CHAPTER EIGHT

Immunity to *Haemonchus contortus* and Vaccine Development

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Contents

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Abstract

Sheep are capable of developing protective immunity to *Haemonchus contortus* through repeated exposure to this parasite, although this immune protection is the result of a complex interaction among age, gender, physiological status, pregnancy, lactation, nutrition and innate and adaptive immunity in the host animal. There are multiple effectors of the protective immune response, which differ depending on the developmental stage of the parasite being targeted, and our understanding of the effector mechanisms has developed considerably in the 2000s. The rational design of vaccines based on 'natural' or 'exposed' antigens depends on an understanding of this exposure-induced immunity. However, the most effective current vaccines rely on protection via the induction of high circulating antibody levels to 'hidden' gut antigens of *H. contortus*. The success of this latter strategy has resulted in the launch of a vaccine, which is based on extracts of the parasite's gut, to aid in the control of *Haemonchus* in Australia. The development of recombinant subunit vaccines based on the components of the successful native vaccine has not yet been achieved and most of the recent successes with recombinant subunit vaccines have focussed on antigens unrelated to the gut antigens. The future integration of an understanding of the immunobiology of this parasite with advances in antigen identification, expression (or synthesis) and presentation is likely to be pivotal to the further development of these recombinant subunit vaccines. Recent progress in each of the components underpinning this integrated approach is summarized in this review.

1. INTRODUCTION

Haemonchus contortus is the most globally important trichostrongylid parasite of small ruminants (sheep and goats) in tropical and subtropical areas, and is a major constraint on ruminant health and production worldwide. The continued and increasing development of resistance to all chemical control options (Kotze and Prichard, 2016) and the persistence of chemical

residues in animal products have highlighted the need for other economically sustainable control options (Newton and Munn, 1999). The development of vaccines against economically important parasitic helminths of humans and ruminants has been a long-term goal of many immunoparasitologists, and vaccination which induced sustained protective immunity would be one of the most cost-effective methods of controlling an infection. For the optimal design of the most effective vaccines and vaccination strategies, the immunological control of a helminth via vaccination should be underpinned by a thorough knowledge of the host—parasite interactions, the immune responses involved in protection and the biology of the parasite itself.

While identifying the key antigens and life stages of the parasite that are targeted by elements of the naturally acquired protective immune response is possible through an integrated immunoproteomic/glycomic and bioinformatics pipeline (eg, Smith et al., 2009), the remaining, substantial challenge is to induce the same levels of immunity, through the same mechanisms, using recombinant or synthetic vaccines. An alternative strategy is to induce vaccine-mediated protection through mechanisms which are not necessarily involved in naturally acquired protective immunity (eg, Smith and Smith, 1996) by immunizing with 'hidden' antigens. In these cases, it is still essential to understand the basis of the immune protection induced by the vaccine, to inform adjuvant selection, immunization strategies, etc.

The aim of this chapter is to review key immunoparasitological studies of *H. contortus* and the current state of knowledge of protective immune responses provoked by infection with this parasite and provide a perspective on the development of vaccines against *H. contortus* and the challenges associated with developing commercially viable vaccines.

2. THE IMMUNOLOGY OF HOST PROTECTION AGAINST *HAEMONCHUS CONTORTUS*

2.1 Background

With repeated parasite exposure, sheep can develop protective immunity to subsequent *H. contortus* infections (Barger et al., 1985; Christie et al., 1978; Miller et al., 1983). The ability of sheep to resist the pathogenic effects of *H. contortus* infection relies on a complex interplay of multiple factors, including the level of nutrition, physiological characteristics, pregnancy status, sex, age and innate and adaptive immunity of the host animal (Andronicos et al., 2010; Hein et al., 2010; Saddiqi et al., 2011). The

immune system is a critical component, capable of mediating protection against these parasitic infections and is inherited (Wakelin, 1985) and polygenic in nature (Kemper et al., 2009). The basis of immunological protection has been investigated and several immune mechanisms have been validated (see Fig. 1). However, recent studies suggest a complex interplay of immune mechanisms, some previously unknown, which are likely to specifically target the larval and/or adult parasitic stages and these are highlighted in the following discussion.

2.2 Critical role of host immune responses in protection

Infection of sheep with *H. contortus* is accompanied by pathological changes in the abomasum associated with flattening and disruption of the mucosa (Nicholls et al., 1985) and hyperplasia of numerous cell lineages, including mucus-producing epithelial cells (Scott et al., 1998, 1999), lymphocytes (Gorrell et al., 1988), mast cells (Miller, 1996), globule leukocytes (GLs; Stankiewicz et al., 1993) and eosinophils (Balic et al., 2006). Such changes are associated with the development of immunity in sheep, and early studies, described briefly later, attempted to demonstrate a critical role for the immune system in acquired immunity to *H. contortus* infection. These studies involved the administration of the general immune suppressant dexamethasone and the cellular transfer (to naïve recipients) or inhibition of critical components of the immune system (in the resistant host).

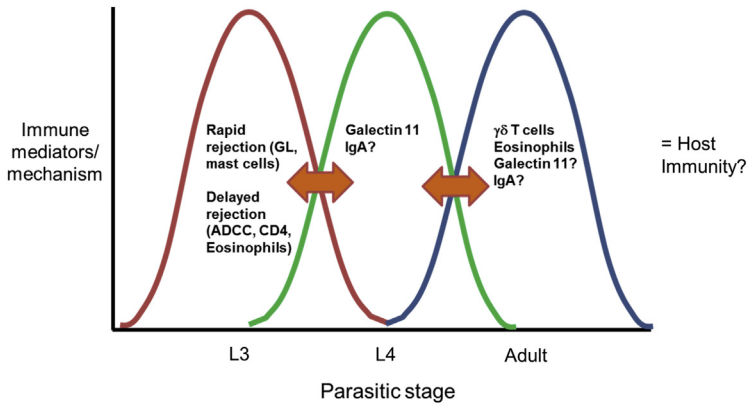

Figure 1 A simple pictorial depiction of the known immune mediators or mechanisms associated with the development of host resistance against *Haemonchus contortus* infection in sheep. The interplays (*orange arrows* (grey in print verisons)) between the immune factors to different larval and adult stages (including unidentified factors to date) are poorly understood.

2.2.1 Administration of dexamethasone

Dexamethasone is a synthetic corticosteroid analogue of the steroid hormone cortisol. Large quantities (eg, 0.5 mg/kg) of corticosteroids exogenously administered to an animal cause non-specific immunosuppression (Plumb, 2005). Administration of dexamethasone to Merino sheep which were genetically resistant to gastrointestinal nematode (GIN) infection demonstrated that immunity to both primary and challenge *H. contortus* infections was decreased, implicating both innate and adaptive immunity, respectively (Adams, 1988; Presson et al., 1988). Dexamethasone administration also abrogated immune protection in sheep which had been rendered immune by prior repeated larval challenges for 10–12 weeks (Huntley et al., 1992). Another study demonstrated that pasture-raised Gulf Coast Native lambs, a breed considered to have natural immunity to *H. contortus* infection, had significantly higher faecal egg counts (FECs) and adult worm counts than untreated sheep when treated with dexamethasone (Pena et al., 2004). These early studies on the abrogation of protection against GINs by using dexamethasone strongly suggested that immunity was an integral part of the protective response against *H. contortus* infection; however, the basis for this immunity was unknown at the time.

2.2.2 Lymphocyte involvement in immunity

Although cortisol has many functions, an effect of large quantities is the non-specific immunosuppression of all leucocyte subsets (Plumb, 2005). Cell depletion and transfer studies suggested that lymphocytes may be important in mediating immune protection against parasites in sheep (Smith et al., 1984). Sets of twin sheep were used as either donors (of whole lymph or purified lymphocytes) or recipient sheep (Smith et al., 1984). The donors were hyperimmunized by repeated infection with *H. contortus* third larval (L3) stage, whereas the recipients remained uninfected. Either gastric lymph or thoracic duct–derived lymphocytes were transferred from donor to recipient sheep, resulting in large reductions in FECs in several of the recipient sheep following a challenge infection (Smith et al., 1984). A further demonstration of lymphocyte involvement in immune protection was through the administration of monoclonal antibodies directed against cell-specific lymphocyte surface antigen(s): CD4 and CD8 cells were depleted in genetically resistant sheep by injection of specific monoclonal antibodies (Gill et al., 1993) and CD4, but not CD8, depletion completely abolished the expression of protection in lambs against *H. contortus* infection, and reduced mucosal mast cell hyperplasia and tissue eosinophilia. A similar depletion of

CD4 T cells resulted in a reduction in the natural protection of Gulf Coast Native sheep against *H. contortus* infection (Pena et al., 2006). The involvement of CD4 cells in vaccine-induced protection was also investigated in Dorset cross-breed sheep. Prior to infection, animals were vaccinated by intramuscular injection with *H. contortus* gut antigen and, from the time of parasite challenge, a subset of the sheep was administered anti-CD4 antibody daily, resulting in significantly higher levels of FECs in these animals at 21 days post-infection (Karanu et al., 1997).

These studies demonstrated that the immune system is critical in mediating immunity to *H. contortus* infection and involved CD4 lymphocyte-mediated mechanism(s). More recently, another lymphocyte subpopulation, $WC1^+$ γδ T cells, has been found to be particularly important in non-specific immunity in the abomasal mucosa of ruminants, and their association with protection against *H. contortus* infections has been reported (Balic et al., 2000a,b; Gonzalez et al., 2011; Munoz-Guzman et al., 2012; Pérez et al., 2008). Further studies, described below, were performed to investigate the functional aspects of lymphocytes, mediator (cytokine) production, and to define the cellular basis for the mediation of immune protection. The infection of ruminants with *H. contortus*, as for other GINs, is associated with elevated type 2 immune responses defined by an increased production of the cytokines interleukin (IL)-4, IL-5 and IL-13; increased parasite-specific immunoglobulin A (IgA) and IgE; and increased tissue eosinophil, mast cell and GL numbers (Balic et al., 2000a,b, 2006; Lacroux et al., 2006). Given the complex nature of the host—parasite relationship and the added complexity of the multi-stage parasite life cycle, evidence as to which parameters are critical in leading to nematode parasite rejection in ruminants is limited, and mechanisms are likely to vary among the different parasite stages. There have been numerous studies investigating the host immune responses to repeated larval infection, particularly those against the L3 stage (which can be readily produced in culture and therefore plentiful), while immunity against the fourth larval (L4) and adult stages is more challenging to investigate, which is reflected in the small number of studies published to date.

2.3 Host immune protection against larval establishment

2.3.1 Mechanisms against L3

Two major immune-mediated mechanisms (rapid and delayed rejection) against the incoming infective L3s have been defined through various studies. Although these two mechanisms can be induced separately in

experimental studies, on pasture these mechanisms are likely to coexist, and rely on the dynamics of exposure and immune competence of the sheep (Hein et al., 2010).

2.3.2 Rapid rejection

One well-established form of host immunity to *H. contortus* infection is the rapid rejection of infective L3s from sheep that have been hypersensitized by repeated larval infections over an extended period, and results in the rejection of a subsequent L3 infection within 48 hours (Balic et al., 2002; Jackson et al., 1988; Miller et al., 1983). This form of rapid larval rejection, also termed 'immune exclusion', is characterized by an immediate hypersensitivity (type I) response and associated with the presence of antibody (IgE), mucosal mast cells and GLs (these latter cells are suggested to be a derivative of mast cells and are also referred to as intra-epithelial mast cells). Antibody-mediated activation of these cells results in increased peristalsis of the gut preventing incoming larvae from establishing in the crypts of the abomasal tissues (Balic et al., 2002; Huntley et al., 1992; Miller et al., 1983; Stankiewicz et al., 1995). While rapid rejection requires the presence of a specific parasitic stage (L3) to be initiated, it is considered a non-specific mechanism, as rejection of unrelated parasites in the same tissue niche or at distal sites further along the gastrointestinal tract can also occur (Dineen et al., 1977; Emery et al., 1993).

Rapid rejection occurs without significant changes in cellular profiles in lymph nodes and the mucosa (Balic et al., 2000a,b) or mucin biosynthesis (Newlands et al., 1990), suggesting that pre-formed mediators, which are able to inhibit larval establishment, are present within cells/cell granules at the luminal surface. Architectural changes in abomasal tissue are clearly visible at the macroscopic level (Kemp et al., 2009). Upon cross-linking of surface-bound IgE, mucosal mast cells degranulate and release preformed mediators, such as histamine, proteases, leukotrienes, neutrophils and eosinophil chemotaxins, resulting in abomasal muscle hyper-motility, gastric hyper-secretion and hyperplasia of goblet cells, increased mucus production and recruitment of innate effector cells (Balic et al., 2002; Meeusen, 1999; Miller et al., 2006). Mucus contains larval inhibitory factors (Douch et al., 1983, 1996), which can be detected at high levels in the faeces of immune sheep (Douch et al., 1983), and their production is strongly associated with GLs (Stankiewicz et al., 1993), suggesting that reduced motility of the L3 aids in the rejection of larvae through peristalsis.

An ex vivo tissue explant model was able to replicate the rapid rejection of larvae ex situ and provided further understanding of key effector

mechanism (Athanasiadou et al., 2008; Jackson et al., 2004; Kemp et al., 2009). Large numbers of mucosal mast cells and GLs were observed in hyper-sensitized tissue, and the ex vivo addition of *H. contortus* L3s to the sensitized tissue caused greater degranulation of mast cells measured by increased levels of histamine (Kemp et al., 2009). Although no difference in eosinophil numbers was reported in this tissue explant model, a role for eosinophils in the rapid rejection of larvae was suggested, as an increased protein level of galectin-14 (a mediator released by eosinophils) was found in the hyper-sensitized tissue (Kemp et al., 2009). Hypothesized roles for galectin-14 include the promotion of cellular adhesion and increased mucus viscosity, which may aid in impeding larval migration and contribute to the mechanism of rapid rejection (Young et al., 2009), although the precise role(s) for galectin-14 need to be established.

2.3.3 Delayed rejection

The second identified immune response against the L3 stage, delayed rejection, is characterized by eosinophil recruitment and a direct role for eosinophil-mediated larval killing through antibody-dependent cell cytotoxicity (ADCC) (Rainbird et al., 1998). Delayed rejection occurs when ingested L3s penetrate the host tissue either in naïve sheep given a primary infection, or in sensitized sheep in which immunity has not yet progressed to the hyper-immune stage or has waned following an extended period of no infection (Balic et al., 2000a,b, 2002, 2006). In each situation, activation of gastric lymph nodes and recruitment of lymphocytes to the abomasal mucosa occur, with the up-regulation and activation of CD4 T cells occurring earlier in immunized compared with naïve animals (Balic et al., 2002). Significant increases in γδ T cells, $CD4^+CD25^+$ T cells, B cells and eosinophils are detected in the abomasal tissues during the delayed rejection response (Balic et al., 2000a,b). A time-sequential study of animals hyper-sensitized to *H. contortus,* given a 12-week rest period, followed by a challenge infection to induce a delayed rejection response, revealed elevated blood and tissue eosinophils and γδ T cells coinciding with the period of rejection of the L3 parasitic stage (Robinson et al., 2010).

A consistent association of tissue eosinophils with delayed rejection has been reported, and eosinophils have been shown to directly kill L3 in vitro, which was enhanced through antibodies and IL-5, suggesting a mechanism involving ADCC (Rainbird et al., 1998). Supporting the in vitro mechanism of killing, eosinophils in vivo are also observed in close proximity to L3 *H. contortus* in animals exposed to a secondary infection and are associated

with structural damage to the larval parasites (Balic et al., 2006). Further support for a role of eosinophils in delayed rejection is the release of the eosinophil-specific molecule, galectin-14, into the gut shortly after parasite challenge, with high galectin-14 expression significantly inversely correlated with subsequent worm burdens (Dunphy et al., 2000, 2002; Robinson et al., 2010, 2011; Young et al., 2009).

2.3.4 Mechanisms against L4s

Although several immune mechanisms have been identified that target the infective L3 stage, few immune mediators have been identified that specifically target the haematophagous L4 and adult stages of *H. contortus*. Following tissue penetration of the host, L3s undergo morphological development, exsheath and form the parasitic L4 stage (Veglia, 1915). Immune protection generated against the L4 stage can result in the induction of hypobiosis (arrested development) or the regulation of L4 feeding. Regulating L4 feeding is considered a major defence mechanism of local IgA in sheep against another abomasal GIN, *Teladorsagia circumcincta* (see Stear et al., 2004). In *T. circumcincta* infections of sheep, high levels of secretory IgA targeting Excretory/Secretory (E/S) products from L4s are consistently associated with reduced L4 length and are hypothesized to help control infection until another immune mechanism develops to regulate parasite numbers (Benothman et al., 2010; Henderson and Stear, 2006; Stear et al., 1999). L4 length is also strongly associated with adult fecundity and reduced parasite length results in a reduced egg-laying capacity (Stear and Bishop, 1999). As such, the mechanism of secretory IgA can also be classified as an indirect immune mechanism against the adult stage. Correlations between IgA and immunity to *H. contortus* have not yet been so firmly established. This may be due to the different nature of parasite feeding, with *T. circumcincta* feeding from the mucosa, whereas *H. contortus* penetrating the tissue to feed on blood. However, it may also be due to limited study of the relationship between IgA and *H. contortus* compared with the large body of information for *T. circumcincta*.

Recent studies, described below, have investigated the role of mammalian galectins, a family of evolutionary conserved glycan-binding proteins, as novel regulators of inflammatory processes, including those induced by parasite infection (Vasta, 2009). Galectin-14 is an eosinophil-specific galectin in sheep and cattle (Dunphy et al., 2002; Young et al., 2009), which has been strongly implicated in playing a protective role against the L3 stage of *H. contortus* (as discussed above in "Section 2.3.3"). Another host galectin,

galectin-11, has been increasingly reported in ruminants (sheep, goats and cattle) infected with parasites (Athanasiadou et al., 2008; Dunphy et al., 2000; Hein et al., 2010; Hoorens et al., 2011; Robinson et al., 2011). Galectin-11 was initially identified as specifically induced in sheep following a primary or secondary challenge with *H. contortus* or *Trichostrongylus vitrinus* (see Dunphy et al., 2000). Galectin-11 was expressed in the nucleus and cytoplasm of the upper epithelial cells lining the gastrointestinal tract, and secreted into the mucus (Dunphy et al., 2000). A kinetic biopsy study investigating the delayed rejection of L3 *H. contortus* showed that galectin-11 was released into the mucus 3–5 days after infection, coinciding with the development and emergence of L4s from the abomasal crypts into the lumen, and was observed to correlate with increased mucus viscosity (Robinson et al., 2010, 2011). The target and role of galectin-11 release are unknown; however, an indirect role in increasing mucus viscosity that impedes parasite establishment and epithelial cell proliferation has been hypothesized (Hoorens et al., 2011; Robinson et al., 2011; Young et al., 2009).

The first demonstration of a galectin directly affecting the parasite was in an in vitro feeding assay, in which galectin-11 inhibited the feeding and exsheathment of *H. contortus* L4s and caused significant inhibition of L4 growth (Preston et al., 2015). Galectin-11 was shown to bind to the L4 and adult stages, but not to the L3 *H. contortus* stage (Preston et al., 2015). Local secretions of IgA are consistently reported as a protective mechanism against *T. circumcincta* by regulating L4 growth (Stear et al., 2004; Strain and Stear, 1999), and it is possible that glycans present on these antibodies interact with galectin-11 to mediate protection against *H. contortus* L4 stage parasites. In addition, a proteomic analysis of sheep gut mucus following *Trichostrongylus colubriformis* challenge has also implicated immune mediators IgA and galectin-11 in the rapid rejection mechanism of immunity (Athanasiadou et al., 2008).

The role of IgA in *H. contortus* infection is uncertain (Strain and Stear, 2001), as is the relationship between IgA and galectin-11, such that further investigations are warranted. The recent successful crystallization of galectin-11 is likely to advance our understanding of ruminant-galectin–parasite interactions and putative parasite ligands for diagnostic and vaccine development (Sakthivel et al., 2015). These recent findings, as well as the proposed effects of galectin-11 on mucous viscosity and epithelial proliferation, suggest that this molecule might have multiple mechanisms of action that target the parasitic stages of *H. contortus*.

2.4 Host immune protection against adult worm infection

Manifestations of immune-based protection against the adult stage of *H. contortus*, as for other GINs, are thought to occur by expulsion, changes in morphology or a reduction in egg-laying capacity (Balic et al., 2000a). A reduction in FECs can be a consequence of reduced female worm burdens and/or reduced egg production in utero. Expulsion of adult parasites usually occurs after repeated larval infections, and is dose dependent (Barger et al., 1985; Barnes and Dobson, 1990). Adult expulsion is thought to occur either through the non-specific mechanism of rapid rejection (see Section 2.3) or through a specific mechanism of acquired immunity (Balic et al., 2000a). Rapid rejection of L3s, once initiated, becomes a non-specific mechanism, which is thought to result in the elimination of residing *H. contortus* adult parasites as well as other GINs established within the same or proximal location in the gut (Miller, 1984; Rothwell, 1989). Other reports suggest that the expulsion of adult *H. contortus* is initiated by a specific immune mechanism, since the rejection of the adult stage does not occur simultaneously with rapid rejection of L3s (Barger et al., 1985). Further evidence supporting specific immunity against adult stages of GINs has come from experiments in which adult *T. colubriformis* were surgically transplanted into hosts that had previously generated immunity against L3 challenges (Emery et al., 1992). Immunity against adult *T. colubriformis* manifested as inhibition of establishment, damaged cuticle and reduced fecundity (Emery et al., 1992).

The exact cellular basis mediating adult worm damage has usually been studied in commercial sheep breeds but is poorly understood. Immune studies of these breeds have shown that adult *H. contortus* infection evokes limited change in leucocyte populations compared with larval infections (Balic et al., 2000b), and suggested that this could be due to the ability of adult parasites to down-regulate the immune response or due to their minimal contact with tissues. In contrast to the intimate contact of the host with tissue-dwelling larvae, adult *H. contortus* worms reside in the lumen and transiently feed at different sites within the abomasum (Hein et al., 2010). Studies of immune function in sheep breeds which are not widely commercially exploited (eg, Rhön sheep, Canaria Hair breed sheep) and which have stronger natural resistance to GIN infections than many of the more widespread commercial breeds, have demonstrated that immunity in these less common breeds can be generated primarily against the adult worm stage (Aumont et al., 2003; Gauly and Erhardt, 2001; González et al., 2008; Zajak et al., 1990). The Canaria Hair Breed sheep, which is native to the Canary

Islands, is refractory to *H. contortus* infection with immunity directed against adult worms and this immunity is independent of larval establishment (González et al., 2008). Preliminary study (González et al., 2011) suggests that the mechanism may involve γδ T cells and eosinophils, cells not previously implicated in immune mechanisms to the adult stage. The exact role of γδ T lymphocytes in infection with adult *H. contortus* is unclear. Whether these cells are involved in natural resistance and/or immunity or if their presence is only a secondary effect of *H. contortus* infection is currently unknown. However, given their association with eosinophils, which have been shown to mediate cytotoxicity to larvae, it is likely these γδ T cells play a functional role through the activation of eosinophils to directly damage the adult parasite and/or stimulate mediators (such as toxic granule proteins and galectin-14), and to reduce viability and enhance the expulsion of *H. contortus*. In addition, galectin-11 expression and direct binding of this molecule to adult *H. contortus* during parasite establishment is observed both in commercial and other sheep breeds (Preston et al., 2015; Piedrafita and González, unpublished data), and might interfere with parasite feeding as demonstrated for L4s. Further work, including inhibition studies of key putative effector cells, such as eosinophils and γδ T cells, might help elucidate their role(s) in immune protection.

2.5 Unknown factors affecting host immunity

As there are several mechanisms of immune protection involving different key effector cells and mediators, it is more than likely that there are multiple reasons for variation in immunity among individual sheep to GIN infections. This statement is supported by genomic data (Andronicos et al., 2010), which report no common mechanisms of immune protection in Merino sheep bred for genetic resistance, but a common mechanism of susceptibility relating to interferon-γ up-regulation (Andronicos et al., 2010). Genetic factors affecting physiological mediators and hormones are also likely to directly or indirectly influence the effectiveness of the immune response (Andronicos et al., 2010; Diez-Tascon et al., 2005; Hickford et al., 2011; Ingham et al., 2008; Keane et al., 2006; Kemper et al., 2011; Rowe et al., 2008, 2009; Sayers and Sweeney, 2005). The preceding sections document key immune mechanisms involved in the cellular basis of immunity against some larval stages, but this understanding is incomplete or may not be the main mechanism of protection across all sheep breeds. The understanding of immune mechanisms targeting larval and adult stages is currently inadequate, and the identification of recent cytotoxic mechanisms directed against

L4 and possibly against adult stages demonstrates the increasing complexity of the immune mechanisms against GINs. The finding that galectin-11 also appears to be ruminant-specific, with a major role in parasite immunity, highlights the limitations of rodent models which underpin much of our understanding of immunity to GIN infections. Given the complex host–parasite interplay and the many mediators involved, more immune mechanisms are likely to be identified in the near future, depending on levels of research investment. Such studies in poorly defined breeds of sheep may provide fertile ground for new and interesting discoveries of mechanisms of natural immunity and novel avenues for parasite control (Piedrafita et al., 2010).

3. DEVELOPMENT OF VACCINES

3.1 Background

The development of commercial vaccines to control parasitic helminths started in the 1950s with the demonstration that infection of calves with radiation-attenuated cattle lungworm (*Dictyocaulus viviparus*) larvae could achieve high levels of protection against challenge infection. This work led to the development of Dictol (now Bovilis Huskvac, MSD Animal Health) which was, until very recently, the only commercially available vaccine for a parasitic nematode of livestock (http://www.worldchanging.gla.ac.uk/article/?id=12). By contrast, the administration of irradiation-attenuated *H. contortus* infective larvae to mature sheep resulted in vaccine-induced protection, but did not give reliable levels of protection in the target age group of young lambs under field conditions (Urquart et al., 1966). Thus, the development of vaccines to control haemonchosis in lambs was focussed on the identification of immunogenic molecules and complexes in the excretory/secretory products (E/S) from the nematode, as well as on cuticular surface and gut-derived 'hidden' antigens (ie, those not recognized by host immune reactions following infection) from the blood-feeding stages. For *Haemonchus*, both E/S and complexes from the luminal border of the intestine have been successfully employed as native vaccines (reviewed by Knox et al., 2003; Newton and Meeusen, 2003; Smith and Zarlenga, 2006). These antigens and complexes have most often been identified as effective vaccines using a pragmatic, iterative approach of successive enrichment of fractions which initially gave some level of protection in preliminary protection trials. Ultimately the most

promising vaccine candidates from these trials have then been produced as recombinant proteins because of the existing paradigm that vaccine commercialization depends on the scale, quality, uniformity and safety that recombinant proteins can offer compared with native immunogens (Geldhof et al., 2007; Knox et al., 2003). This approach, of using recombinant components of native immunogens, has certainly resulted in the development of effective vaccines to control cestode parasites (Lightowlers et al., 2003), but has not yet been successful for an anti-*Haemonchus* vaccine, as discussed in Section 4, below.

3.2 Gut-derived antigens, H-gal-GP

H-gal-GP is a gut-derived, galactose-containing glycoprotein complex which is highly effective when used as a vaccine in lambs, consistently giving $\geq$90% reduction in FECs and $\sim$70% reduction in worm burdens in numerous independent experiments in lambs aged 2–10 months (Smith et al., 1994, 2003a). The major components of H-gal-GP have been identified as a family of zinc metalloproteinases (MEPs) and pepsinogen-like aspartyl proteinases (Newlands et al., 2006; Smith et al., 1999, 2003a,b), with a cystatin, a thrombospondin-like molecule, and a family of galectins also present in the complex (Newlands et al., 1999; Skuce et al., 2001). A review of the contributions of the individual components of H-gal-GP to its protective capacity (Knox et al., 2003) concluded that only one of the zinc MEPs (MEP 3) and the pepsinogen-like molecules were likely to be essential for the protective capacity of the complex. Thus, zinc MEPs and aspartyl proteinases isolated from the complex under non-reducing conditions retained some protective capacity when used as immunogens in lambs, whereas the isolated galectin component (containing Hco-GAL-2), for example, did not, in spite of inducing high levels of antigen-specific circulating antibodies (Newlands et al., 1999; Smith et al., 2003a,b). Thrombospondin and cystatin were considered very unlikely to contribute to protection (Knox et al., 2003), as low amounts of each were present in the complex and a sub-fraction containing components of $<$40 kDa (including cystatin) did not provide protection in vaccine trials in sheep.

High-mass matrix-assisted laser desorption ionization time-of-flight (MALDI-ToF) analysis has determined the overall molecular weight of the H-gal-GP complex to be 981 $\pm$ 10 kDa, which correlates well with the estimated molecular mass from electrophoretic analysis (Smith et al., 1999), and single particle/cryoelectron microscopy revealed the three-dimensional structure of the H-gal-GP complex (Muench et al., 2008).

Overall, the structure represents an arch formed by the two pepsinogens over a base formed by the MEPs, and modelling analysis suggested that the dimensions of the aperture of the arch could accommodate host blood proteins (eg, haemoglobin and albumin), so that both the aspartyl proteinases and the MEPs could access them as substrates (Newlands et al., 2006). The MEPs have hydrophobic domains and are likely to be embedded in the luminal surface of the parasite's intestinal cell membrane (Muench et al., 2008). Immunolocalization of the proteolytic components of H-gal-GP demonstrated their presence on the microvillar surface of the intestinal cells (Knox et al., 2003; Newlands et al., 1999, 2001), supporting their roles in the digestion of the blood meal. Therefore, this complex could form a membrane-bound 'proteolytic machine' analogous to bacterial proteosomes in a particle slightly smaller than foot-and-mouth disease virus, and it has been suggested that it is the inhibition of the proteolytic activity by vaccine-induced antibodies (Ekoja and Smith, 2010) that is responsible for vaccine efficacy (Knox et al., 2003).

Glycosylation is a feature of the H-gal-GP complex, which is rich in β-galactoside sugars (Smith and Smith, 1993), although some elements of the complex (eg, cystatin) lack *N*-linked glycosylation sites (Newlands et al., 2001). Therefore, the H-gal-GP complex is prepared from extracts of adult *H. contortus* by successively removing the water-soluble and membrane-associated proteins, solubilizing the integral membrane proteins and affinity-enriching galactose-containing molecules from the integral membrane proteins by lectin affinity chromatography, followed by gel filtration (Smith et al., 1994). The protective capacity of H-gal-GP is lost if the conformation is destroyed by incubation in reducing conditions, but not by sodium dodecyl sulphate (SDS)—induced dissociation alone (Smith and Smith, 1996). This observation gives support to the proposal that although the components of H-gal-GP are glycosylated and these glycans may contribute to protection, it is the contribution of glycans to the overall conformation of the complex rather than their individual chemical structures that is important for the retention of vaccine efficacy (Knox et al., 2003; Smith and Smith, 1996). When either of the gut-derived antigens H-gal-GP or H11 (see Section 3.3) are used as immunogens, levels of antigen-specific serum IgG are highly correlated with vaccine efficacy, suggesting that the effect of the vaccine is antibody-mediated (Munn et al., 1997; Smith et al., 1999) and a large proportion of the circulating IgG response in lambs vaccinated with native H-gal-GP is directed against the glycan components of the complex (G. Newlands unpublished; cited in Knox et al., 2003).

3.3 Gut-derived antigens, H11

The integral membrane glycoprotein complex, termed H11, is a family of microsomal aminopeptidases present on the intestinal microvilli of the blood-feeding stages of *H. contortus* and, when separated by SDS-polyacrylamide gel electrophoresis (PAGE), runs as a doublet with a mean molecular weight of 110 kDa (Newton and Munn, 1999). Through expressed sequence tag and, more recently, genome analyses, five isoforms of H11 (termed H11, H11-1, H11-2, H11-4 and H11-5) have been described (Newton and Meeusen, 2003; Roberts et al., 2013; Smith et al., 1997). All five isoforms are tandemly arranged in the *H. contortus* genome, suggesting that the gene family has arisen through recent duplication and divergence (Roberts et al., 2013). The members of the H11 family share 62–75% amino acid sequence identity and, combined, give the complex both aminopeptidase A and M-type activities (Newton and Meeusen, 2003; Roberts et al., 2013; Smith et al., 1997). Proteomic analyses of native H11 showed that all five isoforms were present in a typical H11 preparation, and RNA-Seq analyses demonstrated that transcripts representing H11, H11-2 and H11-4 were all highly expressed in the adult gut, whereas H11-5 was expressed in a female-specific manner (Roberts et al., 2013). H11-1 was the most abundant form in infective L3s (Roberts et al., 2013).

H11 is a highly effective protective immunogen and, when the results of a large number of protection trials in different breeds of sheep of varying age and immunocompetence were evaluated, immunization with H11 gave mean reductions in worm burden of 72% (male worms), 82% (female worms) and a 91% reduction in FECs (Newton and Munn, 1999). The potent protective effects of H11 have also been used as a tool to determine the phenotypic effects of silencing genes encoding vaccine candidate proteins in the parasitic stages of *H. contortus* (see Samarasinghe et al., 2011). Thus, exsheathed infective L3s were soaked for 24 hours in double-stranded RNA representing the *H. contortus H11* gene before being used to infect 5-month-old lambs. This treatment led to the silencing of the *H11* transcripts and a 57% reduction in FECs, a 40% reduction in total worm burden and a 64% decrease in aminopeptidase activity in *H. contortus* recovered from infected sheep (Samarasinghe et al., 2011).

As for H-gal-GP, correct conformation and, therefore enzymatic activity, of the H11 complex is important for protection, and the efficacy is reduced in H11 preparations that have been denatured with SDS or with SDS plus the reducing agent dithiothreitol (Munn et al., 1997). Levels of

protection in sheep vaccinated with native H11 are highly correlated with levels of inhibition of H11 aminopeptidase activity by antibodies in the sera from vaccinated animals (Munn et al., 1997). Each H11 isoform also contains potential *N*-linked glycosylation sites and mass spectrometric analyses of the native material have shown *N*-glycans with highly fucosylated core structures including core α1-3 and α1-6 fucosylation which are highly immunogenic (Haslam et al., 1996).

3.4 Barbervax

Of the antigens tested as vaccines to control haemonchosis, only H-gal-GP and H11 have consistently conferred protection in experimental trials at levels that would rival, or exceed, conventional anthelmintic treatment regimes. In addition, preparations of *Haemonchus* integral gut membrane proteins containing both H11 and H-gal-GP, when used as vaccines in sheep in controlled field trials, reduced anaemia, prevented deaths and reduced the contamination of pasture with infective L3s (LeJambre et al., 2008). The demonstration that these antigens could induce protection when administered in microgram quantities opened the door for the commercial exploitation of a native vaccine: Following the acquisition of a good manufacturing licence and 12 field trials in Merino sheep in the Northern Tablelands of New South Wales, in October 2014, the Australian Pesticide and Veterinary Medicines Authority granted permission to sell Barbervax, a vaccine containing *Haemonchus* native integral gut membrane proteins enriched for H-gal-GP and H11. In order to meet local biosecurity regulations, Barbervax is made from *Haemonchus* harvested on an industrial scale from donor sheep at the Albany Laboratory of the Department of Agriculture and Food, Western Australia. The realization of the first vaccine in the world for a nematode parasite of sheep therefore relied on innovations in processing and production technology, rather than novel or emerging biotechnological advances to produce the vaccine in a cost-effective, reproducible and safe manner.

Because the antigens present in Barbervax are hidden antigens, repeated vaccination is required to stimulate high antigen-specific circulating antibody levels but, in areas where haemonchosis is currently controlled by using repeated anthelmintic drenches throughout the grazing season (eg, the Northern Tablelands), the required frequency of application of Barbervax is no higher than that required for drenching (see http://www.wormboss.com.au). If the first three priming injections happen before the risk of *Haemonchus* becomes high in midsummer, two further injections

each at 6-week intervals, should allow protective immunity to be maintained in lambs until late autumn when the risk of *Haemonchus* infection declines (http://barbervax.com.au). A major advantage of the use of the hidden antigens, which make up Barbervax, is that they can produce protective effects in situations where natural immunity is either weak or ineffective, such as in young lambs or in peri-parturient ewes; the effects in this latter groups of animals is of particular importance in the management of transmission of infective L3s to vulnerable lambs early in the season (Andrews et al., 1995; cf. Smith, 2015 in http://www.vetvaccnet.ac.uk/sites/vetnet/files/user-files/research-paper/pdf/02-15/Barbevax-%20Haemonchus%20vaccine.pdf).

3.5 Gut-derived antigens, thiol sepharose—Binding Proteins

Fractionation of the membrane-bound molecules from adult *H. contortus* by peanut lectin affinity chromatography (to remove H-gal-GP) followed by Thiol Sepharose chromatography resulted in a 24-fold enrichment of cysteine proteinase activity in the Thiol Sepharose-binding protein (TSBP) component (Knox et al., 1999). When 10-month-old Greyface/Suffolk cross lambs were immunized with TSBP in Quil A adjuvant and challenged with a bolus of infective *H. contortus* larvae, mean FECs and worm burdens in the immunized lambs were reduced by 82% and 45%, respectively (Knox et al., 1999). Immunolocalization experiments demonstrated that the components of TSBP were located on the intestinal brush border of the adult parasites (Knox et al., 1999). The cysteine proteinases in TSBP are encoded by three genes: *hmcp1, 4* and *6*; possess homology to cathepsin B-like molecules; and are expressed on the microvillar surface of the gut cells (Skuce et al., 1999). In addition to the cysteine proteinases, TSBP contains a glutamate dehydrogenase (GDH) homologue as one of its principal components, but fractionation of the TSBP by anion exchange chromatography demonstrated that it is the cysteine proteinases, which constitute less than 2% of the total protein in TSBP, rather than the GDH which confer protection (Knox et al., 2005). This finding was supported by the demonstration that the cysteine proteinase component of TSBP, when further purified using cystatin affinity chromatography, conferred protection in Greyface/Suffolk cross lambs aged 5 months (leading to a 28—48% reduction in FECs and a 44—46% worm burden reduction), whereas the non-cystatin binding component of TSBP gave no protection in experimental challenge infections (Redmond and Knox, 2004).

3.6 Gut-derived antigens, GA1

Monoclonal antibodies raised against the *H. contortus* gut surface were used to isolate a set of three glycosylated proteins of 46, 52 and 100 kDa by immunoaffinity chromatography (Jasmer et al., 1993). When combined and co-administered with Freund's adjuvant to pigmy goat kids, these proteins induced a significant reduction in abomasal worm burden (60% reduction) (Jasmer et al., 1993). Subsequently, immunoscreening of an *H. contortus* adult cDNA library showed that the 46- and 52-kDa proteins were in fact derived from the 100-kDa polyprotein (Jasmer et al., 1996). The parent 100-kDa molecule was termed gut antigen 1 (GA1), and the two subcomponents named $p46^{GA1}$ and $p52^{GA1}$ (Jasmer et al., 1996). The GA1 protein components were both gut membrane-associated, although only $p52^{GA1}$ possessed a glycosylinositolphospholipid type anchor to the microvillar surface and, although intimately associated with the gut membrane, GA1 proteins were released in E/S products from adult *H. contortus* and were detected in the abomasal mucus from infected goats (Jasmer et al., 1996).

3.7 L3 surface antigen

As the adult stage of *H. contortus* ingests blood from the host, using the 'hidden antigen' approach to target gut-derived antigens, as discussed in Sections 3.1—3.4, has been very effective in reducing parasite burdens and pathological changes. One of the disadvantages of this approach can be the requirement for repeated vaccination to stimulate continuous, high antigen-specific circulating antibody levels. Although our understanding of immune protection towards *H. contortus* is still evolving, the ability of some sheep to develop immunity to *H. contortus* infection has led to optimism that efficacious vaccines are possible using antigens involved in natural immunity. In theory at least, this approach would obviate the need for the multiple boosting requirements of vaccines based on hidden antigens due to a natural boosting of the immune system during re-infection on pasture. Hence, another approach for helminth vaccine development is to target the infective larval stage of the parasite against which natural immunity is commonly directed.

A 70- to 90-kDa antigen, termed *Hc*-sL3, which is specifically recognized by the immune system during a rejection response in *H. contortus*-immune sheep, was shown to be expressed in a stage-specific manner on the surface of L3s (Ashman et al., 1995; Bowles et al., 1995; Raleigh and Meeusen, 1996). Vaccination trials using purified *Hc*-sL3 antigen have shown consistently significant levels of reductions (50—70%) in worm

burden and FECs under a range of vaccination regimes and adjuvants (Jacobs et al., 1999; Piedrafita et al., 2012, 2013). Like Barbervax, the complexity of this antigen (mucin-like) has precluded recombinant antigen formulation, but the simple isolation procedure and abundant access to larval-derived antigens may make this a viable native antigen vaccine.

The importance of adjuvant selection for vaccine-induced immunity against *H. contortus* using antigens involved in natural immunity is exemplified by the vaccine trials with the *Hc*-sL3 antigen. Protection was only achieved when Th2-inducing adjuvants were used [aluminium hydroxide and diethylaminoethyldextran (DEAE)] with no protection observed when Freund's complete adjuvant (FCA) or Quil A were used in the vaccine preparations or when Quil A was mixed with aluminium hydroxide (Jacobs et al., 1999; Piedrafita et al., 2012, 2013; Turnbull et al., 1992). FCA and Quil A induce strong cellular and humoural immune responses. The aluminium-based adjuvants, on the other hand, typically induce much lower antibody responses than Quil A or FCA, but have been reported to be better inducers of Th2-type cytokine responses, including eosinophilia (Cox and Coulter, 1997; Piedrafita et al., 2013). DEAE is a less studied adjuvant but seems to induce both strong antibody and type 2 responses (Piedrafita et al., 2013), which may be useful for a combined adult/larval vaccine. It has been postulated that eosinophil-dependent killing, mediated by antibodies specific to the *Hc*-sL3 antigen, may be an important mechanism in this vaccine-induced immunity, similar to natural immunity, as FECs were negatively correlated with wheal size and tissue eosinophils for the DEAE and aluminium-adjuvanted groups, respectively (Piedrafita et al., 2013).

3.8 Excretory/secretory antigens

Parasite E/S products are a continuous source of natural or exposed antigens (ie, antigens which provoke an immune response during infection) and may be responsible for the development of naturally acquired immunity through continuous exposure and boosting during infection (see Section 2). Nematode E/S products have been investigated as sources of protective antigens for a number of species (eg, Smith et al., 2009) and, for *H. contortus*, the E/S proteins and glycoproteins produced by ex vivo adult worms can confer significant levels of protection when used in vaccination/challenge experiments (eg, see Vervelde et al., 2003). For vaccine antigen discovery for *H. contortus*, the focus has been on two particularly immunogenic E/S proteins of 15 and 24 kDa from a low-molecular-weight subfraction of ex vivo

adult E/S (Schallig and van Leeuwen, 1997; Schallig et al., 1994, 1995; 1997a,b). A preparation of E/S products, highly enriched for the 15 and 24 kDa proteins by anion exchange chromatography, gave substantial levels of protection in 8-month-old Texel sheep when used as a vaccine formulated with the adjuvant dimethyl dioctadecyl ammonium bromide (DDA). This preparation gave a 77% reduction in FECs and an 85% reduction in abomasal worm burden when compared with the adjuvant-only control group (Schallig et al., 1997a). The protective effects of this vaccine were further demonstrated in trials using 9- and 6-month-old sheep, but the vaccine failed to protect younger (3-month-old) lambs against infection (Vervelde et al., 2001). The function of the 15-kDa protein is, as yet, unknown and its sequence contains no homology to known functional domains, although it does possess some sequence similarity to 11- and 30-kDa E/S product vaccine candidate proteins from *T. colubriformis* (see Schallig et al., 1997b). The 24-kDa protein contains an SCP domain and possesses 66% amino acid identity across 223 residues to a C-type single domain activation-associated secreted protein (ASP3) from *Ostertagia ostertagi* (see Visser et al., 2008). The transcripts encoding both the 15- and 24-kDa proteins were only expressed in the parasitic stages of *H. contortus*, suggesting a critical role in host—parasite interactions, which are disrupted by immunization (Schallig et al., 1997b).

In addition to the 15- and 24-kDa E/S proteins, Thiol Sepharose-binding components of adult E/S material have been investigated as prototype vaccines (Bakker et al., 2004). One subfraction of the thiol-binding fraction (the DTT-eluted fraction) contained a range of proteins including MEPs, but no cysteine proteinase activity, and induced reductions of 52% and 50% in FECs and worm burdens, respectively, when coadministered to 9-month-old Zwart-Bles lambs with aluminium hydroxide adjuvant in vaccine/challenge experiments (Bakker et al., 2004). Although these levels of protection were not statistically significant when compared with the adjuvant-only controls, the fecundity (numbers of eggs per female) of worms was significantly reduced when this vaccine was used (Bakker et al., 2004).

The enrichment of the cysteine proteinase components of adult E/S by cystatin affinity chromatography resulted in the purification of a novel cathepsin B-like cysteine proteinase, termed AC-5, which induced reductions of 32% and 36% in FECs and worm burdens, respectively (not significantly different from the adjuvant-only control) when coadministered to 6-month-old Zwart-Bles lambs with aluminium hydroxide adjuvant in vaccine/challenge experiments (De Vries et al., 2009). A feature of both

of these latter reports (Bakker et al., 2004 and De Vries et al., 2009) is that immunization with whole E/S, employing aluminium hydroxide as an adjuvant, failed to confer protection after parasite challenge. This is in contrast to previous experiments in which DDA was employed successfully as the adjuvant for E/S in older lambs (eg, Vervelde et al., 2003) and also in contrast to the induction of significant levels of protection in 3-month-old Zwart-Bles lambs (87% reduction in cumulative FECs) when E/S was coadministered with aluminium hydroxide (Vervelde et al., 2003). In the protected 3-month-old lambs, immunized with E/S in the context of aluminium hydroxide adjuvant, there were significant increases in antibody levels against GalNacβ1,4-(Fucα1,3)GlcNAc, the LDNF glycan antigen, and high levels of antibody which bound the glycan antigen Galα1-3GalNAc, suggesting a role for the high glycan-specific antibody levels in protection in these lambs (van Stijn et al., 2010; Vervelde et al., 2003). The LDNF glycan is also present on MEP 3, a component of the H-gal-GP complex (see Sub-section 3.2), but does not contribute to the protection conferred through the immunization of lambs with H-gal-GP (Geldhof et al., 2005).

4. RECOMBINANT SUBUNIT VACCINES

4.1 Background

Apart from the potentially high costs associated with vaccine production, issues with the use of native antigens for vaccination include quality control and the risk of contaminants that may prohibit export of a vaccine to countries around the world. For this reason, there has been a continuous and concerted effort to produce recombinant or synthetic forms of the antigens. However, in contrast to the successes of native preparations from *H. contortus* as vaccine candidates, there have been, until recently, few reports of success with recombinant vaccines. Where protection has been conferred by immunization with recombinant antigens, partial protection (at levels lower than observed for native antigens) is common in most trials undertaken, and this reduced protective capacity may reflect suboptimal folding or lack of post-translational modifications of the recombinant proteins. The results of trials in which recombinant versions of native vaccine components were used, and trials in which novel recombinant subunit vaccine candidates were employed, are listed in Table 1 and described in the following subsections:

Table 1 Summary of Vaccine Trials for *Haemonchus contortus* Using Recombinant Proteins

Antigen	Expression system	Adjuvant	Route of administration	Effect on FEC[a]	Effect on worm burden[a]	Challenge	References
H11							
H11-1	Baculovirus: *Spodoptera frugiperda* Sf21	None (Sf21 cell extract)	Intramuscular	Unknown	30%[f]	Bolus 15,000 L3	Reszka et al. (2007)
Combined H11-1 H11-4	*Caenorhabditis elegans*	QuilA	Subcutaneous	No[b]	No[b]	Bolus 5000 L3	Roberts et al. (2013)
Combined H11-4 H11-5	*C. elegans*	QuilA	Subcutaneous	No	No	Bolus 5000 L3	Roberts et al. (2013)
H-gal-GP							
HcMEP-1	*Escherichia coli* (GST fusion)	QuilA	Intramuscular	No	No	Bolus 5000 L3	Smith et al. (2003a)
HcMEP-3	*E. coli* (GST fusion)	QuilA	Intramuscular	No	No	Bolus 5000 L3	Smith et al. (2003a)
HcPEP1	*E. coli*	Freund's	Intramuscular	No	No	Bolus 5000 L3	Smith et al. (2003b)
HcPEP1	*E. coli*	QuilA	Intramuscular	No	No	Bolus 5000 L3	Smith et al. (2003b)
Combined HcMEP-1, HcMEP-3, HcMEP-4 HcPEP1	MEPs expressed in *S. frugiperda* Sf9 insect cells; PEP1 in *E. coli*	QuilA	Intramuscular	No	No	Bolus 5000 L3	Cachat et al. (2010)
CYS-1	*E. coli*	QuilA	Intramuscular	No	No	Bolus 5000 L3	Newlands et al. (2001)
Others							
PP2A[c]	*E. coli*	*E. coli* Rosetta 2(DE3)pLysS insoluble fraction	Intranasal	Unknown[d]	No	Bolus 10000 L3 mixed species[e]	Fawzi et al. (2013)

(*Continued*)

Table 1 Summary of Vaccine Trials for *Haemonchus contortus* Using Recombinant Proteins—cont'd

Antigen	Expression system	Adjuvant	Route of administration	Effect on FEC[a]	Effect on worm burden[a]	Challenge	References
Combined *Hco*-gal-m *Hco*-gal-f	*E. coli*	Freund's	Intramuscular	37–48% reduction[f]	41–46% reduction[f]	Bolus 5000 L3	Yanming et al. (2007)
Hc23	*E. coli*	Aluminium hydroxide	Unknown	83% reduction[g]	85% reduction[g]	Bolus 15,000 L3	Fawzi et al. (2015)
HcENO	*E.coli* (thioredoxin fusion)	Montanide ISA 61VG	Intramuscular and subcutaneous	50% reduction	50% reduction	Bolus 5000 L3	Kalyanasud–aram et al. (2015)
Combined Hc15/24	*E. coli*	DDA (± *Trichoplusia ni* extract)	Subcutaneous	0–42% reduction	0–65% reduction	Bolus 5000 L3	Vervelde et al. (2002)
Combined hmcp1, 4 and 6	*E.coli* (GST fusion)	QuliA (+ *E. coli* BL21 extract)	Intramuscular	0–10% reduction	24–38% reduction	Bolus 5000 L3	Redmond and Knox (2004, 2006)
Hc-CPL-1	*C. elegans*	QuilA	Intramuscular	No	No	Bolus 5000 L3	Murray et al. (2007)

All data are from published studies using sheep, unless otherwise stated.
[a]Reduction in FEC or worm burden compared with adjuvant-only control
[b]No = No significant difference ($P < 0.05$) when compared with adjuvant-only control group
[c]PP2Ar is a 76-amino acid portion of the catalytic region of serine/threonine phosphatase 2A from *Angiostrongylus costaricensis*
[d]Reduction in FEC observed compared to adjuvant-only control at some sampling points post-challenge but species composition of nematode eggs unknown so effect cannot be attributed to vaccine efficacy against *Haemonchus contortus*.
[e]4000 *H. contortus* L3; 4000 *T. colubriformis* L3 and 2000 *T. circumcincta* L3
[f]Trial performed in goats
[g]No adjuvant-only control group, comparison is with un-immunized infected group

4.2 Recombinant versions of H-gal-GP components

The success in using the native form of H-gal-GP as a vaccine against haemonchosis in lambs stimulated subsequent research to identify and produce recombinant forms of its components. This initially involved the immunoscreening of cDNA libraries with sera from lambs successfully immunized with native H-gal-GP (eg, Newlands et al., 1999, 2001). In addition, the dissociated complex components were subjected to *N*-terminal sequencing followed by polymerase chain reaction approaches (eg, Smith et al., 2003b) and expression in prokaryotic and, more recently, eukaryotic systems before vaccine efficacy trials in vivo.

4.2.1 Aspartyl proteinases

Urea-mediated dissociation and subsequent fractionation of the H-gal-GP complex yielded a pepsinogen-enriched fraction that retained some of the protective capacity of native H-gal-GP (48% reduction in FECs, compared with 97% for the undissociated H-gal-GP complex) (Smith et al., 2003b). However, immunization of sheep with a bacterially expressed, re-folded, soluble, monomeric, enzymatically inactive recombinant form of the predominant pepsinogen, *Hc*PEP1, led to the production of high levels of antigen-specific IgG in sheep, which bound native HcPEP1 but did not give protection against challenge (Table 1; Smith et al., 2003b). This result indicated that the correct presentation of the conformational epitopes of the pepsinogens in H-gal-GP would be required for their protective capacity, potentially involving their correct glycosylation (Knox et al., 2003; Smith et al., 2003b).

4.2.2 Zinc metalloproteinases

Under non-reducing conditions, H-gal-GP can be fractionated by SDS-PAGE into four major zones of 233, 172, 40 and 31 kDa (Smith et al., 1999). The 233-kDa zone contains three of the MEPS − MEP 1, 2 and 4, whereas MEP 3 is within the 172-kDa zone. Molecular characterization of the MEPs involved in the H-gal-GP complex showed that they were members of the M13 zinc metalloendopeptidase family (EC 3.4.24.11). MEPs 1 and 3 were predicted to possess type II integral membrane protein structure, whereas MEPs 2 and 4 were predicted to be secreted proteins (Newlands et al., 2006). MEP3 was present in the complex as a homodimer, whereas MEP1 and MEP2 formed heterodimers with MEP4. Vaccination of sheep with a combination of all four MEPs, separated from the H-gal-GP

complex by gel filtration in 8 M urea, resulted in significantly reduced FECs (45–50% reduction compared to sheep immunized with adjuvant alone) after parasite challenge, and 26–43% reductions in worm numbers (Smith et al., 2003a). Following the depletion of MEPs 1, 2 and 4 from H-gal-GP, protective capacity of the complex was retained (Smith et al., 2000), indicating that MEP 3 was the most important of the MEPs required for vaccine efficacy. However, when preparations of MEPs 1, 2 and 4, in combination, were compared with MEP 3 in vaccine trials, the effects on FEC reduction in immunized sheep were similar (33% reduction for combined MEPs 1, 2 and 4; 34% reduction for MEP 3), indicating a role for each of the MEP preparations in protection (Smith et al., 2003a). A recombinant version of MEP 1 (rMEP 1) was therefore produced as a glutathione-*S*-transferase (GST) fusion protein in *Escherichia coli* and tested as a protective antigen in vaccination/challenge experiments in sheep, but no protection was evident (Table 1; Smith et al., 2003a). H-gal-GP-specific IgG levels in sera from the sheep immunized with rMEP 1 were significantly lower than those from sheep immunized with the native H-gal-GP complex (Smith et al., 2003a). A pilot vaccination/challenge experiment was also performed using GST fusion, *E. coli*-expressed versions of 41- and 47-kDa subunits of MEP 3, again without success (Table 1; Smith et al., 2003a).

The lack of conformational epitopes on the bacterially expressed versions of MEP 1 and MEP 3, as a result of either incorrect folding or lack of appropriate glycosylation, might have contributed to their lack of efficacy in vaccine trials (Cachat et al., 2010), so an alternative, eukaryotic, expression system based on insect cell cultures (*Spodoptera frugiperda* Sf9 cells) was adopted for the expression of MEPs 1, 3 and 4. A cocktail of these three proteins along with re-folded *E. coli*-expressed PEP1 failed to significantly reduce either FECs or worm burdens (Table 1) when used in vaccination/challenge experiments in 9-month-old sheep (Cachat et al., 2010). Each of the recombinant antigens reacted with IgG from sheep immunized with H-gal-GP and glycosylation of each of the insect cell expressed recombinant MEPs was apparent. However, none of the recombinant molecules exhibited enzymatic activity, suggesting that they were not appropriately folded, and it seems highly unlikely that the quaternary structures required for the protective capacity of H-gal-GP were adopted in the recombinant antigen cocktail (Cachat et al., 2010). The requirement for a combination of correct epitope presentation from each component and the formation of the H-gal-GP structure for optimal protective capacity therefore still represents a huge challenge for molecular, structural and synthetic biology.

4.2.3 Cystatin

Immunoscreening of a cDNA library, prepared from RNA extracted from *H. contortus* harvested 11 days after infection, with serum raised against H-gal-GP, led to the identification of CYS-1, a cysteine proteinase inhibitor of the type 2 cystatin family (Newlands et al., 2001). The transcript encoding this protein possessed a similar expression profile to that of the H-gal-GP galectin *Hco-gal-2*, as it was only present in significant amounts in the blood-feeding L4 and adult stages (Newlands et al., 1999, 2001), and these two molecules were both located in the gut of the parasite, indicating a role for these H-gal-GP components in the unique physiology of these latter parasitic stages. A bacterially expressed recombinant version of CYS-1 was functional as a cysteine proteinase inhibitor, but did not give protection when used as an immunogen in vaccine trials in 6- to 8-month-old lambs, despite inducing high antigen-specific circulating antibody levels (Table 1; Newlands et al., 2001).

4.3 Recombinant versions of H11 components

Initial vaccine trials using combinations of *E. coli*-expressed isoforms of H11 were unsuccessful (Newton and Munn; cited in Knox et al., 2003). Likewise, baculovirus-insect cell expressed, enzymatically active H11 isoforms also failed to protect sheep in initial immunization/challenge experiments (Newton and Meeusen, 2003). In later experiments, a baculovirus-insect cell expressed recombinant version of H11-1 with aminopeptidase A activity induced a modest level of protection (30% reduction in worm burden; Table 1) when used in vaccine/challenge studies in 5-month-old Merino sheep (Reszka et al., 2007), but a GST-fusion version of the same protein failed to give significant protection (Reszka et al., 2007). The failure of recombinant H11 proteins to induce protection has been ascribed to differential post-translational modifications, incorrect folding, the induction of low avidity antibodies or the absence of non-H11, contaminating molecules which may have been critical for the efficacy of the native vaccine (Newton and Meeusen, 2003). This latter suggestion was endorsed by Smith and Zarlenga (2006) who suggested that a combination of digestive enzymes rather than H11 aminopeptidases alone is required for vaccine efficacy.

To address the issues of conformation and post-translational modifications of recombinant forms of H11 and their effects on vaccine efficacy, a range of alternative eukaryotic systems has been employed. These approaches include both DNA vaccination to induce the host cells to produce H11 and the expression of H11 isoforms in the nematode *Caenorhabditis*

elegans. A DNA vaccine expressing H11 plus the host cytokine IL-2 was used to immunize goats (see Section 4.5) and induced high levels of antigen-specific serum IgG and, following challenge with L3, cumulative mean FECs and worm burdens were reduced by 57% and 47%, respectively (Zhao et al., 2012).

The presence of functional α1-3 and α1-6 fucosyltransferases and core α1-3 and α1-6 fucosylation structures in *C. elegans* suggested that transgenic forms of these nematodes, transformed with expression cassettes featuring H11 isoforms with gut-directed promoters and suitable signal peptides, should produce recombinant proteins with more appropriate glycosylation than in other eukaryotic expression systems (Murray et al., 2007; Roberts et al., 2013). Using this system, Roberts et al. (2013) produced enzymatically active recombinant forms of H11-1, H11-4 and H11-5, and glycosylation was confirmed by both lectin binding and MALDI-ToF tandem mass spectrometry, which demonstrated the presence of high-mannose structures, highly fucosylated pauci-mannose like glycans (including α1-3 and α1-6 fucosylation) and evidence of phosphocholine. Serum IgG from sheep immunized with native H11 preparations bound the *C. elegans*-expressed recombinant forms of H11 and, as is the case for native H11, the majority of this binding was to glycans (Roberts et al., 2013). In spite of the similarities in glycosylation between native and recombinant H11 molecules and the evidence for correct folding of the recombinant H11 molecules, vaccine/challenge trials in sheep using combinations of rH11-1 and rH11-4 together or rH11-4 and rH11-5 together did not give any protection. Native H11 appears to be a dimer with a combined mass of around 230 kDa (as determined by size exclusion chromatography), suggesting the possibility of a quaternary structural element to protection that may be missing when using combinations of two or more recombinant versions (Newlands, personal communication).

4.4 Recombinant proteins – others

4.4.1 15- and 24-kDa ES proteins

Protection studies with combined native 15- and 24-kDa E/S proteins gave substantial levels of protection in 8-month-old Texel sheep (Schallig et al., 1997a). Bacterially expressed recombinant versions of both proteins (rHc15 and rHc24; Table 1) initially gave promising results when combined and co-administered to 7- to 9-month-old sheep with DDA (with and without an extract of insect cells derived from *Trichoplusia ni*) as an adjuvant, resulting in a 38–42% reduction in FECs and a 55–65% reduction in worm burden

compared with the adjuvant-only control (Vervelde et al., 2002). Subsequent attempts to repeat these results in 3-month-old lambs and in 9-month-old sheep did not result in reductions in FECs or worm burden (Vervelde et al., 2002).

4.4.2 Galectins

Although protection trials with recombinant forms of Hco-GAL-2, the H-gal-GP-associated galectin, have not been performed in sheep, it would seem unlikely that this recombinant galectin would be protective, as the purified native molecule did not give protection in vaccine trials (Newlands et al., 1999). In contrast, recombinant versions of two isoforms (termed *Hco*-gal-m and *Hco*-gal-f because they were derived from male and female worms, respectively) of a different putative immunomodulatory galectin from *H. contortus* (Sun et al., 2007; Wang et al., 2014), induced partial protection in vaccine trials in 9- to 10-month-old goats when administered in Freund's adjuvant (Yanming et al., 2007) (Table 1).

4.4.3 Hc23

Hc23 is an exposed antigen of unknown function which constitutes ~1.8% of the total aqueous somatic extract of adult *H. contortus* (Fawzi et al., 2014). A pilot study using the native protein suggested that Hc23 may be used as an effective vaccine antigen when injected into 4- to 5-month-old lambs: reductions in FECs and abomasal worm burdens were 70% and 67%, respectively, when native Hc23 was co-administered with aluminium hydroxide, and 85% and 87%, respectively, when co-administered with bacterial lipopolysaccharide/inactivated bacteria as adjuvant (Fawzi et al., 2014). It should be noted, however, that these values relate to reductions compared with an un-immunized challenge group rather than control groups administered the appropriate adjuvant only (Fawzi et al., 2014). A recombinant form of Hc23, expressed in *E. coli* and co-administered with aluminium hydroxide, was also effective in reducing FECs and worm burden by 83% and 85%, respectively (Table 1) in vaccination/challenge trials in 4- to 5-month-old Assaf breed lambs (Fawzi et al., 2015). Again, this trial suffered from a lack of appropriate (adjuvant-immunized only) controls, but the findings offer some hope for the use of recombinant proteins as vaccines to control haemonchosis.

4.4.4 Enolase

Enolase (2-phosphoglycerate hydratase; EC 4.2.1.11) is a cytosolic enzyme performing the penultimate step in glycolysis. In spite of its cytosolic

localization and function, enolase has been detected in E/S of adult *H. contortus* (see Yatsuda et al., 2003) and antibodies in sera from experimentally infected goats bound both native and recombinant versions of the enzyme, indicating exposure of the host to the protein during infection (Han et al., 2012a). In other helminth species, enolase has been shown to be a component of both the E/S material and the tegument of the adult worm (Wang et al., 2011). In the Chinese liver fluke *Clonorchis sinensis*, tegumental enolase is able to bind host plasminogen, potentially facilitating tissue invasion (Wang et al., 2011). A recombinant form of the *H. contortus* enolase HcENO, expressed in *E. coli* and co-administered as a water-in-oil emulsion with the adjuvant Montanide ISA 61 VG, was effective in halving both FECs and worm burden in vaccination/challenge trials in 6-month-old Mecheri breed lambs (Table 1; Kalyanasundaram et al., 2015).

4.4.5 Cysteine proteinases

Cysteine proteinases purified from TSBP derived from adult *H. contortus* confer significant levels of protection against experimental challenge infection (Redmond and Knox, 2004; Knox et al., 2005), and immunoscreening of cDNA libraries with sera from protected lambs identified three major cathepsin B-like cysteine proteinases (hmcp1, 4, and 6) in TSBP (Skuce et al., 1999). When expressed as GST fusion recombinant proteins in *E. coli* and used to immunize Suffolk/Greyface cross sheep, a cocktail of the three proteins conferred partial protection, with reductions in worm burden of up to 38% (Table 1; Redmond and Knox, 2004). When the same cocktail of recombinant proteins was used, but without fusion partners on the recombinant proteins, reductions of 27% and 29% in FECs and worm burdens, respectively, were observed (Redmond and Knox, 2006).

In each of the protection trials using hmcp1, 4 and 6, the recombinant proteins were expressed in an insoluble form, as inactive proteinases. In contrast, Hc-CPL-1, a functionally active cathepsin L-like cysteine proteinase from *H. contortus*, was expressed in *C. elegans* and was able to rescue yolk protein processing in *C. elegans* loss-of-function mutants, demonstrating correct folding and activation of the recombinant enzyme (Britton and Murray, 2002). Cysteine proteinase activity of the glycosylated *C. elegans*-expressed Hc-CPL-1 was further confirmed using cathepsin L-specific substrates (Murray et al., 2007) and immunization of 5-month-old Suffolk lambs with this form of Hc-CPL-1 induced antibodies which bound the native protein (Murray et al., 2007). In spite of this, immunized lambs were not protected when infected with *H. contortus* larvae in an experimental challenge

(Table 1; Murray et al., 2007), although it should be noted that native Hc-CPL-1 has not been tested in protection trials in sheep, such that this molecule might not have been a suitable vaccine candidate antigen for *H. contortus*.

4.5 DNA vaccination

DNA vaccination as a means to control parasitic disease in production animals is still in its infancy, and the high levels of antigen-specific antibody required for successful protection against *H. contortus* in lambs might be difficult to reach using this technology (cf. Scheerlinck et al., 2004). Recently, however, several reports have been published describing partial protection against *Haemonchus* in goats following DNA vaccination. Vaccination of 8- to 10-month-old goats with plasmid constructs containing three fragments encoding sections of H11-1 and caprine IL-2 conferred partial, but statistically significant, protection after challenge (a 57% reduction in FECs and a 47% reduction in worm burden) compared with goats that had received only phosphate-buffered saline (PBS) injections (Zhao et al., 2012). Immunization of goats of the same age with DNA encoding HC29, an *H. contortus* glutathione peroxidase (GPX), led to high levels of HC29-specific serum IgG and IgA and increases in the numbers of $CD4^+$ T lymphocytes as well as 36% reductions in FECs and worm burdens compared with goats which had received only PBS (Sun et al., 2011).

Further DNA vaccination trials in goats, employing ubiquitously expressed proteins as the target antigens, have also shown modest levels of protection. For instance, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; a cytosolic enzyme involved in glycolysis) from *H. contortus* (HcGAPDH) has been assessed in DNA vaccination studies in 9- to 10-month-old goats (Han et al., 2012b). The administration of HcGAPDH in plasmid pVAX1 conferred a modest level of protection (35% reduction in FECs and a 38% reduction in worm burden), accompanied by significantly increased levels of antigen-specific serum IgG and IgA as well as an increase in $CD4^+$ T lymphocyte numbers compared with goats that had received only PBS (Han et al., 2012b). Very similar results were obtained when DNA encoding *H. contortus* actin was used as an immunogen instead of HcGAPDH (Yan et al., 2014), and immunization of goats with DNA encoding a further structural protein, *H. contortus* Dim-1, conferred slightly higher levels of protection (a 46% reduction in FECs and a 51% reduction in worm burden) compared with goats which had received PBS alone (Yan et al., 2013).

5. CONCLUDING REMARKS

The success of the hidden antigen approach in controlling *H. contortus* has led to the development of a commercially available vaccine, which is proving effective in the field. The mechanism of action of this vaccine operates through induction of high antigen-specific circulating IgG levels that need to be stimulated by repeated immunizations during the risk period for haemonchosis. As such, the protective mechanism does not mirror natural immunity, though natural immunity may develop in the vaccinated hosts from exposure to infective larvae during the growing season. The future development of further immunoprophylactics to control *H. contortus* and other GINs will likely depend on understanding the development of immune resistance to underpin both rational vaccine development and the enhanced efficacy of some of the prototype vaccines described herein. This review highlights the complexity of the immune response in the relationship between the *H. contortus* and its host animal, and, as for other helminth–host systems, this complexity is not surprising, given the multiple developmental stages with distinct niches and antigen profiles associated with individual developmental stages. The recent recognition of galectins in resistance development as well as sheep breeds with diverse immune responses against different stages of the parasite (eg, larvae *versus* adult) emphasize the challenges for the development of an efficacious commercial vaccine. In addition, the substantial levels of inter- and intra-population genetic variation, and thus antigenic diversity, within *H. contortus* (see Blouin et al., 1995; Hussain et al., 2014; Redman et al., 2008) as well as the inherent differences in the genetic backgrounds, and thus immune responses and effectors, of individual definitive ruminant host animals (Bishop, 2012), are also likely to play a role in differential vaccine responsiveness.

The immune factors affecting host resistance have been investigated in vaccine trials in which protective efficacy has been correlated with immune parameters. For the hidden antigen approach, this protection is often strongly associated with the induction of antibody responses. In contrast, parallel vaccine studies, investigating cellular changes considered necessary for the development of natural immunity, have been limited. This has been partly due to small numbers of protective molecules investigated to date and partly due to limited immunological analysis carried out in these experiments, as they are logistically difficult to perform. As such whether vaccine-induced efficacy and natural immunity are interdependent as would

be expected remains unclear. In addition, selecting the right adjuvant is critical, but our understanding of immune modulation of adjuvants remains in its infancy. This highlights the need for further studies to advance our understanding of essential requirements for a broader base in vaccine antigen identification and efficacy.

Subunit recombinant vaccines are highly favoured because of the commercial applicability of such approaches (Section 4). In spite of recent encouraging advances in the recombinant production of *Haemonchus* antigens, their appropriate selection and expression remain the major challenges. If antigen selection is to be based on effective native complexes, it is vital that the effective component(s) of those complexes are fully identified and understood. In this regard, it is notable that H11, the most potent native vaccine produced thus far (Smith et al., 1993), was originally a very minor contaminant of the effective contortin-enriched preparation (CEP) that heralded *Haemonchus* vaccine research (Munn, 1977; Munn et al., 1987). While the potent immunogenicity of H11 in CEP was resolved by immunoblotting (Smith and Munn, 1990), modern proteomic technologies should be capable of detecting femto- to attomolar quantities of immunogenic components in effective native vaccine preparations, though reproducing these as effective recombinant vaccines remains a challenge. In the early 1990s, the potential for expressing *H. contortus* antigens in *C. elegans* was recognized (eg, Jasmer et al., 1993) and the subsequent production of enzymically active, correctly folded, glycosylated *Haemonchus* antigens in *C. elegans* in sufficient quantity for vaccine testing (eg, Murray et al., 2007; Roberts et al., 2013) might have opened the door for the production of effective recombinant versions of the most protective native antigens. However, this avenue has not yet delivered a successful outcome, potentially because of the need for quaternary structures in the most complex of the native, effective vaccine antigens. In contrast, encouraging levels of protection have been achieved in pilot trials using bacterially expressed versions of single antigens, such as Hc23 (Fawzi et al., 2015), suggesting that the prospects of developing recombinant vaccines based on protective native molecules may need to be focussed on simple antigens rather than complexes such as H-gal-GP until developments in genome editing, structural and synthetic biology can enable synthesis of the complex quaternary structures needed for protection using H11 and H-gal-GP.

Given these challenges, it is important to ask, what do we realistically expect from a vaccine? It is now prudent to suggest that *Haemonchus*-specific vaccines will play an essential role as one tool for parasite control, but it is

also clear that such a vaccine only targets one (albeit highly pathogenic) species of GIN infecting sheep in commercial livestock production systems. In terms of vaccine efficacy required for Barbervax or any other vaccine to control *Haemonchus*, there is now acceptance, at least amongst the scientists involved, that vaccines to control parasitic nematodes will not achieve the sterile immunity associated with vaccines developed for the control of bacterial and viral diseases (Emery, 1996; Knox et al., 2003). It is also highly unlikely that vaccines will attain the efficacy expected of anthelmintics, but modelling studies comparing the relative benefits of vaccination against a conventional anthelmintic control program (Dobson et al., 2011) suggest that, if vaccines achieve $\geq$65% efficacy, they should deliver substantial benefits in the control of haemonchosis in lambs. Thus, 65% vaccine efficacy in lambs would lead to a mortality rate of 4.5% compared with 27.7% in unvaccinated lambs receiving four anthelmintic treatments in regions with high infection pressure (Dobson et al., 2011; Smith, 2015 in http://www.vetvaccnet.ac.uk/sites/vetnet/files/user-files/research-paper/pdf/02-15/Barbevax-%20Haemonchus%20vaccine.pdf).

Issues relating to antigenic diversity in nematodes as well as stage-specific immune responses and 'non-responder' animals suggest that cocktail vaccines may be required (eg, Nisbet et al., 2013). The incorporation of antigens from multiple developmental stages has long been purported as being essential for the generation of efficacious vaccines. Of course, the identification of such antigens is challenging, requiring the isolation of various developmental stages of the parasite from the natural host, the identification and assessment of a large number of antigens and the financial investment that is then required for validation. The use of recombinant subunit cocktail vaccines developed in this way also adds to the cost and complexity of vaccine manufacture, and is no guarantee of success (Willadsen, 2008). A vaccine targeting multiple stages of *H. contortus* based on native antigens could be an alternative approach. For example, a vaccine which targets *Hc*-sL3 (L3 surface antigen) used in combination with the vaccine targeting hidden gut antigens in the L4 and adult stages of *H. contortus* (e.g., Barbervax) might provide protection in young lambs early in the season, while allowing the development of enhanced exposure-related natural immunity by immunization with the L3 surface antigen, diminishing the requirement for boosting later in the season. Clearly, vaccine development against GIN parasites is challenging but is currently looking brighter for *H. contortus* than for other species. The pay-off for the successful vaccine development against GINs will be long-term benefits in a world

where increasing animal productivity, rather than increasing animal numbers, will be essential.

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