBB lambs, tissue recruitment of effector cells, and local and systemic antibody responses remained similar between the two breeds. The BBB lambs again proved that this breed is highly resistant to *H. contortus* but since the regulation of the parasite populations seems to occur very quickly in these animals, the concurrent involvement of an innate immune response cannot be excluded. In contrast, the INRA 401 lambs showed an enhanced response in terms of regulation of the parasite's lifecycle after re-infection, although a correlation in the aspects of immune response examined in this study could not be demonstrated.

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Publication No. 5 (in preparation)

Eosinophils in *Haemonchus contortus*-infected resistant and susceptible breeds of sheep: abomasal tissue recruitment and *in vitro* functional state

Abstract

In our previous study, despite higher values of blood eosinophilia and IL-5 expression in the resistant breed, *H. contortus*-resistant (Barbados Black Belly (BBB)) and susceptible (INRA 401 (INRA)) breeds of sheep have demonstrated similar abomasal fundic mucosal eosinophilia 4 and 30 days post infection. Recruitment of eosinophil in tissues is a dynamic process. Hence, absence of difference irrespective of the resistance status of the breeds may be attributed to two important factors among others: (a) inappropriate necropsy timing and (b) existence of possible differences in the functional activity of eosinophils between the two breeds. In the present study, a comparative study was performed where animals were killed 16 days after primary-infection to measure abomasal tissue eosinophilia. Moreover, blood eosinophils from the two breeds were compared *in vitro* for their *H. contortus* larvae-immobilizing ability before and after infection. Eosinophil peroxidase (EPO) assay was also performed for the resistant breed. The results show that: (1) Three out of 4 BBB sheep had very high tissue eosinophilia compared to the INRA sheep. (2) While tissue eosinophilia was more marked in the pyloric than in the fundic region of the abomasum, intraepithelial eosinophil recruitment follows parasite burden, being higher in the pyloric than in the fundic region and in the INRA than in the BBB breed. (3) No significant difference was observed in the *in vitro* larval immobilizing potential of eosinophils between the two breeds. Larval immobilization was enhanced over time and more efficient in the presence of immune serum in BBB while no such tendency was observed for the INRA breed. A similar pattern was registered for EPO in the resistant breed where maximal absorbance was attained on the third week of the infection. Collectively, the results indicate that (a) eosinophil activity is enhanced rapidly in the course of infection in the resistant breed and (b) both necropsy dates and sampling site can affect data interpretations when measuring such parameters of dynamic nature as tissue eosinophil recruitment.

Key words: *Haemonchus contortus*, sheep, eosinophil, larval mobility, eosinophil peroxidase

Introduction

Eosinophils are considered key elements in the response against helminth infections (Balic *et al.,* 2000; Dawkins *et al*., 1989; Pfeffer *et al*., 1996). Sheep eosinophils are potentially lethal to *H. contortus* infective larvae *in vitro* (Terefe *et al*., 2007; Rainbird *et al*., 1998). Similar observations were reported on *Onchocerca volvulus* infective larvae (Strote *et al*., 1990) and *Trichinella spiralis* newborn larvae (Kazura and Grove, 1978) in mice. *In vivo* works have described the close association between eosinophils and death of *H. contortus* larvae in the abomasal mucosa of sheep (Balic *et al*., 2006) or of *Nippostrongylus brasiliensis* and *Litomosoides sigmodontis* in the skin and pleural cavity of mice respectively (Daly *et al*., 1999; Martin *et al*., 2000). IL-5 and eotaxin play central roles in promoting bone marrow eosinophil mobilization and trafficking to tissues (Palframan *et al*., 1998; Mould *et al*., 1997; Matthews *et al*., 1998, Shalit *et al*., 1995). Eosinophil chemotactic factors have also been found in a wide range of parasites including *H. contortus* and *Ostertagia ostertagi* (Klesius *et al.,* 1989; Wildblood *et al.,* 2005).

In a previous study, in spite of differences in parasite burden and female worm development and fecundity, blood eosinophilia and IL-5 mRNA expression in the abomasal lymph node, resistant (Barbados Black Belly) and susceptible (INRA 401) breeds of sheep had equivalent tissue eosinophilia at 4 and 30 days post-infection (Terefe *et al*., submitted to Parasite Immunology). Inappropriate necropsy timing was suggested as one possible cause for the absence of difference in the number of tissue eosinophils between the two breeds. This was supported by the fact that peak blood eosinophilia occurred between days 10 and 20 after infection. In addition, the functional activity of eosinophils could be another driving force contributing to the difference in the levels of resistance to *H. contortus*. Hence, the present study was initiated to assess the recruitment of eosinophils in the abomasal tissue at day 16 post-infection, in the Barbados Black Belly and INRA 401 breeds of sheep during a primary infection by *H. contortus*. In addition, the larval killing ability of blood eosinophils derived from the two breeds was compared *in vitro*, and eosinophil peroxidase assay was performed for the resistant breed.

Material and methods

Animals and parasites

Eight-month-old male INRA 401 (INRA) and Barbados Black Belly (BBB) sheep reared and maintained worm-free were used in this experiment. Four animals from each breed were infected with 10000 infective larvae of *H. contortus* (Humeau strain) and two others (INRA) were kept as uninfected controls. All sheep received concentrate feed adapted to their age, and hay and water *ad libitum*. Animals were handled following European Union recommendations for animal welfare under the supervision of the local INRA ethics committee.

Blood, bone marrow and tissue eosinophil counts

Blood samples were collected in EDTA-coated tubes at days 0, 3, 7, 10, 14 and 16 post-infection and processed for eosinophil counting as previously described (Terefe *et al*., 2005). Four hundred bone marrow leucocytes were counted at x1000 magnification from May Grünwald-Giemsa stained smears prepared from the sacrum immediately after necropsy, and percentage of eosinophils was determined. Abomasal tissues from the fundic and pyloric regions were paraffin-embedded and 5 µm sections were mounted on glass slides. Eosinophils were counted from haematoxylin-carbol-chromotrope stained sections (Lendrum, 1944) in ten randomly selected microscopic fields at x400 magnification.

Preparation of eosinophil-enriched blood leucocytes

Eosinophil-enriched blood leucocytes were isolated at D_0, D_7 and D_{14} post-infection using the method described previously (Terefe *et al*., 2007). Suspensions containing more than 40% eosinophils were considered for the *in vitro* culture. Cell viability was verified using trypan blue exclusion stain. Immune serum was obtained from a sheep (Lacaune breed) that has been repeatedly infected with *H. contortus*. This animal was selected among 33 similarly infected sheep based on its high level of serum IgG (ELISA) against L3 crude extract. Foetal lamb serum was used for comparison as a negative serum. These two sera were used throughout the *in vitro* tests.

In vitro L3-eosinophil co-culture and larval mobility

Three hundred exsheathed L3 in 200 µl RPMI (Ref. 21875 GIBCO, Cergy-Pontoise, France) complete medium (supplemented with 1% penicillin-streptomycin and 10% FCS) and 200 µl eosinophil-enriched cell fraction containing 1x 10^5 eosinophils were incubated with 100 µl of either immune serum (IMS) or foetal lamb serum (FLS) in a 24 well test plate (Ref. 92024, TPP, Geneva, Switzerland) in triplicate. After overnight incubation at 37 °C and 5% CO_2 atmosphere, motility of 100 larvae was examined from each well under an inverted microscope. Larvae were considered mobile if any part of the body was seen moving. An aliquot was taken in a different culture plate from each representative well and stained for three hours using equal volume of eosin Y 0.5% (Ref. 6766009, Thermo Electron Co., U.S.A.) to identify the types of cells (especially eosinophils) adhering to the larvae.

Worm count

Animals were killed on day 16 post-infection by intravenous barbiturate followed by exsanguinations. Immediately after necropsy, the contents and washings of the abomasum were collected and processed as described previously (Terefe *et al*. 2005) and worms were counted in 10% aliquot of the collected materials.

Eosinophil peroxidase (EPO) assay

In a separate study, another four Black Belly sheep of similar age were infected with the same dose of *H. contortus* L3. Blood samples were collected at regular intervals for eosinophil isolation. The isolation technique was as described above, except that RPMI medium was replaced by sterile PBS. Approximately, 5x10^6 cells/500 µl were lysed with 0.2% triton X-100 and stored at -80 °C. Eosinophil peroxidase assay was performed according to the method described by Adamko *et al*. (2004) with slight modifications. Briefly, a substrate solution containing 3 mM o-phenylenediamine (OPD) dihydrochloride , 6 mM KBr and 8 µM H_2O_2 was prepared in a 50 mM Hepes (pH 7) with (R^+) or without (R^-) 120 µM of the EPO-inhibitor, resorcinol. A 1:1 volume of the substrate solution and the cellular suspension was mixed and incubated in the dark at 23°C for 30 minutes. The reaction was stopped by adding 500µl of 4M H_2SO_4. 150µl of the coloured solution was distributed in triplicate in a flat-bottomed 96 well micro-plate and the colour absorbance was read at 490

nm. Data were converted to fixed number of eosinophils ($1x10^5$/sample) before analysis. The difference between R^- and R^+ represents the absorbance for EPO. Serum samples were also analysed at regular intervals for EPO in a similar manner to above.

Statistical analysis

The kinetics of blood eosinophil counts were compared between the two breeds using analysis of variance with repeated values (SYSTAT software). Comparisons of values for worm count (between breeds) and tissue eosinophil counts between breeds and between abomasal regions were made using the Kruskal-Wallis non-parametric tests (SYSTAT software). Microsoft excel program was used for calculating correlation coefficients, and to perform X^2 tests in the comparison of larval mobility between breeds, between sampling dates and between presence of FLS and IMS. In all cases, $P < 0.05$ was considered significant.

Results

Eosinophils in blood and bone marrow

Significantly elevated blood eosinophilia was seen before and after infection in the BBB sheep compared to that in the INRA breed ($P<0.05$). Peak level was attained at D_{14} post-infection in the BBB (Figure 1) while it was still increasing steadily in the INRA. No remarkable difference was observed between the two breeds for the percentage of bone marrow eosinophils (9.5±1.2% and 8.5±3.7% for BBB and INRA respectively).

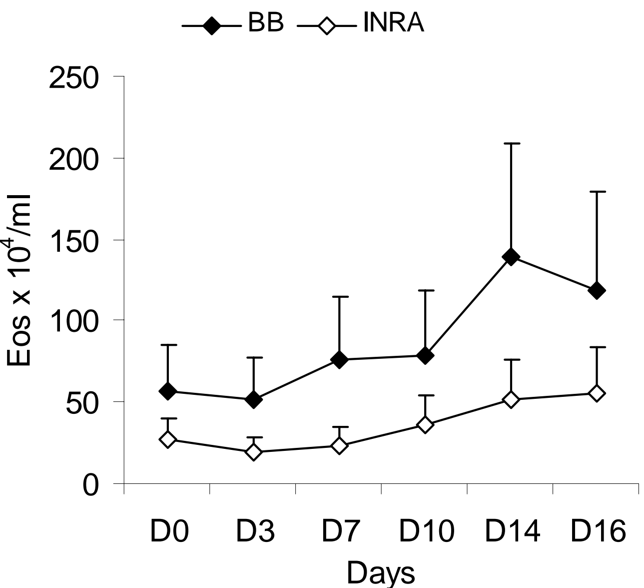

Figure 1. Kinetics of blood eosinophils (Eos) in the Barbados Black belly (BBB) and INRA 401 (INRA) breeds of sheep infected with 10^4 L3 of *H. contortus* at day 0.

Abomasal eosinophils

Very few eosinophils were seen in the abomasal tissue of uninfected control animals (Figure 2A) while they are recruited in large number into the abomasa of all infected sheep (Table 1). Three out of four BBB sheep had higher eosinophil numbers in the pyloric lamina propria (LP) compared to INRA sheep. Eosinophils were more densely populated in the pyloric than in the fundic region in both breeds in all the three layers of the abomasal wall (epithelial layer, lamina propria and submucosa) examined ($P < 0.05$ in all cases). Numbers of intraepithelial eosinophils (Table 1, Figure 2B) tend to be higher in INRA than in BBB ($P = 0.07$). In the lamina propria (Figure 2C, D) and submucosa (Figure 2E, F) of infected animals, adhesions to endothelial cells and massive extravasations of eosinophils were observed particularly in the pyloric region.

Table 1. Counts of intraepithelial, lamina propria and submucosal eosinophils in fundic and pyloric regions of the abomasum in 10 randomly selected microscopic fields at x400 magnification. Values are given as mean and standard deviations (SD). Statistical comparisons were made between two abomasal regions (fundic and pyloric), and between the two breeds.

Breed	Intraepithelial		Lamina propria		Submucosa	
	Fundus (Mean & SD)	Pylorus* (Mean & SD)	Fundus (Mean & SD)	Pylorus* (Mean & SD)	Fundus (Mean & SD	Pylorus* (Mean & SD)
BBB	0.5 ± 0.58	17.0 ± 16.4	167 ± 150	653 ± 325	143 ±82	333 ± 124
INRA	0.8 ± 0.96	51.8 ± 42.3	115 ± 101	300 ± 120	56 ± 56	217 ± 165

* Values for the pyloric region are always higher than values for the fundic region (P < 0.05).

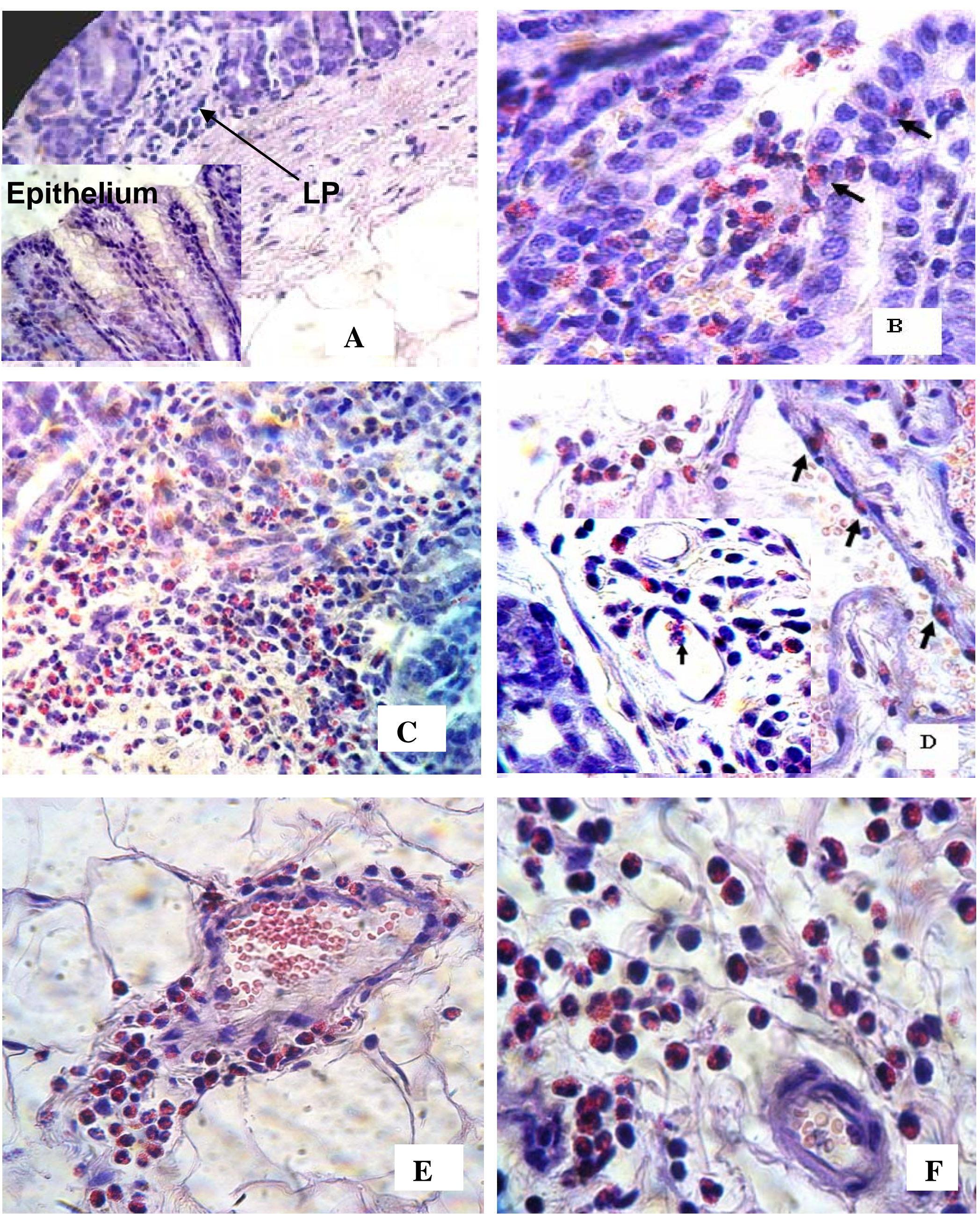

Figure 2. Tissue recruitment of eosinophils in sheep abomasum. The abomasal tissue is almost devoid of eosinophils in the non-infected sheep (A). Intraepithelial (B, arrows), lamina propria (pink staining cells, C) and submucosal (F) eosinophils are shown for infected abomasa. Eosinophils appear to adhere to and traverse the endothelial linings of blood vessels (D, arrows) in the lamina propria and submucosa. Submucosal vessels are highly engorged with eosinophil-rich leucocytes (E), which probably gave rise to massive submucosal eosinophil infiltration (F).

Worm count

Worm counts were significantly higher in the INRA compared to those in BBB sheep (Table 2). Among the four BBB sheep, only two animals were found to harbour worms. Most of the worms were observed closely attached to the pyloric mucosal surface in all infected animals while a few, some of them wandering, worms were seen in the fundic region (data not shown). Almost all worms recovered were at immature stage (with very few late L4 stages). No eggs were found in faecal materials collected from all animals at the time of autopsy. Worm burden was positively correlated to eosinophil numbers in fundic lamina propria and pyloric epithelium in the INRA breed, and in pyloric submucosa in the BBB sheep ($P < 0.05$).

Table 2. Mean *H. contortus* worm count at D_{16} post infection. Animals were infected with 10000 L3 at D_0.

Sheep breed	**Worm burden ± SD**
INRA	4535 ± 754
BBB	180 ± 309

Larval mobility tests in vitro

There was no significant difference in the larval immobilizing activity of eosinophils between the two breeds (Figure 4). Despite the presence of contaminating cells in our eosinophil preparations, staining with eosin Y directly in the culture medium showed that the vast majority of cells attached to the larvae were eosinophils (Figure 3). Compared to the pre-infection level (D_0), enhanced larvae-immobilizing activity was observed (only in the presence of IMS) with eosinophils derived from the BBB breed at D_{14}, and the difference was of borderline significance ($P = 0.06$). Furthermore, 14 days post-infection in the same breed, eosinophils were more effective with IMS than with FLS ($P < 0.05$) while the contributions of the two sera were similar at D_0 and D_7.

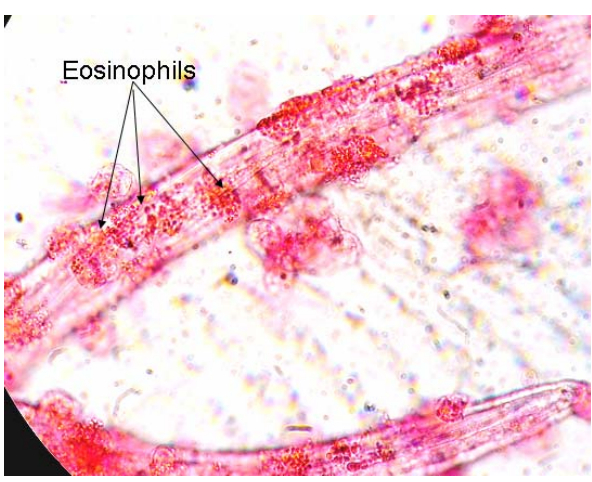

Figure 3. Eosinophil adherence on *H. contortus* infective larvae after 24 hrs of co-incubation in the presence of either IMS or FLS. (Original magnification: x1000)

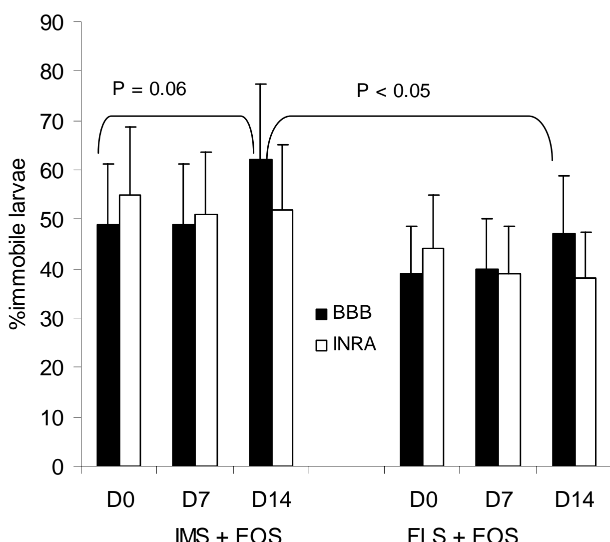

Figure 4. Percentages of immobile larvae 24 hrs after culture with eosinophil-enriched (EOS) blood leucocytes isolated at days 0, 7 and 14 in the presence of FLS or IMS. Motility was assessed on 100 larvae. Percentages of immobile larvae in control wells (incubated with complete medium only) were 10, 15 and 16 respectively for days 0, 7 and 14 after infection.

Eosinophil peroxidase

Having seen the time-dependent tendency in the larvae immobilizing ability of eosinophils from the BBB sheep in the preceding *in vitro* tests, we have decided to perform an additional experimental infection of 4 BBB sheep of similar age to those in the previous experiment, to further explore if there was a change in the contents of these cells overtime. It was observed that the absorbance for eosinophil peroxidase increases from D_0 to D_{22} followed by a sharp decline thereafter (Figure 5). Mean absorbance at D_{22} was significantly higher than the mean at D_0 ($P < 0.05$). EPO in serum was extremely low with optical densities between 0 and 0.08 (data not shown).

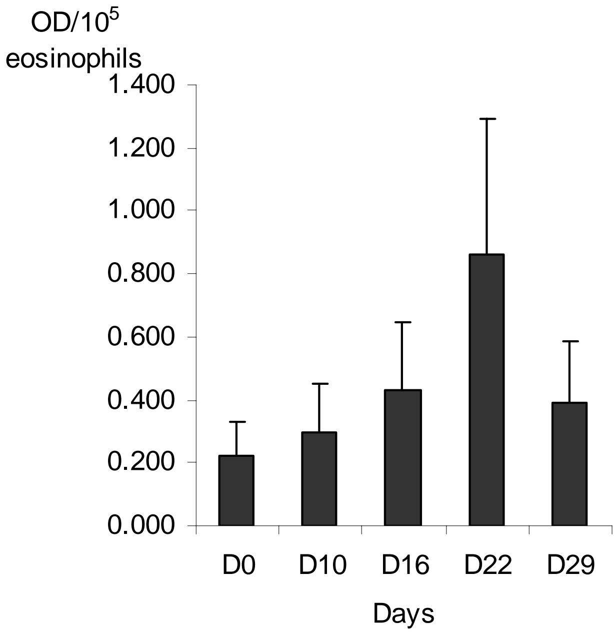

Figure 5. Mean optical density (OD) for eosinophil peroxidase assay in 4 BBB sheep infected with 10000 *H. contortus* L3. Values are presented as mean of differences between values of optical densities (OD) without and with resorcinol. 120 µM resorcinol was used to inhibit EPO activity.

Discussion

In the present study, in agreement with our previous findings, very few worms were recovered from BBB compared to INRA sheep, and this is in accord with the findings of Aumont *et al*. (2003) and Gruner *et al*. (2003).

Mean blood eosinophilia was significantly higher in the resistant than in the susceptible breed. Similar trends have been reported to be characteristic of resistant lines of sheep in various studies (Hooda *et al*., 1999; Hohenhaus and Outteridge, 1995; Dawkins *et al*., 1989). The equivalent percentage of bone marrow eosinophils observed for the two breeds may indicate that although the BBB produces more eosinophils, these cells may not be accumulated at the site of production but are rapidly released into the systemic circulation.

There was a trend for higher number of eosinophils in the pyloric lamina propria in the BBB compared to that in the INRA breed. Such tendency was evident in the fundic region neither in this study (D_{16}) nor in our previous report where animals were sacrificed 4 and 30 days after infection, and only fundic mucosal samples were examined. Absence of statistical differences between sheep of different genetic backgrounds for abomasal tissue eosinophilia has been documented (Amarante *et al*., 2005, 1999; Bricarello *et al*., 2002). However, most of these studies were realised by repeated immunisation through natural infection where the level of worms acquired by each animal could not be controlled, making comparisons difficult. Both normal and parasite induced eosinophilia are dependent on the expression of IL-5 (Hogan *et al*., 2000; Sher *et al*., 1990) and IL-4 (Chen *et al*., 2004). In our previous study, it was indicated that the BBB had higher levels of IL-4 and IL-5 gene expressions in the abomasal lymph node, which suggests that these cytokines might have partly contributed for the high blood eosinophilia and relatively increased eosinophil recruitment in the abomasal tissue in this breed.

During primary infection, worms can possibly be attacked by eosinophils at two different occasions: one is when the L4 is in the abomasal glands, and the second is when the immature and adult stages are feeding on the mucosal surfaces. Interestingly, in all abomasal tissue layers examined at D_{16} post-infection, eosinophils were more densely populated in the pyloric region than in the fundic region. It was also observed during necropsy that more worms were attached to the former than the latter region of the abomasum. From this, it can be suggested

that at least at this particular stage of the infection, eosinophil recruitment follows parasite localisation in the abomasum. Upon arrival, infective larvae localize the fundic glands for their development until they re-emerge onto the mucosal surface. This is evident particularly from the structural and functional changes observed at this region during invasion by infective larvae (Scott *et al*., 1998). However, once they leave the glands they appear to migrate to new locations (pyloric region) (Dash, 1985, Rahman and Collins, 1990), probably in search of less hostile areas. The presence of an already sensitized immune system (IL-5, eotaxin) and the possibility of chemotaxis by worm secretions (Wildblood *et al*., 2005; Klesius *et al*., 1989) seem to allow eosinophils to be mobilised towards this new location while their number in the fundic region gradually wanes because they are no more important at this site. This is supported by the fact that most blood vessels at the pyloric region were engorged with large number of eosinophils, which are seen massively traversing the endothelial layer into the connective tissue spaces of the lamina propria and the submucosa. This phenomenon was less prominent in the fundic region. Intraepithelial eosinophils are more numerous in the pyloric region than in the fundic region suggesting that their mobilization depends on parasite density at this particular period. They are presumably mobilised to attack worms on the surface of the abomasal mucosa (El-Malky *et al*., 2003) but were unable to effectively remove the already developed worms (immature or adult stages). Although it is not clear at what stage much of the worms were expelled, it is evident that even in animals in which worms were not found, significant numbers of eosinophils have been recruited into the lamina propria and the submucosa of the pyloric region suggesting that the parasites were there some time before necropsy.

To see if there was any difference between the two breeds in the ability of their eosinophils to immobilise/kill *H. contortus* infective larvae, an *in vitro* test was realized. The result shows that there was no difference in the larval immobilizing ability of eosinophils between the two breeds. Instead, for eosinophils derived from the resistant breed, larval immobilizing ability at D_{14} was found enhanced in the presence of immune serum compared to the pre-infection level. Furthermore, eosinophils at day 14 from this breed were more efficient in the presence of IMS than when incubated with FLS. These, collectively suggest rapid changes in the functional (activation) state of these cells as infection advances and enhanced responsiveness to serum elements, especially *H. contortus* L3-specific

immunoglobulins. Blood eosinophils can be sensitized or activated either *in vitro* or *in vivo* by mechanisms such as immunoglobulin and cytokine binding (Bochner, 2000; Yamaguchi *et al*., 1988; Takafuji *et al*., 1998, Capron *et al*., 1981; Matsumoto *et al*., 1998; Mawhorter *et al*., 1996) resulting in upregulation of surface activation molecules and specific receptors. Activation of eosinophils at inflammatory sites, and subsequent release of granule proteins is also a common phenomenon associated to tissue eosinophils (Hua *et al*., 1990, Sugaya *et al*., 1997). Whether, eosinophils are activated differently in the abomasal tissues of the two breeds of sheep remains to be elucidated. On the other hand, the non-negligible reduction in larval mobility when larvae were incubated with eosinophil-enriched leucocytes in the presence of FLS indicates that factors other than specific antibody, probably complement are also involved in the immobilization/killing of larvae. Complement is known to play important roles in innate and adaptive immunity (Morgan *et al*., 2005; Shin *et al*., 2001). It was shown that complement in FLS can be activated by the alternative pathway in the presence of worms such as *T. vitrinus* (Stankiewicz *et al*., 1981) infective larvae *in vitro*, and their effect is maximal on *N. brasiliensis* infective larvae but appear to diminish as larvae develop within the murine host (Giacomin *et al*., 2005). In our previous *in vitro* study (Terefe *et al*., 2007), we have demonstrated that heating immune or non-immune sera, or incubating eosinophil-larvae mixtures without serum drastically reduces the larval immobilisation ability of eosinophils strengthening the hypothesis that complement has a role in the cell-mediated toxicity to *H. contortus* infective larvae.

Eosinophil peroxidase and other granule proteins are capable of damaging/killing larvae of various helminth parasites (Hamann *et al*., 1990; Ackerman *et al*., 1985). Therefore, the time-dependent increase in the level of EPO in the Black Belly sheep may indicate a similarly increasing larval killing potential of eosinophils although this can be influenced by the degranulation process.

In conclusion, abomasal eosinophil recruitment tends to be higher in the resistant breed, but cell distribution seems to follow parasite localization in different regions of the organ. At least at D_{16}, intraepithelial eosinophilia is dependent on worm burden. Although lack of data for the INRA sheep has prohibited us to show trends for EPO assay, it is reasonable to suggest that infection by *H. contortus* results in not only an increase in number but also a change in the quality and functional state of eosinophils especially in the resistant breed.

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3.2. L’âge de l’animal comme facteur de variation

Les agneaux sont considérés comme plus sensibles aux infestations par les nématodes gastro-intestinaux. Cette expérimentation a été réalisée pour savoir s’il existe une association entre l’âge des animaux et la réponse éosinophilique sanguine en présence ou non d’infestation par *H. contortus*. Les résultats obtenus indiquent une augmentation de l’éosinophilie sanguine avant et après primo-infestation et une diminution concomitante de l’intensité de l’excrétion d’œufs avec l’âge de l’animal.

3.2. Animal age as a factor of variation

Eosinophils are considered important elements in the response to gastrointestinal parasitism. Young animals are said to be more susceptible to most GI parasitic infections and other disease problems. This experiment was realised to see if such association exists between animal age and blood eosinophilic responses in the presence and absence of infection by *Haemonchus contortus*. The results show an increase in blood eosinophilia and a decrease in FEC as age of the animal advances.

Age-dependent blood eosinophilia in the Barbados Black Belly sheep with and without *Haemonchus contortus* infection

Introduction

The Barbados Black Belly (BBB) breed of sheep is resistant to gastrointestinal parasites such as *Haemonchus contortus* (Aumont *et al*., 2003, Gruner *et al*., 2003, Yazwinski *et al*., 1980). However, the underlying mechanism is poorly described. In our previous studies on this breed, we have noticed better Th2 polarised cytokine gene expressions and higher blood/tissue eosinophilic responses compared to the relatively susceptible breed, INRA 401. These were consistently accompanied by highly reduced values for different parasitological parameters in the BBB. The present work is a supplement to these studies. The objective was to show the trend in blood eosinophilia from young age to maturity in the BBB breed of sheep in the presence or absence of primary infections with *H. contortus*.

Materials and methods

Animals and Parasites

Twelve Barbados Black Belly sheep of similar initial age were divided into three groups of 4 animals such that the first group was infected at the age of 4 months, the second at the age of 10 months and the third at 14 months of age. Animals were infected orally with 10000 *H. contortus* (Humeau strain) infective larvae. Another 4 BBB sheep were kept uninfected to assess their blood eosinophilia when they attain the age of 4, 10 and 14 months.

Blood Eosinophils count

Blood samples were collected at regular intervals in EDTA-coated tubes and eosinophils were counted according to the method described previously (Terefe *et al*. 2005).

Faecal egg count

Faecal egg counts (FEC) were performed for infected animals according to the modified McMaster technique (Raynaud, 1970).

Results

Blood eosinophilia

The number of circulating eosinophils increases with increasing age in non-infected animals (Figure 1), and mean values for the different age groups were significantly different ($P<0.05$). In infected animals, higher eosinophil counts were registered in relatively older animals (Figure 2), and the mean for 14 month-old sheep was significantly higher than that of 4 month-old sheep ($P<0.05$).

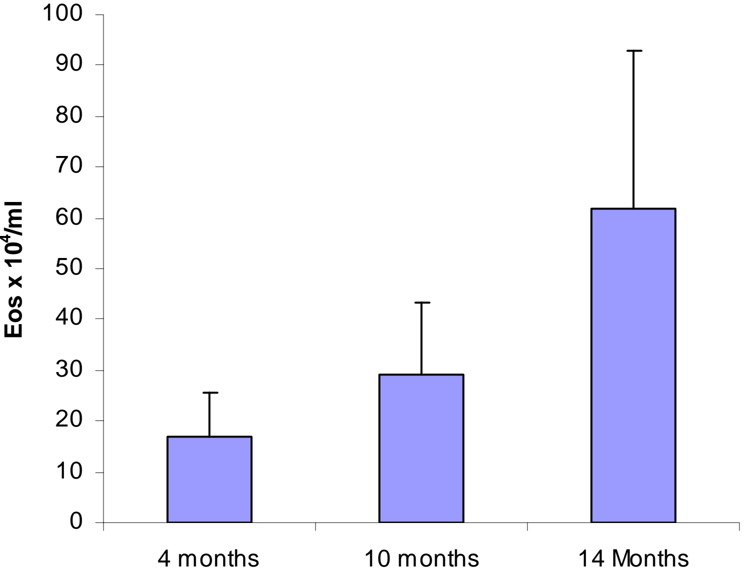

Figure 1. Patterns of blood eosinophilia in four non-infected Black Belly breed of sheep at 4, 10 and 14 months of age. Values are presented as mean of three consecutive weekly counts for each age. Error bars indicate individual variations.

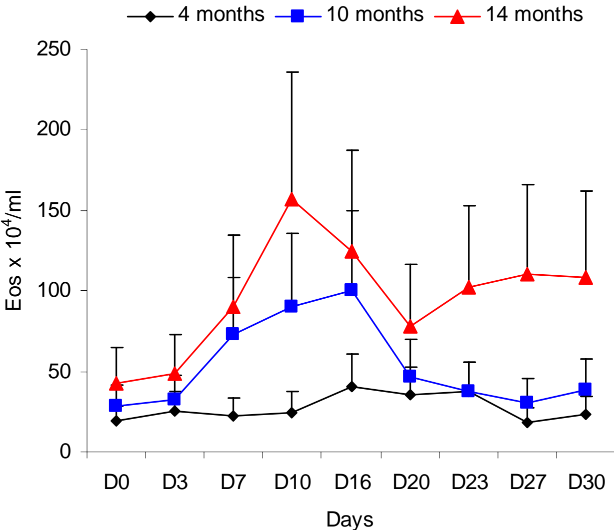

Figure 2. Changes in blood eosinophilia after primary infection of 4, 10 and 14 month-old BBB sheep. Data are presented as means for 4 sheep in each age group. Error bars indicate individual variations.

Faecal egg count

Faecal egg counts (FEC) were drastically lower in older animals than in younger ones ($P<0.05$). Among the 14 month-old sheep, only one animal was positive for faecal egg count. FEC was negatively correlated to blood eosinophilia ($P<0.05$) in the period between days 24 and 29 post-infection.

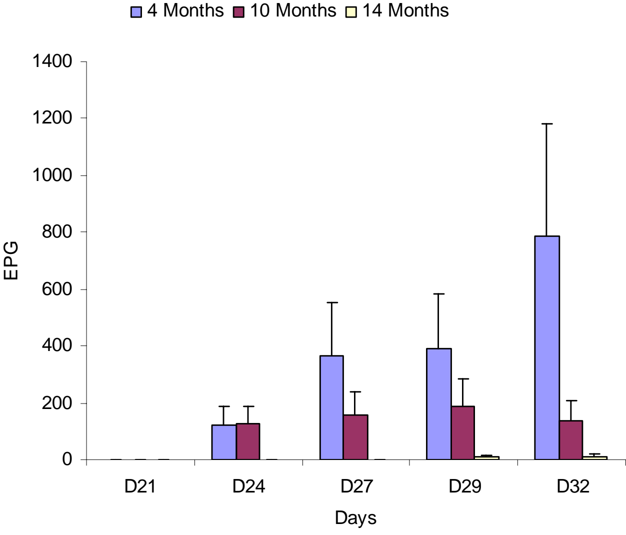

Figure 3. Mean FEC in *H. contortus*-infected 4, 10 and 14 month-old BBB sheep. Animals were infected with 10000 L3 at day 0 (D_0)

Discussion

Very little information is available to explain the age related eosinophilia and resistance to helminth infections in sheep. Protection in lambs vaccinated with *H. contortus* antigens was found related to animal age with increased serum IgE, blood eosinophilia and tissue mast cell and eosinophil numbers in older animals (Kooyman, *et al*., 2000). Lower resistance to gastro-intestinal helminth infections in young ruminants can be attributed to immunological hyporesponsiveness and/or insufficient exposure to pathogens to develop immunity (Colditz *et al*., 1996; Manton *et al*., 1962). In this study, patterns of blood eosinophilia in non-infected BBB sheep were assessed at 4, 10 and 14 months of age. The results show that blood eosinophilia increases with age of the animal suggesting the development of immunological maturity as the age of the animal advances. However, for how long in the lifetime of the animal can the increase continue remains undetermined.

When age-matched BBB sheep were infected with the same dose of *H. contortus* infective larvae, it was observed that the age-dependent increase in blood eosinophilia was well respected, but with highly pronounced values as a result of the infection. Concomitant with the rise in blood eosinophilia, there was a dramatic reduction in FEC, whereby values for 14 month-old sheep were close to zero suggesting complete elimination of the parasite and/or inability to produce eggs. In our previous studies, during primary infection in BBB sheep, *H. contortus* worm burden and female worm size and fecundity were found greatly diminished when compared to the susceptible (INRA 401) breed of sheep. However, FEC in the previous studies, which were realised on relatively younger animals were far higher than the values presented here for the 14-month-old sheep indicating the influence of age in the resistance to *H. contortus* infection (Vlassoff *et al*. 2001; Gruner *et al*. 2003).

Although, a number of factors could participate in such age related immune responsiveness, negative correlations observed between blood eosinophilia and FEC could suggest the importance of eosinophils in the development of resistance to *H. contortus* infection.

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PART IV. GENERAL DISCUSSION AND PERSPECTIVE

1. RATIONALE

The development of immunologically well-defined laboratory models (*in vitro* and *in vivo*) has allowed significant advances to be made in understanding the immunological basis of susceptibility and resistance to intestinal nematode infections. Experiments using nematode parasites such as *Trichuris muris, Heligmosomoides polygyrus, Nippostrongylus brasiliensis* and *Trichinella spiralis* have allowed analysis of the cellular and molecular interactions involved in the generation and regulation of immune responses during infection (Behm and Ovington, 2000; Meeusen and Balic, 2000). They have provided insights into the nature of protective effector responses operating in the gastrointestinal microenvironment, which result in worm expulsion and confer host resistance. With the discovery of IL-5 as a key mediator of eosinopoiesis, a number of murine models of IL-5-dependent eosinophil depletion or overproduction have been used to examine the *in vivo* role of eosinophils in helminth infections (Behm and Ovington, 2000). A significant reduction in the level of eosinophilia after treatment of mice with monoclonal antibodies that neutralise IL-5 favoured the development of *Onchocerca lienalis* (Folkard *et al*., 1996) but had no effect on *T. muris* (Betts and Else, 1999). Similarly, IL-5 transgenic mice expressing high degree of eosinophilia rapidly reject *N. brasiliensis* infection (Shin *et al*., 1997) but are not different from wild type mice during *T. spiralis* infection (Hokibara *et al*., 1997). The discrepancy in various studies examining the role of eosinophils in protective immunity in different animal models of parasitic infections may be attributed to the redundancy of effector immune responses or to the basic differences in the animal models used.

Recently, important advances have been made in obtaining tools to dissect the ruminant immune responses to nematode infections and our understanding of this response has increased considerably in the last years. For example, monoclonal antibodies specific for ovine IgE have become available (Kooyman *et al*., 1997), several cytokines (such as IL-3, IL-4, IL-5, IL-5) have been cloned, sequenced and expressed (Wood and Seow, 1996) and potential protective antigens have been identified (Newton and Munn, 1999). Accordingly, similar immunological mechanisms to most natural murine models are said to be involved

during GI parasitism in large animals such as sheep. The blood feeding abomasal parasite of sheep, *Haemonchus contortus,* is reported to induce Th2-polarized immune response with characteristic effector cells and antibody responses (Lacroux *et al*., 2006). However, the protective role of these intricate immune effector mechanisms is often difficult to demonstrate. Differences in experimental protocols often account for variations in the outcomes of various studies where animal breed, parasite isolate and dose, frequency of infections, parameters under study and sampling schedules are different.

The experimental protocols adopted to study the role of eosinophils in sheep in this thesis include two classical techniques, *in vitro* and *in vivo*. They were designed in such a way that: one technique supports the other, they allow comparisons between successive experiments, avoid biases arising from number and isolate of infective larvae used, timing of parameter assessment etc. and ensure similar qualitative and quantitative measurements employed throughout the study period.

In all *in vitro* studies, eosinophils of approximately similar density (<1.090 g/ml) were used so that comparisons in their functional activity (larvae immobilisation/killing) could be possible. It is believed that the density of eosinophils depends on their activation status, hypodense eosinophils being more activated than normodense eosinophils (Owen *et al*., 1991; Yukawa *et al*., 1989). *In vitro* efficiency of eosinophils is also known to depend on the presence of various serum elements such as specific antibodies and complement (Rainbird *et al*., 1998; Capron *et al*., 1987; Khalife *et al*., 1985). Hence, the inclusion of immune and non-immune sera as well as heat inactivation of serum-complement has allowed us to determine the optimal conditions required for eosinophil-mediated larval immobilization and killing. Staining with eosin Y has further permitted us to see the extent of eosinophil adhesion to larvae *in vitro*, and avoided resorting to techniques that are more sophisticated. Furthermore, the intra-abomasal administration of exsheathed larvae incubated with eosinophils and other blood cells has greatly supported the confirmation of our *in vitro* observations and served as a link between *in vitro* and *in vivo* methods. For example, when newly excysted juvenile *Fasciola hepatica* were incubated with rat cells and serum, cells were seen to adhere to the flukes, but when the flukes were transferred to the peritoneal cavity of naïve rats, recovery from the livers three weeks later was unaffected by the treatment (Doy and Hughes, 1982).

On the contrary, viability of worms was greatly reduced when mice were administered with schistosomula of *S. mansoni* cultured *in vitro* with eosinophils (Dessein *et al*., 1983).

In vivo, several experimental infections were performed in order to evaluate the development of the parasite in sheep and the diverse immune response mounted against them. Animal age and parasite strain can influence the outcome of infection (Aumont *et al*., 2003; Colditz *et al*., 1996; Bellaby *et al*., 1995; Manton *et al*., 1962). Hence, attempts were made to minimize variations in the age of experimental animals. A single strain of *H. contortus* (Humeau strain maintained in a susceptible breed of sheep), and larvae of approximately similar age after culture were used. Parasite development and expression of anti-parasitic immunity are dynamic processes. Hence, examinations of different parasitological and immunological parameters were made at approximately regular time intervals. The identification of IL-5 as a key growth and differentiation factor responsible for the generation of eosinophils in the bone marrow (Ishihara *et al*., 2000), and the subsequent cloning and expression of the gene encoding IL-5 (Mertens *et al*., 1996) allowed the production of specific reagents to study eosinophil responses *in vivo*. While the use of transgenic mice and mice with targeted gene deletions especially for IL-5 has allowed an in-depth exploration of the effect of increasing or decreasing eosinophilia, such tools are yet to be developed for sheep. In our experimental studies, attempts were made to follow similar approaches but with available alternative means.

First, increased blood eosinophilia was induced by the administration of *Oestrus ovis* larvae (Yacob *et al*., 2002) prior to nematode infection. *O. ovis* and *H. contortus* are phylogenetically distinct (Diptera and Strongylida respectively), occupy different anatomical niches (naso-sinusal cavity and abomasum respectively) and depend on different nutrient sources (mucous secretions and blood respectively) hence direct competitive interference is assumed to be practically inexistent. This allows us to monitor the effect of increased eosinophilia on *H. contortus* development in the abomasum with very minimal interference from the nasal bot. Second, the inverse approach was made by using the non-steroid anti-allergic drug, sodium cromoglycate (SCG). Different from corticosteroids that suppress the general immune system, SCG targets mainly mast cells (Nemmar *et al*., 2004; Shin *et al*., 2004; Hoshino and Nakamura, 1997), which are one of the early sources of various inflammatory mediators and cytokines among which is IL-5 (Bischoff, 1996; Okayama *et al*.,

1995). As is the case for human gastrointestinal allergy, blocking mast cell degranulation by SCG was assumed to consequently reduce levels of IL-5 and eosinophilia at least in the early phase of nematode infection. The third approach was to compare two naturally resistant and susceptible breeds of sheep, the former being characterized by high blood eosinophilia both before and after infection compared to the latter.

In spite of all efforts made to standardize our methodology, limitations, which deserve some explanation, have been encountered. The availability of acceptably pure and viable eosinophils is an essential prerequisite for the study of their role *in vitro*. However, the commonly applied techniques (density gradient centrifugation and immunomagnetic methods) to isolate eosinophils from blood of other species did not appear to fit to isolating pure sheep eosinophils. The human anti-CD16 antibody failed to recognize sheep neutrophils during repeated trials to isolate eosinophils by the immunomagnetic method (Hansel *et al*., 1991). Using the low speed percoll density gradient centrifugation described by Woldehiwet *et al*. (2003) we were able to isolate between 40 and 60% eosinophils, the maximum being at around peak blood eosinophilia following infection. The difficulty in isolating pure eosinophils has forced us to investigate the level of interference by contaminating cells mainly lymphocytes and neutrophils *in vitro* and this was further extended to verification through intra-abomasal infection of sheep with larvae incubated with the different cell types. This method was originally designed to isolate sheep granulocytes (mixture of neutrophils and eosinophils) during studies on *Anaplasma phagocytophilum* where, according to the authors' observations, a layer of eosinophils was found under the mononuclear layer at the percoll-PBS interface. However, to our knowledge, there is no further report on the utilization of this technique for isolating sheep eosinophils. The failure to isolate pure eosinophils has also made us to include some precautionary measures during our eosinophil peroxidase assays. Neutrophils are known to produce the granule protein myeloperoxidase (MPO), which has about 70% homology to EPO (O'Brien, 2000) at the amino acid sequence level. The interference of MPO in our EPO assay was thus minimized by increasing working pH to 7 and a parallel employment of the EPO specific inhibitor, resorcinol. The activity of MPO to oxidize H_2O_2 in the presence of halides is minimal at pH 7, while inhibiting EPO activity by resorcinol allows determining the reaction contributed by the presence of MPO (Schneider and Issekutz, 1996).

Lack of sufficient number of experimental animals that could fulfil our requirements was another problem encountered during our studies. Our studies required animals raised worm-free, and these were generously provided by the INRA research station (domaine INRA de la Sapinière) where ewe lambs were always kept for breeding purposes. All experimental groups were constructed of 4-6 animals. Sample size varied depending on the number of ram lambs born in each season. Except in one case where we were able to incorporate both male and female sheep, the rest of experimental infections have been performed on male animals. Although, the general immune mechanism could be similar, differences that could arise from differences in hormonal and reproductive factors could not be determined.

2. EOSINOPHILS ARE CAPABLE OF KILLING *H. CONTORTUS* INFECTIVE LARVAE *IN VITRO*

The potential of sheep blood eosinophils to kill *H. contortus* infective larvae has been demonstrated by their ability to greatly reduce larval mobility *in vitro* and the failure of such larvae incubated with eosinophil-enriched leucocytes to establish after being deposited directly in the abomasal lumen. The majority of cells closely adherent to larval surface *in vitro* were eosinophils as confirmed by eosin staining and electron microscopic examinations. In addition, the effect of neutrophils and lymphocytes on larval mobility was minimal although the former has shown some adherence to the larvae. The intra-abomasal administration of larvae, after incubation *in vitro* with eosinophil-enriched leucocytes derived from primary-infected sheep, has clearly demonstrated that not only the larvae that were considered immobile but also the majority of those larvae that were initially counted as mobile were incapable of establishing in a susceptible host. Better worm establishment was observed when larvae were incubated with serum alone or with neutrophils and lymphocytes as compared to those incubated with eosinophils. This is in line with the observations of Dessein et al. (1983) on *Schistosoma mansoni* in mice. Evidences of *in vitro* eosinophil-dependent killing of larvae of several helminth species have long been documented in rodent models (Butterworth, 1984; Kazura and Grove, 1978). Purified eosinophil granule proteins such as eosinophil peroxidase are also capable of killing schistosomula of *S. mansoni in vitro* (Jong *et al*., 1981).

Although, eosinophils possess extremely potent mediators that can damage and kill infective larval stages of most if not all helminth parasites, a number of factors appear to determine the functionality of these cells. We have demonstrated that eosinophils alone are less efficient in adhering to and in reducing the mobility of *H. contortus* larvae *in vitro*. The addition of serum (irrespective of the status of the serum, immune or non-immune) results in a dramatic increase in the larval immobilizing ability of these cells, but serum alone was not sufficient to immobilise *H. contortus* L3. Therefore, it was assumed that serum components are essential for sheep eosinophils to adhere to infective larvae and subsequently cause significant immobility *in vitro*. This is in agreement with a previous work (Rainbird *et al*., 1998) in which eosinophils derived from antigen-inoculated mammary gland of sheep had immobilised *H. contortus* infective larvae in the presence of immune serum and complement. Furthermore, heating the immune or non-immune serum (complement inactivation at 56°C, 1hr) has been found to greatly reduce eosinophil adherence and their effect on the larvae. In addition, significant reduction in larval mobility was observed when larvae were incubated with eosinophils in the presence of foetal lamb serum (FLS). These, collectively suggest that sheep eosinophils can function with serum components (such as complement) other than larvae-specific antibodies. Complement dependent eosinophil-mediated larval killing was reported in mice implanted with diffusion chambers containing *S. stercoralis* L3 (Brigandi *et al*., 1996). In a study on *T. spiralis* in mice, it was found that newborn larvae are more efficient in complement activation (primarily via the alternative pathway) than the infective stage and adult parasites (Hong *et al*., 1992). Complement binding on *T.spiralis* and *N. brasiliensis* infective larvae *in vitro* has also been shown by direct immunofluorescent staining (Shin *et al*., 2001; Stankiewicz *et al*., 1989). On the other hand, Brigandi, *et al*. (1996) have demonstrated in *S. stercoralis*-infected mice that eosinophils, complement (C3) and immunoglobulin (IgM) act in concert in the development of resistance to the parasite, and complement activation was via the classical pathway. However, the importance of complement in the cell-mediated immune response appear to be stage-specific in that complement activation and leukocyte adherence were greatly reduced as *N. brasiliensis* develops within the murine host (Giacomin *et al*., 2005). In addition, ultrastructural study of the interaction between eosinophils and third and fourth stage larvae of *Onchocerca volvulus*

showed that rapid adherence of eosinophils to and degranulation onto the L3 was observed while no interaction was seen with fourth stage larvae (Strote *et al*., 1990).

The fact that eosinophils can function effectively in the apparent absence of worm-specific antibodies generally suggests their involvement in the innate immunity against *H. contortus* infection in sheep. Granulocytes such as eosinophils as well as complement proteins are considered important components of both innate and adaptive immune responses (Morgan *et al*., 2005; Herbert *et al*., 2000).

The activation status may also influence the potential of eosinophils to cause larval killing. *In vitro* comparisons of the larval killing efficiency of eosinophils from resistant and susceptible breeds of sheep after primary infection have shown that eosinophils from the resistant breed at D_{14} were more responsive to immune serum (IMS) than those at D_0 and more efficient with IMS than with FLS. This may suggest enhanced activation of these cells when compared to their pre-infection levels. Activated eosinophils from human subjects and rodent species are known to upregulate surface receptors for immunoglobulins, complement and cytokines (Bochner, 2000; Takafuji *et al*., 1998; Mawhorter *et al*., 1996; Capron, *et al*., 1987, 1981; Khalife, *et al*., 1985). However, whether sheep eosinophils have similar composition of receptor molecules is yet to be demonstrated. Rainbird *et al*. (1998) have also shown that primed eosinophils obtained from repeated intra-mammary infusions of *H. contortus* L3 were more potent in reducing larval mobility *in vitro* than unprimed eosinophils. We have also demonstrated that the quantity of EPO increases over time in a resistant sheep infected with *H. contortus*. In asthmatic subjects, activated blood eosinophils are reported to elaborate increased quantity of cationic proteins such as eosinophil-derived neurotoxin (Sedgwick, *et al*., 2004), and EPO and other granule proteins are important in the defence against helminth infections in mice (Specht, *et al*., 2006; Buys *et al*., 1981).

Therefore, it is possible that an increase in the levels of activation and stored toxic granule proteins could be responsible for the time-dependent increase in larval immobilizing ability of sheep (at least in the resistant breed) eosinophils.

3. EOSINOPHILS DURING EXPERIMENTAL *H. CONTORTUS* INFECTIONS IN SHEEP

Parasites use host resources for their own benefit that, in turn, can decrease host fitness through reductions in growth, reproduction and survival. To mitigate these effects, animals have evolved complex immune systems that include innate and adaptive components. Because each stage of parasite development can be antigenically distinct, the host response to helminth infection is often characterized by a series of discrete immune responses that evolve at different times during the course of infection, and can be expressed in terms of immunological (cytokines, effector cells and antibodies) and parasitological (worm count, development and fecundity) parameters. The composition and levels of these parameters vary according to breed (Amarante *et al*., 2004), age (Colditz *et al*., 1996), sex (Gauly *et al*., 2006) and physiological status (Tembely *et al*., 1998) of the animals. On the other hand, parasites have evolved with various strategies to modulate and circumvent the host's innate and adaptive immune responses. Examples are the secretion of proteins by *Schistosoma* eggs that bind certain chemokines (Smith *et al*., 2005) and by *H. contortus* and *O. ostertagi* that serve as chemoatractant for tissue inflammatory cells (Wildblood *et al.,* 2005; Klesius *et al.,* 1989).

We have demonstrated that infections of sheep with *H. contortus* trigger the development of a Th2-biased immune response with characteristic cytokine (IL-4, IL-5 and IL-13) gene expressions. The Barbados Black Belly (BBB) breed of sheep known for its resistance to this parasite was found to have more pronounced and persistent gene expressions for these cytokines (more importantly IL-5) as compared to the susceptible breed INRA 401 (INRA). In all the experimental infections undertaken during our studies, an increase in eosinophil recruitment in blood and abomasal tissues was the hallmark of *H. contortus* infection in sheep irrespective of the breed. Gene expression for IL-5 in the BBB was found positively correlated to eosinophil number in the abomasal fundic mucosa. This is characteristic of most if not all helminth infections in mammalian hosts (Behm and Ovington, 2000; Meeusen and Balic, 2000). The resistant breed had higher blood eosinophil levels throughout the infection periods, and relatively more eosinophil infiltration in the abomasal pyloric lamina propria two weeks post-primary infection than the susceptible breed. Similar findings were reported where several independently selected lines of sheep bred for increased resistance to gastrointestinal

parasite infections also showed greater eosinophilic responses after infection compared with random-bred or low-responder flocks (Buddle *et al*., 1992; Gill *et al*., 1991; Dawkins *et al*., 1989). However, this correlation was observed only after priming of sheep with natural or experimental infections. Yet, the involvement of these cells in the resistance to *H. contortus* in sheep may depend on various factors. Stage of infection and immunization history may determine levels of eosinophil activation and tissue recruitment, and progress of worm development as well.

Early expulsion and/or killing of worms in the gastrointestinal tract can occur at different stages. In a comparative study in resistant (BBB) and susceptible (INRA) breeds of sheep, animals were killed 4 days post infection to see the immune response in relation to worm establishment following infection with 10,000 L3. It was found that very few eosinophils and mast cells were recruited into the abomasal mucosa together with low levels of cytokine gene expressions at this stage. Yet, a substantial difference in worm number (exclusively L4) was observed between the two breeds. The same was true for re-infected groups at day 4, but with a slight increase in cell number (eosinophils and mast cells) and insignificant improvement in worm expulsion when compared to primary-infected sheep. This could mean that the small number of resident/recruited eosinophils and other inflammatory cells from the resistant breed were more efficient in reducing parasite burden at this very early stage. Alternatively, such a difference may have been caused by other factors (level of L3 exsheathment in the rumen and effect of abomasal mucus secretions etc.) well before the L3 penetrated the gastric glands. In one of our *in vitro* studies, we have demonstrated that in the early stage of infection, there was no difference in the larval killing activity of eosinophils between resistant and susceptible breeds, but whether a similar scenario is true for tissue residing eosinophils remains to be elucidated. Whereas in most natural host-parasite systems non-specific inflammation is minimal, this is significantly increased in unnatural or nonpermissive hosts, even in closely related rodent species. For example, primary infection of *Strongyloides ratti* in rats induces a minimal cellular response in the skin and repeated priming infections are required to induce immediate and pronounced cellular changes (including eosinophil recruitment) and immunity (Moqbel, 1980). In contrast, mice exhibit a significant inflammation in the skin after primary infections with *S. ratti* and eosinophils and neutrophils are recruited within hours after a secondary exposure, resulting in larval killing by 24 hours (Dawkins *et al*., 1981). Hence, for

eosinophils to be involved in early larval killing in tissues, repeated immunizations seem essential so that they can rapidly be mobilized in sufficient number towards the site of parasite invasion before the parasite develops to advanced stages. Balic *et al*. (2006) reported that following repeated natural and experimental immunizations, the administration of a large number of *H. contortus* infective larvae (10^6) in sheep resulted in a massive mobilization of eosinophils around the invading worms 24-48 hours after larvae administration. Large numbers of eosinophils were found attached to dying or damaged larvae in the abomasal mucosa.

For effective larval establishment in the abomasal mucosa to occur the infective larvae must be exsheathed, a process that commonly occurs in the fore-stomach (rumen). Evidences from some previous studies show that a proportion of the third stage larvae of *H. contortus* cannot leave their sheath (Dakkak and Dorchies, 1984, Dakkak *et al*., 1981) and hence are incapable of feeding and surviving in the abomasum. Mucus secretions may also play their share in promoting L3 exclusion (Claerebout *et al*., 1999; Harrison *et al*., 1999; Newlands *et al*., 1990), collectively suggesting the presence of other potential factors that can influence early worm expulsion in the presence or absence of major involvement of eosinophils after primary infection of sheep with *H. contortus*. Whether these factors are employed differently in the two breeds is yet to be investigated.

At a later stage of *H. contortus* infection, the situation may take a different course. Exsheathed infective larvae that have managed to penetrate the abomasal wall, subsequently lodge into abomasal glands and moult within 2-3 days to the next larval stage (L4). The abomasal inflammatory responses mediated by cellular (eosinophils, mast cells) and humoral (immunoglobulins) components will have some time to be mobilized to the area and combat the invading larvae. *H. contortus* L3, by preference, develops in the first instance in the glands of the fundic mucosa (Rahman and Collins, 1990). The consistent finding of eosinophil infiltration and mastocytosis in this region of the abomasum 16 and 30 days after infection indicates the deployment of defensive forces to the area some time before the worms were withdrawn from the glands. Treatment of infected animals with anthelmintics caused a significant decline in blood eosinophil numbers suggesting the presence of the parasite in the abomasum is essential to induce eosinophil mobilization. Though we were not able to demonstrate the interaction between eosinophils and tissue larvae at early stage, data from our

in vitro observations strongly support that these cells have undoubtedly battled with some if not all of tissue stage larvae.

The late L4 stage, after a maximum of 7-11 days stay in the glands, emerges into the lumen and evolves into the immature (L5) and then adult stages (Rahman and Collins, 1990) that are less accessible to cellular damage compared to the tissue phase larvae. In murine models, repeated administration of rabbit anti-mouse eosinophil serum to *T. spiralis*-infected mice had no effect on spontaneous expulsion of adult worms from the small intestine, but the numbers of larvae in the muscles were doubled suggesting the importance of eosinophils in the resistance to the systemic phase of trichinosis (Grove and Mahmoud, 1977). Furthermore, Kazura and Grove (1978) have reported stage-specific antibody-dependent eosinophil-mediated destruction of *T. spiralis* where mouse eosinophils were able to damage the newborn larvae but not muscle larvae or adult parasites *in vitro*.

However, little is known on the mechanism by which sheep regulate lumen dwelling stages of this parasite. Eosinophils were found to follow parasite localization, being more densely populated in the region where more worms are located. Visual observations strongly suggested that the pyloric region of the abomasal luminal surface is a preferred location for immature and adult stages of *H. contortus*. Our observations at day 16, and retrospective analysis of data from other experimental infections in which we examined both fundic and pyloric mucosal tissues for eosinophil counts at day 30 post-infection (Publication No. 3) consistently show significantly higher number of eosinophils in the pyloric region. The presence of an already sensitized immune system (IL-5, eotaxin) and the possibility of chemotaxis by worm secretions (Wildblood *et al*., 2005; Klesius *et al*., 1989) seem to allow eosinophils to be mobilised towards this new location while their number in the fundic region gradually wanes because they are no longer important at this site. This, together with the recruitment of intraepithelial eosinophils and globule leucocytes reported in our studies and other previous works (Balic *et al*., 2006, 2000; El-Malky *et al*., 2003), may potentially contribute to late-stage-worm expulsion and regulation of their life cycle, probably in concert with immunoglobulins and complement (Capron *et al*., 1987; Khalife *et al*., 1985). Although we were not able to demonstrate appreciable levels of *H. contortus*-specific IgA antibody in the abomasal mucous during our studies, IgA-induced eosinophil degranulation (Abu-Ghazaleh *et al*., 1989) may explain the concerted action of eosinophils and immunoglobulins.

The importance of mucosal IgA in the reduction of worm size, worm number and FEC has also been suggested from negative correlations observed in various studies in sheep (Henderson and Stear, 2006; Amarante *et al*., 2005) and may explain the role of immunoglobulins in GI parasitism. Blood and/or abomasal eosinophil numbers were correlated to worm burden positively at day 16 and negatively at day 30 post primary infections collectively indicating an *in vivo* effect of eosinophils during primary infections may be a cumulative process rather than an instantaneous action as simulated in the *in vitro* tests. Negative correlations observed in most instances between blood and tissue eosinophilia, and parasite traits such as FEC, female worm length and fecundity may also suggest that eosinophils are important in regulating the development and fecundity of worms that have survived immune rejection (killing). These results are in agreement with a number of previous reports (Amarante *et al*., 2005; Stear *et al*., 2002; Doligalska *et al*., 1999). In this respect, when animals of different age groups were compared, FECs were inversely related to blood eosinophilia and age of the animals. Similar findings were reported after vaccinating sheep of different age-groups where older animals demonstrated higher antibody titres and eosinophilia, and increased protection compared to younger animals (Vervelde *et al*., 2001; Kooyman *et al*., 2000; Salman and Duncan, 1984).

To further explore the roles of eosinophils during *H. contortus* infection, two independent experimental infections were carried out, one in which blood eosinophilia was increased prior to nematode larvae administration and the other in which development of blood eosinophilia was suppressed during infection.

Prior exposure of a susceptible breed of sheep to *O. ovis* larvae few weeks before the arrival of *H. contortus* has resulted in an increase in the number of circulating and abomasal mucosal eosinophils compared to those animals infected with the nematode alone. Interestingly, in animals, which have received the nasal bot alone, a slight but significant increase in the number of resident abomasal mucosal eosinophils was noted suggesting a change in the microenvironment of the abomasum, which could potentially influence the incoming *H. contortus* larvae. Parallel to these observations, it was found that in co-infected animals, *H. contortus* female worm length and fecundity were greatly reduced compared to the mono-infected group but worm number was not affected by the concurrent infection. This could be either due to the increase in number and/or the enhanced non-specific activation of

eosinophils caused by the presence of *O. ovis* larvae in the nasal cavity although the contributions of other cells and immune components cannot be ruled out. Here, numbers of blood and tissue eosinophils were negatively correlated to worm number, FEC, female worm length and fecundity while mast cell and globule leucocytes were negatively associated to abomasal worm burden. It seems that, if worms arrive in an environment where an increased number of eosinophils is consistently prevalent, it is likely that their life cycle could be affected. Similar observations were reported where the development of *Trichostrongylus colubriformis* is suppressed by the presence of *O. ovis* (Yacob *et al*., 2002). Likewise, some IL-5 transgenic mice expressing a high degree of eosinophilia are known to rapidly expel helminth infections and/or influence worm development and fecundity in several instances (Martin *et al*., 2000; Daly *et al*., 1999; Shin *et al*., 1997).

On the other hand, treatment of a resistant breed of sheep (BBB) with the non-steroid anti-allergic drug, SCG, has caused a significant reduction in bone marrow eosinophil production (counted 30 days after nematode infection) and a transient reduction in the level of blood eosinophilia between days 12 and 18. In addition, numbers of abomasal mast cells and globule leucocytes were reduced, but tissue eosinophil number was similar between treated and non-treated groups 30 days post-infection. Such reductions in inflammatory responses tend to favour increased FEC and worm number after infection of sheep with *H. contortus*, although the difference was not significant when compared to the non-treated group. Treatment level was based on the dose prescribed for human subjects. Therefore, the amount of drug administered might have been less than what is required to cause a substantial immune suppression and thus to result in a significant difference between treated and non-treated groups. Alternatively, it might simply be due to the involvement of other immune components that have possibly compensated for the reduction in the eosinophil and mast cell responses. In mouse models, where animals were depleted of their eosinophils by anti-IL-5 monoclonal antibodies or by targeted gene deletions for IL-5 and/or IL-5R, there are instances where parasite development was favoured (Herbert, *et al*., 2000; Rotman *et al*., 1996). There are also reports where mice have rejected challenge infections normally compared to non-treated or their wild type counter parts (Betts and Else, 1999; Herndon and Kayes, 1992). It is possible that other host defence mechanisms against helminths are sufficiently redundant that eosinophil ablation is not deleterious (Weller, 1997).

4. CONCLUSION AND PERSPECTIVE

Many indications from the *in vitro* studies show that sheep eosinophils are capable of killing *H. contortus* infective larvae often in concert with complement and antibodies. This ability, as reflected by larval immobilization, appears to be similar between resistant and susceptible breeds at least until two weeks post-infection. However, eosinophil activation and hence their potency seems enhanced in a relatively short period during primary infection in the resistant breed. Whether the level of eosinophil activation and larval killing ability could be maintained, enhanced or downregulated with time beyond day 16 remain a matter of further investigation.

In vivo, early expulsion of a certain proportion of larvae from the abomasum in the primary infection is dependent on factors that favour innate immunity, which may or may not involve eosinophil-mediated resistance depending on the time course of the infection. In the later stage of *H. contortus* infection, where tissue- and lumen-dwelling stages are exposed to increasing levels of cellular and humoral immune responses, eosinophils could largely participate, in a time-dependent manner, in the regulation of worm survival and development. The dramatic increase in the number of eosinophils in blood and tissues, the significant reduction in worm development when eosinophil number and activation was enhanced together with the consistent statistical correlations observed, strongly support the protective role of these cells during *H. contortus* infection. Furthermore, the high blood eosinophilia in the resistant breed and the increase in eosinophil number with increasing age, both being associated to enhanced resistance, may suggest enhanced immunological competence.

Although, efforts have been made to generate as much information as possible (in a limited time frame) on the potential role of eosinophils in the immune response to *H. contortus* infections in sheep, a lot more remains to be done before a plausible conclusion can be drawn.

Observations in the early phase of primary infection suggested that non-eosinophil factors could be involved in worm rejection. Hence, further studies are needed to dissect the innate immune response at the mucosal level, including secretory and motor functions, both of which can be important in early worm expulsion. It is also essential to see larval exsheathment potential in resistant and susceptible breeds of sheep as this could partly

contribute to the early elimination of infective larvae from the gut. This can be done by comparing the number of exsheathed larvae arriving at the omaso-abomasal orifice after oral infection (Dakkak *et al*. 1981). A study was conducted on the fate of exsheathed *H. contortus* infective larvae injected directly into the abomasal lumen of naïve and hyperimmune sheep (Miller *et al*., 1983). It was found that 90% of the larvae were lost within 48 hours of challenge from the immune sheep while 50% of larvae entered glandular tissue within four hours of challenge. Hence assessing the establishment potential of intra-abomasally administered exsheathed larvae could also provide valuable information.

To obtain a better image of eosinophil-parasite interactions *in vitro*, improving our eosinophil isolation method seems essential in the future. For this, the fluorescence-activated cell-sorting technique (FACS) that can be used for identifying and separating different cell types (Takizawa *et al*., 2006) may be tried with careful scrutiny on cell viability. Once this is achieved, the individual contribution of complement proteins, immunoglobulins and IL-5 to eosinophil activation and adherence to larvae can be assayed by using either commercially available or locally purified proteins of each kind. Aided by immunofluorescent techniques such as for complement, the adsorption of these elements on larval surface can be traced (Shin *et al*., 2001). While human eosinophils are known to express a plethora of surface receptors for complement, immunoglobulins, cytokines and chemokines, little is described for sheep eosinophils (Pastoret *et al*., 1998) suggesting the requirement for further investigation. The use of available monoclonal antibodies against known receptor molecules from other species (human, mice) may provide some insight into the cooperative action of eosinophils and important serum proteins (complement, specific antibodies, cytokines and chemokines).

Currently, transgenic or gene knockout sheep are not available with regard to *in vivo* studies on eosinophils and associated cytokines (such as IL-5) and chemokines (such as eotaxin). However, the use of anti-IL-5 and anti-eotaxin monoclonal antibodies and purified IL-5 and eotaxin can be envisaged to be of use in the study of eosinophil production and tissue recruitment. Ovine IL-5 is known to have 79% and 65% similarity to human IL-5 at DNA and protein levels respectively (Pastoret *et al*., 1998). Recent studies in the treatment of asthma and other allergic diseases in human subjects have identified the potential of anti-IL-5 antibodies, and peripheral and tissue eosinophilia have been reported to be successfully reduced in treated individuals with the antibody (Sutton *et al*., 2005). In the absence of such

tools specific to sheep, this could serve as possible starting material for future studies although the outcome could not be predicted due to species differences. Bao *et al*. (1996) have described the detection of sheep intestinal lamina propria eosinophils expressing IL-5 mRNA by in situ hybridization techniques using a murine IL-5 riboprobe and subsequent staining with eosin. The application of such methods on the abomasal eosinophils in conjunction with the expression of activation markers such as receptor molecules for the above immune mediators will have paramount importance in the understanding of eosinophil functions *in vivo*.

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TRENDS AND CHALLENGES IN THE EFFECTIVE AND SUSTAINABLE CONTROL OF *HAEMONCHUS CONTORTUS* INFECTION IN SHEEP. REVIEW

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Summary:

Haemonchosis, with its very wide distribution, has become a very important production constraint in sheep farms in tropical, subtropical and temperate regions worldwide. Various intrinsic and extrinsic factors determine the survival of *Haemonchus contortus* and hence the development of the disease in the animal. In general, control of gastrointestinal nematode infestation in sheep relies heavily on anthelmintic treatments. However, the indiscriminate use of these drugs has led to the widespread emergence of drug resistant strains of parasites, that has necessitated the development and use of various parasite control methods such as grazing management, biological agents and vaccines and the selection of resistant breeds of animals, with or without moderate use of anthelmintics. The ultimate goal of such control programs is to enhance productivity, while minimising risks regarding drug resistance and consumer and environmental concerns. This review attempts to highlight the different methods employed in the control of haemonchosis in sheep and the practical limitations associated with both control programs and the internal and external factors associated with the parasite and its microenvironment.

KEY WORDS : *Haemonchus contortus*, sheep, anthelmintic, biological control, breed resistance.

Résumé : MOYENS DE LUTTE CONTRE *HAEMONCHUS CONTORTUS* CHEZ LE MOUTON. REVUE BIBLIOGRAPHIQUE

*L'hémonchose est une infestation parasitaire très répandue chez les petits ruminants et est un facteur limitant important de la production aussi bien dans les régions tropicales que subtropicales, voire tempérées. Divers facteurs intrinsèques ou extrinsèques déterminent la survie d'*Haemonchus contortus *et donc l'épidémiologie de l'infestation. La lutte contre les nématodoses gastro-intestinales du mouton repose principalement sur l'emploi d'anthelminthiques. Cependant, leur emploi à grande échelle a abouti au développement de résistances qui obligent à recourir à d'autres méthodes de lutte par la gestion du pâturage, par l'utilisation de champignon nématophage, ou par la sélection de souches ovines naturellement résistantes à* H. contortus. *L'objectif de ces moyens de contrôle est d'augmenter la productivité, tout en limitant les risques de résistance et les risques potentiels pour l'environnement ou les consommateurs. Cette revue présente les différentes méthodes utilisées pour la lutte contre l'hémonchose et souligne leurs limites, en particulier dans la pratique de l'élevage.*

MOTS CLÉS : Haemonchus contortus, *mouton, anthelminthique, contrôle biologique, race résistante.*

INTRODUCTION

While in temperate regions, the severity of gastrointestinal (GI) parasitic diseases in most livestock farms is now minimised through the seasonal use of anthelmintics and pasture management, the problem still persists in the vast majority of tropical and subtropical regions. Among the GI parasites, *Haemonchus contortus* is the species with greatest pathologic and economic importance. This nematode is a blood feeding abomasal parasite of sheep and goats but can circulate in other ruminant species such as cattle and reindeer (Jacquiet *et al.*, 1998; Achi *et al.*, 2003; Hrabok *et al.*, 2006). The importance of haemonchosis may be explained by: the parasite's ability to produce large numbers of eggs, which results in extensive pasture contamination; the blood-sucking nature of the nematode that causes variable degrees of anaemia, loss of production and mortality in lambs and kids in situations where few or no control measures are used; and its ability to survive adverse climatic conditions through hypobiosis (Gatongi *et al.*, 1998; Waller *et al.*, 2004). The nature of the parasite, host genetic and physiological factors, as well as environmental determinants, greatly influence the degree of infestation and thus appropriate control strategies.

For many years, several methods have been used to reduce the threats resulting from gastrointestinal nematode infestations in general, and haemonchosis in particular, in small ruminants. The use of anthelmintics is the most extensively employed method of control of GI nematodes. However, because of such reasons as emergence of drug resistant strains and problems related to drug residues in food of animal origin and ecotoxicity, a significant amount of research is focusing

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on alternative or complementary control measures (De Liguoro *et al.*, 1996; Strong *et al.*, 1996; El-Makawy *et al.*, 2006). This review is intended to highlight some of the control strategies and associated challenges encountered in limiting the effects of haemonchosis in sheep.

FACTORS AFFECTING *H. CONTORTUS* POPULATIONS

Intrinsic factors (the parasite)

As for any disease process, the parasite, the environment and the host are in a dynamic interaction, the outcome of which depends on various determinants. *Haemonchus contortus* is a highly prolific, blood-feeding parasite with various strategies to escape adverse climatic conditions and immune reactions of the host. A mature female can produce 5,000-7,000 eggs per day while *Trichostrongylus* species produce only 100-200 eggs per day (Coyne *et al.*, 1991a, b). This allows *H. contortus* an advantage over other parasites in that it can easily contaminate grazing areas and may survive in its small ruminant hosts through frequent and rapid reinfestations. Variations in the degree of infectivity of different *H. contortus* isolates have already been documented. A comparison in infectivity between *H. contortus* isolates from France with those from the West Indies (Guadeloupe island) in two breeds of sheep, namely the Black Belly and the INRA 401, has shown that the latter (sympatric isolate) established better than the former (allopatric isolate) in the Black Belly (Aumont *et al.*, 2003) suggesting that it is important to take into account parasite genetic diversity in different agro-ecological zones.

After being ingested, the infective stage (L3) successively moults to L4 and L5 (immature adult) and then matures to the adult stage in the abomasum. With the exception of the L3, all other stages of development feed on blood resulting in a variable degree of anaemia which results not only from the blood consumed by the parasites but also due to haemorrhage after the parasites detach from their feeding sites. *H. contortus* is known to produce calcium and a clotting factor binding substance known as calreticulin (Suchitra & Joshi, 2005), enabling the parasite to feed easily on host blood and in so doing, cause haemorrhagic lesions. Studies of field parasitism and experimental larval infestations have also identified the morphological and physiological effects of abomasal nematodes: nodule development, mucous cell hyperplasia, superficial epithelial damage, reduced acid secretion and increased serum gastrin and pepsinogen concentrations (Simpson, 1997; Scott *et al.*, 1999). When both internal and external conditions are favourable, the pre-patent period for *H. contortus* in sheep is between two and three weeks. However, in extreme degrees of temperatures and relative humidity, the L4 stage may enter into a phase of arrested development or hypobiosis (Reinecke, 1989; Urquhart *et al.*, 1996, Miller *et al.*, 1998). Hypobiosis usually follows the onset of cold autumn/winter conditions in the northern hemisphere (Waller *et al.*, 2004) or very dry conditions in the subtropics and tropics (Gatongi *et a.*, 1998). This might be an environmental stimulus received by the free-living infective stages prior to ingestion by the host, or it could be an evolutionary parasitic adaptation to avoid adverse climatic conditions for survival of the free-living stages by a significant number of parasites remaining as sexually immature stages in the host until more favourable conditions return. Furthermore, Jacquiet *et al.* (1995) also demonstrated that in some extreme climatic conditions, such as in the desert areas of Mauritania, adult *H. contortus* were capable of surviving for up to 50 weeks in the host which guaranteed the perpetuation of the species from one wet season to another.

Extrinsic factors

The environment

Factors including temperature, rainfall, humidity and vegetation cover, influence patterns of parasite development (Coyne *et al.*, 1992; Krecek *et al.*, 1992; Tembely *et al.*, 1997). In most tropical and sub-tropical countries, temperatures in the environment are permanently favourable for larval development. The ideal temperature range for larval development of many nematode species in the microclimate of the pasture or vegetation is between 22 and 26°C while the optimal humidity is close to 100 %. Desiccation from lack of rainfall kills eggs and larvae rapidly, and is the most lethal of all climatic factors. Wet seasons favour the growth of vegetation which in turn attracts grazing sheep and goats leading, not only to further contamination of pasture, but also providing an opportunity for the existing infective larvae to encounter their favourite hosts. By pasture larval assessment in Ghana (Agyei, 1997) and by the use of tracer lambs in Kenya (Wanyangu *et al.*, 1997), it was found that very few or no *H. contortus* infective larvae were available during dry periods while numbers of larvae were high in the rainy seasons and shortly after. Under optimal conditions (high humidity and warm temperatures), the developmental process for *H. contortus* requires about 7-10 days while in cooler temperatures it may be prolonged. In experiments where larvae were cultured from faecal material under ideal laboratory conditions, only 24 to 44 % of the eggs were found to develop to the third larval stage, 10 to 25 % of the eggs did not hatch after 10 days

of incubation while 40 to 55 % of the eggs were assumed to be non-viable (personal observations). Below 5° C, movement and metabolism of L3 is minimal favouring prolonged survival as these larvae are enclosed in a double sheath and thus unable to feed to continuously renew their energy.

NATURE OF THE HOST

• Host breed and immune status

The pre-patent period of *H. contortus* can vary according to the breed and age of infested hosts. Evidence from different experimental infestations have already demonstrated that the first *H. contortus* eggs appear in the faeces of the susceptible INRA 401 breed of sheep, as soon as days 17 and 21 post infestation, instead of 21 and 28 days in the resistant Black Belly breed (personal observation). Though it is still not clear how natural selection might shape patterns of immuno-responsiveness in terms of type and strength of response, different breeds of sheep express different susceptibility to gastro-intestinal parasitic infestations. In this respect, the Santa Ines (Amarante *et al.*, 2004), Barbados Black Belly (Aumont *et al.*, 2003; Gruner *et al.*, 2003) and Texel (Good *et al.*, 2006) breeds of sheep appear to be more resistant to infestation with *Haemonchus contortus* compared with Suffolk and Ile-de-France, INRA 401, and Suffolk breeds respectively. This was shown by reductions in faecal egg count (FEC) and/or worm number, slower worm development and reduced fecundity. Genetic variations in the resistance to *H. contortus* within sheep flocks have also been demonstrated and used in breeding schemes in Australia (Albers & Gray, 1987; Woolaston & Baker, 1996). The development of immunity to gastrointestinal (GI) parasitic infections appears to depend on the host's ability to mobilise both cellular and humoral immune effectors locally at the site of infection and systemically in the blood circulation. *Haemonchus contortus* infestation in sheep is known to elicit a Th2-polarised immune response characterised by Th2 cytokines (Lacroux *et al.*, 2006), the recruitment of higher number of eosinophils, mast cells and globule leucocytes (Balic *et al.*, 2000, 2006) and the development of local and circulating antibody responses (Schallig *et al.*, 1995; Gòmez-Muñoz *et al.*, 1999).

• Host age and sex factors

In addition to genetic factors, animals of different ages and sex respond differently to parasitic infections under similar management conditions. Young animals are generally more susceptible to parasitic diseases than mature animals. It is believed that the lower resistance to disease in young ruminants is partly due to immunological hyporesponsiveness, and is not simply a consequence of their not having been exposed sufficiently to pathogens to develop immunity (Manton *et al.*, 1962; Colditz *et al.*, 1996). Innate immunity, often age-related, is also considered important in many cases. This may be due to physico-chemical differences in the gut environment in adult, compared with young hosts (Mulcahy *et al.*, 2004). In four, 12 and 16 months old Black Belly sheep that have never been exposed to helminth infections, we have observed that *H. contortus* faecal egg excretion following primary exposure was markedly affected by the age of the animals, which was in turn strongly associated with an age-dependent increasing level of blood eosinophilia (unpublished observations). The contribution of the stress associated with weaning in delaying the development of protective immune responses to *H. contortus* and *T. colubriformis* has been documented (Watson & Gill, 1991). On the other hand, previous exposure to *H. contortus* infestation could result in enhanced resistance to subsequent homologous infestations. Gauly *et al.* (2002) for Rhön and Merino land lambs, Aumont *et al.* (2003) for Black Belly and INRA 401 lambs, and Gamble & Zajac (1992) for St. Croix and Dorset lambs, reported that animals infested for the second time have demonstrated better resistance. This may be due to the alteration of immunological and physicochemical mechanisms that even though incapable of controlling the primary infection, is nevertheless able to influence the challenge infection.

The phenomenon of the peri-parturient rise (PPR) in nematode egg output is also of great importance in the epidemiology of GI nematodes of sheep. A temporary loss of acquired immunity to *H. contortus* at around the time of parturition and during lactation has been associated with a marked increase in faecal egg count in ewes. Woolaston (1992), Romjali *et al.* (1997) and Tembely *et al.* (1998) have reported that the PPR in FEC started two to four weeks before lambing and continued into lactation in the post-parturition period. This may be related to increased susceptibility to new infestations and enhanced prolificacy of female parasites arising from stress associated to pregnancy, increased milk production and nutritional factors. The inhibition of immunity during lactation places the ewes at a greater risk of parasitic disease and production loss. A significant reduction in haematocrit and serum albumin concentration in post-parturient ewes that had been experimentally infected with *H. contortus* has been reported (Thomas & Ali, 1983). Furthermore, the PPR in FEC could also contribute to increased pasture contamination. Hence, animals in such a physiological state require special attention in order to avoid losses.

• Nutritional status

There is substantial evidence for a beneficial role of a good plane of nutrition in the resistance or resilience of sheep to GI nematode infections. According to Coop and Kyriazakis (2001), nutrition can influence the

development and consequences of parasitism in three different ways: 1) it can increase the ability of the host to cope with the adverse consequences of parasitism (resilience); 2) it can improve the ability of the host to contain and eventually to overcome parasitism (resistance) by limiting the establishment, development and fecundity of the parasites; and/or 3) it can directly affect the parasite population through affecting the intake of certain antiparasitic compounds. Haile *et al*. (2004) have demonstrated that parasitic infestation adversely reduced dry matter intake and apparent digestibility in Menz and Horo breeds of sheep and that these were improved by high quality feed supplementation such as cotton seed cake and urea-molasses mixtures. Similarly, highly metabolizable protein diets have been shown to increase resistance of Ile-de-France and Santa Ines lambs against *H. contortus* (Bricarello *et al*., 2005). Well-fed animals can withstand the harmful effects of GI parasitism, can remain reasonably productive and may require less anthelmintic treatments when compared with undernourished animals (Knox *et al*., 2006). The major problem in this respect is that haemonchosis is more prevalent in regions where animal feed resources are very scarce and/or improperly managed and therefore insufficient to satisfy the demand throughout the year.

CONTROL OF HAEMONCHOSIS AND ITS CHALLENGES

The aim of most parasite control strategies is not to totally eliminate the parasites in livestock, but to keep the population under a threshold, above which it would otherwise inflict harmful effects on the host population (Larsen, 2000). Any parasite control method aimed at minimising a given parasitic population must consider the basic disease determinants briefly described above. The relative success or failure of any control strategies can be judged in terms of immediate and/or long term objectives, the ultimate goal being increase production, minimising risks regarding drug resistance and consumer and environment associated problems. Generally, nematode control strategies can be directed against the parasite in the host and/or in the environment.

TARGETING THE PARASITE IN THE HOST

Chemotherapy and chemoprophylaxis

Anthelmintic drugs are commonly used either for prophylactic purposes, in which the timing of treatment is based on a knowledge of the epidemiology, or for therapeutic purposes to treat existing infections or clinical outbreaks. Since the advent of modern anthelmintics, tremendous advances have been made in the use of various preparations for different species of animals against diverse spectrum of parasites. The relative success of these drugs depends on their ease of administration, extension of action after administration and period and/or frequency of application based on the epidemiology of the disease problem. In most cases, anthelmintics are administered *per os* in the form of solution, paste or bolus but some of them can be given *via* other routes. In temperate areas, priority is usually given to strategic treatments rather than to a regular interval dosing with anthelmintics. Animals at risk, such as weaned lambs, are often only treated during the first grazing season. In some arid areas where haemonchosis is a problem, first season animals are treated at least twice during the rainy season, four weeks after the onset of the rains and at the end of the rains (Urquhart *et al*., 1996). An additional treatment at the culmination of the wet season may sometimes be necessary. Various drugs have been shown to be successful (almost 100 %) in eliminating *H. contortus* during their early periods of utilisation, and some still remain effective in different parts of the world (Table I). Hence, in the traditional sense of chemotherapy-chemoprophylaxis, we have probably achieved the maximum effect of what is possible from excellent anthelmintics developed by the pharmaceutical industry since 1960, *i.e.* from thiabendazole through levamisole and morantel tartrate, to more advanced benzimidazoles and to the avermectins and milbemycins (Williams, 1997).

For various reasons, however, the efficacy of such valuable and very effective drugs is endangered. Their long term utilisation, inappropriate handling and underdosage may be some of the reasons for their reduced efficacy and for the increasing development of drug resistance. On the other hand, where these drugs are not easily accessible either because of economic reasons or scarcity of veterinary services, as in most parts of Africa, animals die as a result of acute haemonchosis or develop a chronic form of the disease resulting in

Chemical group	Anthelmintics	Prescribed dose
Imidazothiazoles	Levamisole	7.5 mg/kg (Andrews, 2000)
Benzimidazoles	Albendazole	5 mg/kg
	Fenbendazole	5 mg/kg
	Oxfendazole	5 mg/kg (Mckenna & Watson, 1987)
Macrocyclic lactones (avermectins)	Ivermectin	0.2 mg/kg
	Moxidectin	0.2 mg/kg
Salicylanilides	Closantel	10 mg/kg (Uppal *et al*., 1993)

Table I. – List of some recommended drugs against haemonchosis in sheep (Bowman, 1999).

marked loss of body weight and consequent reduced production. This adds another constraint to the already existing poor production performance of small ruminants in such regions.

Risk of drug resistance

Development of drug resistance by populations of *H. contortus* in sheep and goats to repeated applications of benzimidazoles, levamisole and ivermectin, has already been demonstrated (Table II). In most cases where resistance against various anthelmintics has been reported, closantel remained the only efficient drug available, signalling the urgent need to develop alternative measures. *H. contortus* strains resistant to one group of drugs may also be resistant to other groups, which suggests the existence of multiple resistance to the major anthelmintic drugs currently available. In an experimental study, Waruiru (1997) tested the efficacy of closantel, albendazole, levamisole and ivermectin against ivermectin resistant and susceptible isolates of *H. contortus* in sheep. A very impressive result was obtained where all these drugs were almost 100 % effective against ivermectin susceptible isolates, while only closantel proved efficacious on the ivermectin resistant strain. Further alarming findings were also reported, where such resistance in *H. contortus* was found to be inherited as either dominant or recessive traits. According to Le Jambre *et al.* (2000), a completely dominant autosomal trait governs the resistance of *H. contortus* larvae to avermectin while they suggested that in adult worms the expression of resistance was sex-influenced. On the other hand, resistance to levamisole and benzimidazoles has been reported to be inherited as an incomplete recessive autosomal trait (Sangster *et al.*, 1998) and at least two genetic loci (beta-tubulin genes) are involved (Beech *et al.*, 1994). Highly prolific species such as *H. contortus* with short life expectancy of adult worms have a higher risk of developing diverse resistance-alleles due to spontaneous mutations than the less prolific *T. colubriformis* (Silvestre & Humbert, 2002).

Therefore, if effective parasitic treatment with the existing drugs is to continue, more efficient and strategic dosing regimes must be practiced in order to enhance the efficacy or prolong the useful lives of the currently available anthelmintic compounds. Reduction of feed intake before oral anthelmintic treatment slows ruminant digesta flow and premature drug removal (Ali & Hennessy, 1995), whereas administering the normal dose over several hours rather than increasing the amount of drug (Sangster *et al.*, 1991) prolongs availability. Improved drug delivery systems such as the use of chemicals or physical carriers (salts, oils, etc) that reduce drug absorption and metabolism, and that can specifically direct large quantities of active products to the sites of parasite habitat must be adopted (Hennessy, 1997), but these must be cost effective.

OTHER TREATMENT METHODS

Alternative treatment measures such as the use of copper oxide wire particles and medicinal plant extracts have long been used against parasitic diseases with varying apparent success. Briefly, administration of 2.5 to 5 gm of copper oxide wire particles in sheep was shown to reduce *H. contortus* faecal egg counts (Knox, 2002). However, besides its limited usefulness, the use of 4 gm of these wire particles in late pregnancy was reported to threaten the life of multiple born offspring (Burke *et al.*, 2005). On the other hand, plant extracts such as condensed tannins, which are secondary tanniferous plant metabolites, have been found to reduce *H. contortus* faecal egg counts and the number of eggs per female worm in goats (Paolini *et al.*, 2003) and the faecal egg counts, worm number and fecundity of *T. colubriformis* in sheep (Athanasiadou *et al.*, 2000). Similarly, flower extracts of *Calotropis procera* have shown excellent anthelmintic activity against *H. contortus* in sheep (Iqbal *et al.*, 2005). Despite the possible existence of a wide variety of plants species potentially able to control gastrointestinal parasites, the difficulty in the selection of potential candidates and extraction of the active ingredients essential for nematode killing without compromising the health of the animal is still a problem which hinders the development of these resources.

	Country	**Anthelmintics**	**Reference**
Africa	Ethiopia	Albendazole, tetramizole, ivermectin	Sissay *et al.*, 2006
	South Africa	Almost all groups	van Wyk *et al.*, 1997
Europe	France	Benzimidazoles, levamisole	Chartier *et al.*, 1998
	Great Britain	Benzimidazoles	Coles, 1998
Asia	Malaysia	Benzimidazoles, levamisole, closantel, ivermectin	Chandrawathani *et al.,* 1999
South America	Argentina	Benzimidazoles, levamisole, ivermectin	Eddi *et al.*, 1996
	Uraguay	Benzimidazoles, levamisole, ivermectin	Nari *et al.*, 1996
Australia	Australia	Benzimidazoles, avermectin	Green *et al.*, 1981 Le Jambre *et al.*, 1995

Table II. – Some examples of drugs to which resistant strains of *H. contortus* were reported in different countries.

WHICH WAY FORWARD?

Anthelmintics are not always available to all livestock breeders. When they are easily accessible, their usefulness is increasingly hampered by the development of drug resistance. At present, there appears to be no new chemical class of anthelmintics with a unique mode of action, and hence the chemical active products that are currently available are all that we are likely to have for the foreseeable future (Hennessy, 1997). One recently discovered compound, emodepside, which inhibits pharyngeal pumping of nematodes via latrophilin-like receptors (Harder *et al.*, 2003), is very expensive and is only in use for limited animal species such as cats.

Every available option should be exploited to minimise anthelmintic usage. The FAMACHA system developed by South African researchers could be one of the best methods for reducing the necessity of regular anthelmintic treatments against *H. contortus* infections of sheep thereby minimising both the risk of the development of drug resistance and the cost of treatment. This method is based upon the management of *H. contortus* infestation in sheep and goats, through the clinical identification of developing anaemia in individual animals within a flock (van Wyk & Bath, 2002). Accordingly, trials over several seasons showed that most sheep under severe *Haemonchus contortus* challenge required no or only one treatment over a full summer season in contrast to the usual repeated medication of all animals in the flock. An important feature of the system is that it is easy to use at all levels of the farming community. The visual appraisal of anaemia is linked to an identification chart; therefore literacy is not a requirement and the system can be applied throughout the moist tropics/subtropics of the world where *H. contortus* is endemic (Waller, 1999; van Wyk & Bath, 2002). However, while applying the FAMACHA system, one should not ignore other important parasite infestations that may or may not be accompanied by detectable anaemia.

Apart from the use of chemical medications in livestock, other measures targeting mainly the environment but the host are also in practice in order to address the problem of drug resistance and to accommodate the trend towards organic farming designed to produce consumer and environmentally friendly animal products.

TARGETING THE MICROENVIRONMENT

Grazing strategies

Alternate grazing of different host species (Barger, 1997) and alternation of grazing and cropping are management techniques that can provide safe pasture and give economic advantage when combined with anthelmintics. Studies in the wet tropical climates of several Pacific Island countries showed that peak larval concentrations of *H. contortus* and *Trichostrongylus* species occurred on pasture about one week after contamination, but fell to barely detectable levels within nine weeks (Banks *et al.*, 1990). Based on these results, a rotational grazing system was designed, that has resulted in a significant reduction in faecal egg counts as well as in the number of anthelmintic treatments needed per year. However, in many parts of Africa, communal pastoral systems do not allow for regulated grazing as a means of lowering exposure to infective larvae on pasture. Growing human populations and livestock densities, coupled with the frequent drought in some regions, necessitate unregulated animal movement in search of green pasture and drinking water (Abule *et al.*, 2005). In spite of such prevailing chronic problems, particularly in most African countries, livestock owners should be informed of the benefits of conservation of the relatively abundant forage and water available during the rainy seasons. This would reduce the increased susceptibility to parasites due to malnutrition and subsequently reduce exposure to parasites during the period when pastures are scarce and animals are obliged to graze very close to the ground, which predisposes them to pick up more parasite larvae. Exploitation of refugia through alternate grazing of cattle and sheep (Barger, 1997), or sheep and goats (Sissay *et al.*, 2006) to reduce pasture levels of infective larvae or dilute populations of drug resistant strains of parasites, could be of great value in any management program.

Biological control: use of fungal spores

This is a method in which biological agents can be used to reduce the populations of parasites either on pasture or in the host and by so doing minimise the frequency of anthelmintic usage. One example of biological control against gastrointestinal nematodes is the use of some species of nematophagous fungi with the potential to reduce nematode larval populations on pasture by using these either as their main source of nutrients or as a supplement to a saprophytic existence. There are a number of reviews on this topic by Larsen (2006) and Waller & Faedo (1996). Two groups of such fungi have been identified: there are predacious fungi which produce adhesive or non-adhesive nematode-trapping structures and endoparasitic fungi that infect nematodes or their eggs. Among the endoparasitic fungi, those reported to infect *H. contortus* are *Drechmeria coniospora* and *Harposporium anguillulae* while *Arthrobotrys oligospora* and *A. robusta* are predacious fungi of different species of *Haemonchus* (Larsen, 2000). A significant breakthrough in this area was

reported by a number of studies using the species, *Duddingtonia flagrans* (Waller *et al.*, 2001; Fontenot *et al.*, 2003; Chandrawathani *et al.*, 2004; Paraud *et al.*, 2005). This predacious fungus produces three dimensional sticky networks, which tightly traps free-living nematode larvae in the faeces ultimately resulting in their death.

Unlike difficulties associated with the use of other species of fungi, several authors have reported the successful passage of *D. flagrans* chlamydospores in the faeces of sheep after oral drenching (Larson, 2000). Despite its appreciable degree of efficacy, this method of parasite control is still not widely applicable. This may be attributed largely to the requirement for continuous oral or in-feed dosing with fungal spores to achieve the desired level of efficacy (Terrill *et al.*, 2004; Paraud *et al.*, 2005). Also the chlamydospores have a relatively short shelf life (less than one week) in a moist environment, which enables the fungal spores to start to germinate and become vulnerable to degradation during their passage through the animal host (Larsen, 2006).

IMPROVING HOST RESISTANCE

VACCINATION

Control of gastrointestinal parasites by vaccination has been a long-term objective of many parasite research programs. Ideally, vaccines should have a high efficacy and be commercially viable for their proposed use in the livestock sector. A number of GI nematode proteins have been tested as potential vaccine products. In general, these molecules have been divided into two categories. Those termed "natural antigens" or "conventional antigens" are recognised by the host during an infection and are targets of the naturally acquired immune response; the molecules, which are normally not recognised, or which do not induce an immune response during a natural infection but which may serve as targets of the immune response generated against them, are termed "concealed" or "hidden" antigens (Klei, 1997). Natural antigens are constituted mainly of worm surface antigens or excretion/secretion products. Vaccines for *H. contortus* based on natural antigens can generate some level of protection which, although likely to significantly reduce pasture contamination, may not be sufficient to protect young lambs from severe haemonchosis (Newton, 1995).

The majority of concealed antigens of GI parasites described so far are components of epithelial cell surface membranes of the digestive tract of *Haemonchus contortus*. Antibodies directed against these molecules following immunisation and ingestion of blood by the parasites, have proven to be effective in reducing worm burdens (Jasmer & McGuire, 1991; Smith, 1993). In these early studies, a serum transfer experiment suggested that the effector mechanism was serum antibody which bound to the brush border membrane of the parasites intestinal cells; sheep that had acquired immunity to previous *H. contortus* exposure did not recognise the gut membrane proteins, suggesting that these are normally hidden from the host. A more comprehensive review on gut-associated membrane antigens is given by Knox & Smith (2001). One of these molecules, H11, is a 110-kDa integral membrane protein expressed on the intestinal microvilli of the parasitic stages of *H. contortus* and homologues have been identified in *Teladorsagia circumcincta* (Smith *et al.*, 2001). This molecule has been cloned and characterised as an aminopeptidase localised in the brush border of the epithelial cells. The H11 vaccine is apparently effective in all age groups of sheep and against different isolates of *H. contortus* (Jasmer & McGuire, 1991; Newton *et al.*, 1995). Other gut associated antigens such as the 1000kDa *Haemonchus* galactose-containing glycoprotein complex (H-gal-GP) and the 46 and 52 kDa (P46 and P52) glycoproteins have excellent efficacy in reducing FECs (Faecal egg count) and worm burdens (Smith *et al.*, 2000). The cDNAs encoding H11 as well as most of the components of H-gal-GP have been expressed in *E. coli* but, unfortunately, none of these recombinant proteins has been reported to be protective (Knox & Smith, 2001).

Regardless of promising results achieved over the years, especially in terms of vaccine efficacy, we are still waiting for the release of a commercial product. However, its haematophagous nature makes *H. contortus* more prone to gut-associated vaccines compared to other GI nematode parasites; the complex nature of its antigens, involving extensive glycosylation, has probably precluded their molecular cloning at a commercial level. Also, the requirement for various adjuvants and repeated injections that could raise the cost of vaccination, have created considerable difficulties in the realisation of GI nematode vaccines (Klei, 1997; Knox & Smith, 2001).

BREEDING FOR RESISTANCE

The other promising angle, both for the developed and the developing livestock sector, is the selection of breeds or lines of sheep for parasite resistance. There is a sizable body of evidence for the existence of genetic variation in resistance to gastrointestinal nematode parasites both between and within breeds (Gray, 1997) and selection for parasite resistance have been successfully demonstrated in Australia and New Zealand (Barger, 1989; *Bisset et al.*, 1996; Woolaston & Baker, 1996). The benefits of such selection arise from the effects of having fewer and less developed worms

or greatly reduced faecal egg counts, which in turn leads to a reduced impact on production, a decreased requirement for chemical control and a reduced contamination of pasture by infective larvae. In this respect, although possibly lacking the productivity and performance capacity of their counterparts in temperate regions, a number of indigenous tropical breeds of livestock have the genetic ability to tolerate or resist disease, a potential developed through natural selection. The long term exposure to GI nematodes in endemic areas coupled with their adaptation to harsh environmental conditions and low levels of nutrition have allowed them to survive in the regions in which they exist. Some examples of relatively resistant breeds have already been given in this text, but it is likely that there are many other, as yet untested, breeds. Similarly, within populations of animals genetically determined differences in parasite resistance has been reported (Woolaston, 1992). Such animals may serve as a potential nucleus for selecting *Haemonchus*-resistant sheep. A number of markers such as FEC, worm burden, peripheral eosinophil count and serum antibody level have been used to identify animals with increased resistance to infection (Douch *et al.*, 1996), and the results using FEC as a marker are promising. However, as a selection trait, FEC has practical limitations and its use may incur production penalties through withholding drench treatment for prolonged periods. Furthermore, FEC is also influenced by the level and composition of a natural nematode challenge and the expression of the immune response (Douch *et al.*, 1996). Moreno *et al.* (2006) have detected a QTL for resistance to *H. contortus* on ovine chromosome 5 in the INRA 401 x Barbados Black Belly back cross lines. The proximal most likely location of this QTL corresponded to the IL-3/IL-4/IL-5 region. As these cytokines are characteristic of Th2 type immune responses (Lacroux *et al.*, 2006), studies of both the cellular and humoral responses will be of paramount importance in the understanding of the mechanism of resistance (Gill, 1991; Schallig *et al.*, 1995; Colditz *et al.*, 1996; Balic *et al.*, 2000).

Hence, there is still much to be done to understand the mechanisms underlying differences both between and within breeds of sheep in terms of their resistance to helminth infections in order to identify the best markers of resistance for use during the selection processes.

CONCLUSION

Haemonchosis with its very wide distribution has become a very important production constraint in sheep farms in temperate, tropical and subtropical regions of the world. Various intrinsic and extrinsic factors determine the survival of the parasite and hence the development of disease in the animal host. The indiscriminate use of anthelmintics has often led to the widespread emergence of drug resistant strains of *H. contortus*. This has encouraged the use of various parasite control methods such as grazing management, biological agents, vaccines and selected breeds with or without moderate use of anthelmintics. The ultimate goal of such control programs is to enhance productivity while minimising the risk of developing drug resistance and addressing consumer and environmentally associated problems. The problem of haemonchosis in sheep is most severe in tropical areas where the potential of livestock production is very high but unfortunately largely unexploited because of lack of knowledge, complex lifestyles and economic reasons. Hence, any proposed control strategy must take into account the practicalities in the successful application of the program and the economic benefits to the livestock owner. Throughout the temperate regions of the world, grazing management combined with the moderate use of anthelmintics has been used to improve the efficiency of parasite control in livestock. In comparison, there are relatively few examples of such schemes in the tropics/subtropics largely due to reasons specific to each agro-ecology. Therefore, intensive epidemiological surveys are required to adapt available grazing management methods to the existing farming systems of the area. Vaccines, "ethnoveterinary" products and biological control agents are unlikely to be commercialised in the near future. Though these could be of great benefit to the large sheep industries of the world and consequently to the consumers, the prospect of such products reaching poor sheep farmers in the tropics/subtropics is questionable for reasons such as lack of veterinary services and availability. In the short term, selection of sheep for resistance to GI parasitism seems to be the most promising and potentially successful answer to the problem of haemonchosis in the tropics and would involve the maximum exploitation of locally available genetic resources. To make further progress however, the mechanisms governing resistance of different breeds of sheep has to be better understood and due consideration should be given to intensifying research efforts in this area.

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Rôle des éosinophiles dans la régulation des populations d'*Haemonchus contortus* chez le mouton.

Résumé

Les éosinophiles sont des cellules effectrices recrutées dans le sang et dans les tissus lors d'infestations par des helminthes. Nos études expérimentales avaient pour but d'explorer le rôle de ces cellules dans la résistance des moutons à *Haemonchus contortus*. In vitro, les éosinophiles de mouton étaient capables de tuer des larves infestantes d'*H. contortus* en présence de sérum immun ou non. Dans les études in vivo, (1) les infestations expérimentales étaient accompagnées par une réponse immune de type Th2, plus prononcée et plus soutenue dans le temps chez une race résistante que dans une race sensible, (2) les corrélations entre les éosinophilies sanguine et tissulaire d'une part et les paramètres parasitologiques d'autre part étaient positives 15 jours puis négatives 30 jours post-infestation, (3) dans les premiers jours d'une primo-infestation, la participation des éosinophiles dans le rejet des larves semblait minimale, (4) le développement des vers et la fécondité des vers femelles étaient réduites dans un environnement riche en éosinophiles sanguins et tissulaires. L'ensemble de ces résultats indique que les éosinophiles participent à la réponse immune des moutons infestés par *H. contortus*. Cependant, leur importance relative en présence d'autres éléments de la réponse immune dépend de facteurs comme la présence de complément et d'anticorps spécifiques, de la durée et de la fréquence des expositions au parasite.

Mots clés : Mouton, *Haemonchus contortus*, éosinophile, *in vitro, in vivo.*

Role of eosinophils in the regulation of *H. contortus* infection in sheep

Abstract

Eosinophils are effector cells that are consistently recruited in both blood and infected tissues during helminth infections. Experimental studies in this thesis explore the role of eosinophils in the resistance to *H. contortus* infection in sheep. Our results from *in vitro* studies show that sheep eosinophils are capable of killing *H. contortus* infective larvae in the presence of immune or non-immune sera. Data from the series of experimental infections undertaken in the course of our studies indicate the following observations: (1) *H. contortus* infection causes Th2-biazed immune response, which is more pronounced in a resistant breed, (2) correlations between blood and tissue eosinophilia and parasitological parameters were time-dependent, positive two weeks and negative 30 days post-infection, (3) in the early primary infection, the involvement of eosinophils in parasite rejection appears minimal, as tissue recruitment is a relatively late phenomenon and (4) worm development and fecundity are reduced when infections occur in an environment where blood and tissue eosinophilia are high. These results collectively indicate that eosinophils definitely involve in the innate and acquired resistance to *H. contortus* infection in sheep, but their relative importance in the presence of other immune components, appears to depend on factors such as availability of complement and specific antibody systems, and duration and frequency of infection.

Key words: Sheep, *Haemonchus contortus*, eosinophil, *in vitro, in vivo.*