Development of vaccines against gastrointestinal nematodes

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SUMMARY

Vaccination against complex metazoan parasites has become a reality with the development and registration of recombinant protein-based vaccines against the cattle tick *Boophilus microplus* and the sheep cestode *Taenia ovis*. Progress towards the development of similar vaccines against gastrointestinal nematodes, primarily of ruminants, is outlined within a framework of defining the practical requirements for successful vaccination, antigen selection, recombinant protein production and antigen delivery, be it mucosal delivery or DNA vaccination. Antigen selection strategies include the fractionation of complex, but protective, parasite extracts, the use of antibody probes, evaluation of excretory-secretory components and gut-expressed hidden antigens as well as antigens targeted on the basis of function such as enzyme activity. The difficulties being encountered in recombinant protein production and their solution are discussed as are the requirements for successful antigen delivery. Recent technological developments such as the use of functional genomics to identify new vaccine candidates and DNA vaccination to present the selected antigen to the host immune system are discussed and are anticipated to have a profound effect on vaccine development in the future.

Key words: Gastrointestinal nematodes, vaccination, antigens, DNA vaccines.

INTRODUCTION

Cattle and sheep are continuously exposed to nematode infection and gradually become resistant to reinfection. This latter fact provides the basis for believing that vaccination is feasible. However, the acquisition of immunity is dependent on numerous different influences. Notwithstanding the complexity of the immune response these influences include breed, the genetic make-up of individuals, age and the differing characteristics of co-infecting nematode species. In addition, management factors such as provision of clean pasture and adequate nutrition can greatly influence attempts to control infection. This brief list could be extended and all of these factors need to be considered when devising vaccination strategies. But why bother with vaccination? Are not currently available anthelmintic drugs quite adequate? Anthelmintic resistance is a stark reality as is increasing consumer awareness of food safety with regard to drug residues in meat products. Problems of resistance and consumer hostility are likely to be encountered by new generations of anthelmintics, if these are developed at all because of the escalating costs which have to be offset by profitability in the market place. However, the current utility of existing anthelmintics, although limited in many regions of the world, provides a window of opportunity for the development of alternative strategies. Recent research shows that it is possible to stimulate practically useful protective immunity against blood-feeding nematode species by vaccination, but not, as yet, with recombinant proteins. Results obtained with non blood-feeding species are less encouraging for reasons which will be discussed.

It is clear that most progress has been made in developing vaccines against gastrointestinal nematodes of sheep despite the fact that cattle are economically more important. This disparity reflects the relative costs of vaccine trials in sheep and cattle and the relative lack of definition of protective immune responses in cattle.

Vaccine development faces several fundamental challenges not least of which is the isolation of native antigens from non blood-feeders which elicit protective immunity if delivered to the immune system in an appropriate manner. In an appropriate manner? Larval and adult gastrointestinal nematodes stimulate mucosal immune responses and are unlikely to be susceptible to systemic vaccination regimes with the exception of blood-feeding species. This points to three fundamental requirements (1) definition of the mucosal immune mechanisms which result in worm expulsion, (2) identification of worm antigens which stimulate these responses and (3) the development of mucosal antigen delivery systems. Without the latter, how do we know that we have not already isolated the appropriate antigens but have not been able to evaluate efficacy properly? Then there is the problem of recombinant protein production. Not as simple as it may appear but more on that later. Finally, to replace anthelmintics, a subunit vaccine will have to be effective against a number of co-infecting nematodes, be cost-effective and its administration will have to fit in with farm management practices. This review, while sum-

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marizing different rationales and progress made to date in identifying and administering potential nematode antigens as vaccine candidates, will also consider future research directions which are required if multivalent vaccines are to become a practical reality replacing, or at least, reducing anthelmintic usage.

VACCINE ATTRIBUTES AND EFFICACY

When considering the practical development of a vaccine against gastrointestinal nematodes it is necessary to define the required 'performance characteristics' of the vaccine. These issues have been addressed in several recent reviews (e.g. Smith & Munn, 1990; Emery, McLure & Wagland, 1993; Newton, 1995; Emery, 1996; Miller, 1996; Meeusen, 1996; Klei, 1997; Munn, 1997; Smith, 1999). The first question to be addressed is the degree of protection required. Perhaps, the best practical definition of required efficacy is 'reducing parasitism below that which causes a significant production loss' (Klei, 1997). It is unlikely that anti-parasite vaccines will attain the almost 100% efficacy associated with new anthelmintics and bacterial/viral vaccines (Emery, 1996), but evidence obtained by using computer models of population dynamics of host–parasite interactions (Barnes & Dobson, 1990) indicates that adequate control can be achieved with vaccine efficacies well below 100% (Barnes, Dobson & Barger, 1995). For example, conventional vaccines, i.e. vaccines based on antigens recognized during the course of natural infection, were predicted to be superior to standard anthelmintic programmes with vaccine efficacies of 60% in 80% of the flock. These figures were obtained on the assumption that sheep naturally acquire immunity to re-infection when exposed to parasites on pasture. This was assumed to be more beneficial in the case of vaccines based on conventional antigens compared to those based on hidden antigens and this was upheld by the model which predicted a required vaccine efficacy of 80% in 80% of the flock. However, this prediction may be somewhat inaccurate in that lambs which were vaccinated against *Haemonchus contortus* using a defined hidden antigen acquired immunity to re-infection following natural exposure to infection (Smith & Smith, 1993). Notwithstanding, it is clear that sterile immunity is not a prerequisite for a nematode vaccine and, in fact, may prove detrimental in the long term. Next, it is necessary that a commercially viable vaccine must be efficacious against all the principal nematode species co-infecting the gastrointestinal tract. This statement may not apply to Southern Hemisphere countries where anthelmintic resistance in populations of *H. contortus* is particularly prevalent. Here, a vaccine providing protection against this parasite alone is likely to have a significant niche market. However, in general terms, we need to be aiming for vaccines with the same cross-species efficacy of existing anthlemintics to ensure product uptake by the agricultural industry. This may be achieved by identifying protective antigens shared by different nematode species or by developing vaccine formulations containing several different species-specific antigens. This requirement may be facilitated by exploitation of the demonstrable cross-specificity of rapid rejection of incoming larvae from immune animals, an effect promulgated distally but not proximally and consistent with the specific recognition of a parasite antigen followed by a non-specific effector phase (Emery, McClure & Wagland, 1993). This may present future opportunities when the precise mechanisms of worm rejection are further defined. Any vaccine must protect young weaner stock, a difficulty to date only overcome using 'hidden antigen'-based vaccines against the blood feeder, *H. contortus*, progress discussed in more detail below. Finally, an effective vaccine will reduce pasture contamination in successive seasons (Emery *et al*. 1993).

EPIDEMIOLOGICAL AND IMMUNOLOGICAL CONSIDERATIONS

Nematode infections occur on a seasonal basis and this seasonality can be attributed to factors affecting contamination of the environment and those controlling development and survival of the free-living stages of the parasites (Urquhart *et al*. 1987). Differing seasonality between species has potential implications for multivalent vaccine development. For example, in Britain, pasture contamination with *Nematodirus battus* larvae shows an abrupt increase in May and June while contamination with *Teladorsagia circumcincta* peaks between July and October; *Trichostongylus* spp. become prevalent in late summer and early autumn. Therefore, exposure to nematode infection is effectively continuous, but growing lambs will require protection against infection with different nematode species depending on the time of year. An efficacious vaccine would have to provide early protection against *Nematodirus*, and, at the same time, prime immunity to incoming *T. circumcincta* and *Trichostrongylus* spp. later in the grazing season. Pasture contamination will be affected by the ability of larvae on pasture to survive adverse environmental conditions, by arrested larval development in the host, parasite fecundity, stocking density and the immune status of the host. In the case of the latter, the periparturient relaxation of immunity is particularly significant in that it results in an increased level of pasture contamination when the numbers of susceptible young stock are greatest.

Any vaccination regime will have to circumvent the phenomenon of immunological unresponsiveness (generally < 3–6 months of age) of young stock to

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incoming nematode infections. The reasons for this are unclear and may be influenced by parasite or host species (McClure *et al*. 1995) and the degree and rate of exposure to infection but, given that production losses primarily accrue due to infection in young stock, it provides a major hurdle to vaccine development. Protective immunity takes several months to develop and can result in the rejection of incoming larvae, arrested larval development, reduced fecundity and the expulsion of adult worms.

Host susceptibility to nematode infections is, in part, genetically determined to the extent that selective breeding has been investigated as a means of controlling ovine nematodes (Windon, 1990). Nematode resistance, the ability of the host to reduce establishment and/or delay development, as judged by faecal egg counts, has a heritability of about 0·3 in Romney and Merino sheep. Work has progressed to examine the immune mechanisms underlying within- and between-breed differences in resistance and susceptibility which may help to identify phenotypic and genetic markers for these traits. (Beh & Maddox, 1996; Douch *et al*. 1996). Bissett & Morris (1996) argued that the benefits which may accrue following selection based on resistance would be derived from reduced pasture contamination and that resilience, i.e. the ability to maintain relatively undepressed production in the face of parasite challenge, may be an equally useful criterion for selection. Selective breeding programmes will, undoubtedly, contribute to nematode control in the medium term but they are unlikely to have the broadly based and relatively short-term applicability to counteract developing anthelmintic resistance.

The nutritional status of the host has a profound influence on its ability to mount a successful immune response and this, in turn, is likely to affect individual responses to vaccination. Protein and/or nitrogen supplementation of the diet increases the rate of acquisition of immunity and increases resistance to re-infection (Coop & Holmes, 1996; Knox & Steel, 1996). In addition, the periparturient rise in faecal egg output can be dramatically reduced by dietary protein supplementation. Addition of molybdenum to the diet of lambs given trickle challenge infections of either *H. contortus* or *Trichostrongylus vitrinus* markedly reduced worm burdens at the end of the experiment compared to lambs to given a standard diet (Suttle *et al*. 1992*a*, *b*). Mean worm burdens were reduced by 78%, a reduction as good as any obtained by vaccination with a purified antigen(s).

VACCINATION WITH WHOLE WORM MATERIAL AND RADIATION-ATTENUATED PARASITES

Rats have been successfully vaccinated against *Strongyloides ratti* by giving a total of 16000 heat-killed larvae in 13 subcutaneous injections at intervals of 3 days, a regime which gave resistance to challenge infection equivalent to that induced by a single challenge with 1000 living larvae (Sheldon, 1937). However, the approach proved largely unsuccessful against other infections including *Ancylostoma caninum* in mice, *Ascaris suum* in guinea pigs (Kerr, 1938) and *Trichinella spiralis* in rats (Bachman & Molina, 1933). These early experiments led to the conclusion that the protective antigens were not present in sufficient quantity in extracts of dead parasites but might be actively secreted by the living parasite (Clegg & Smith, 1978).

Parasites attenuated by exposure to X-radiation do not achieve patency, do not induce significant pathology but do stimulate immune responses which can be host protective. This approach has resulted in the development of two commercially available anti-nematode vaccines for the control of lungworm in cattle (*Dictyocaulus viviparus*, Jarrett, *et al*. 1958) and sheep *D. filaria*, Sharma, Bhat & Dhar, 1988). In addition, the same approach was effectively applied to the vaccination of dogs against the intestinal nematode *A. caninum* (Miller, 1971) but the resultant commercial product failed due to respiratory side effects, short shelf life and, in some cases, lack of sterile immunity (Miller, 1978). Irradiated larval vaccines protected mature sheep (> 6 months old) against *H. contortus* (Urquhart *et al*. 1966) or *T. colubriformis* (Gregg *et al*. 1978) but, as in natural infection, immature animals did not develop immunity (Urquhart *et al*. 1966; Gregg *et al*. 1978; Smith & Angus, 1980). Pigs have also been protected against *T. spiralis* infection by vaccination with radiation-attenuated muscle larvae (Cabrera & Gould, 1964). Subsequent work has focused on identifying parasite antigens which stimulate protective immunity.

VACCINATION WITH PARASITE ANTIGENS

Nematodes are multicellular organisms with complex lifecycles. Many parasite proteins are recognized by the host during infection, often in a stage-specific manner, but many of the responses to these antigens will have no functional significance in terms of resistance to re-infection (O'Donnell *et al*. 1989). Four main approaches for identifying parasite antigens which stimulate protective immunity have been defined by Emery & Wagland (1991) and these definitions form the basis for the sub-headings used below. These approaches, though different, are often complementary. Antigens can be selected on an empirical basis where parasite material is fractionated by conventional protein chemistry techniques followed by successive immunization trials. Empirical selection tends to be both time consuming and costly so more rational approaches are being applied, where molecules are selected on the basis of

their contribution to parasite survival. Antigens studied to date can be defined as conventional or covert (hidden). Conventional antigens are recognized by the host in the course of natural infection, would augment host natural immunity, but may have limited utility due to selection pressure associated with the host immune response (Emery & Wagland, 1991). Covert antigens are not normally accessible to the host immune system and are less likely to be subjected to selection pressure, reducing the risk of antigenic variation. However, repeated vaccination may be required as specific host immune responses would not be boosted by subsequent natural infections. To date, the antigens tested have been purified in small quantities from parasite extracts or *in vitro* culture fluids (ES). Emery & Wagland (1991) estimated that three donor lambs were required to provide sufficient purified antigen to vaccinate one sheep. ES antigens are even more difficult to harvest, requiring donor animals for the provision of parasites. ES antigens exhibit stage-specificity and are often difficult to purify from complex media while protein yields are poor, adult *T. colubiformis* producing about 1 mg ES protein per 25000 worms per day (Emery, 1996). Therefore, commercial vaccine production will be dependent on the successful application of recombinant DNA technology, be it recombinant protein production, vaccine vectors or DNA vaccination.

Biochemical fractionation of complex parasite extracts

This approach involves the progressively more refined fractionation of crude, but protective, parasite extracts or ES. Fractionation is achieved using parasite extracts prepared in a variety of solvents combined with relatively standard chromatographic and electrophoretic methodologies. The solvent used to prepare the initial extract, be it an aqueous medium such as PBS or detergents such as Triton or Tween, can have a profound effect on the antigens isolated and the complexity of the resultant extract.

The basic approaches to the isolation of protective antigens in parasite extracts are exemplified by a series of experiments in Australia which led to the identification of tropomyosin as a potentially useful antigen for stimulating protective immunity in sheep against *T. colubriformis* and *H. contortus* infections. Immunization with whole worm homogenates from fourth larval stage *T. colubriformis* accelerated expulsion of the same parasite from guinea pigs (Rothwell & Love, 1974) and homogenate subfractions obtained using SDS–PAGE, which still comprised a complex mix of proteins, had a similar effect (O'Donnell *et al*. 1985). A PBS/deoxycholate extract from third larval stage *T. colubriformis* comprised four protein components, one of which, a 41 kDa protein, induced 43–51 % protection against infection in guinea pigs. Partial amino acid sequence analysis showed this protein was tropomyosin and a cDNA encoding this protein was isolated using oligonucleotide primers based on this sequence (Cobon *et al*. 1989; O'Donnell *et al*. 1989). A 27 kDa subunit was expressed as a β-galactosidase fusion protein in *E. coli* which produced accelerated worm expulsion in guinea pigs following challenge infection. A homologue from *H. contortus*, isolated by DNA hybridization, significantly protected sheep against challenge infection (Cobon *et al*. 1989).

A slightly different approach was used to identify a protective protein fraction from *Oesophagostomum radiatum* which infects the large intestine of calves. Calves acquire strong resistance to re-infection and the protective response primarily targets the late fourth larval stage (Roberts, Elek & Keith 1962). Protective immunity was induced by vaccination with L_4 or adult parasite whole worm homogenates or using excretory gland homogenates from the adult parasite (Keith & Bremner, 1973). Adult parasite extracts were resolved into four fractions using gel filtration, and ELISA analysis showed that antibodies from naturally infected calves predominantly reacted with antigens eluting in the void volume (East, Berrie & Fitzgerald, 1989). Calves immunized with this fraction were significantly protected against challenge infection (East *et al*. 1989). High molecular weight antigen preparations have also been used to vaccinate against *Heligmosomoides polygyrus* (*Nematospiroides dubius*) infection in mice (Monroy & Dobson, 1987), *T. colubriformis* (O'Donnell *et al*. 1985) and *H. contortus* (Neilson & Van de Walle, 1987) in sheep with varying degrees of success.

Isolation of parasite molecules essential for parasite maintenance within the host

This category includes molecules released by the parasite during *in vitro* culture (ES) which are either known to be released or presumed to be released *in vivo*. ES have been ascribed numerous roles including host penetration, parasite feeding and evasion of host anti-parasite immune responses and include enzymes such as proteases, acetylcholinesterases and superoxide dismutases (reviewed by Knox, 1998). In addition, molecules with the capacity to directly alter the local gastrointestinal environment have been identified including a homologue of a porcine intestinal peptide, valosin, secreted by *T. colubriformis* (Savin *et al*. 1990) and a serine proteinase inhibitor with homology to human leukocyte elastase inhibitor from L_4 and adult *Trichostrongylus vitrinus* (MacLennan, K. & Knox, D. P., unpublished).

ES may be derived from the parasite surface, from specialized secretory glands or as by-products of parasite digestion and represent the major antigenic and functional challenge to the host (Lightowlers &

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Rickard, 1988). ES are often released in a stage-specific manner. Despite extensive study and significant early successes, surprisingly few ES components have been evaluated as potential as potential protective immunogens.

Antigens purified from L_3 and adult ES from *H. contortus* and *T. colubriformis* have shown considerable potential in trials primarily conducted by Australian workers (Emery, 1996). *T. colubriformis* L_4 and adult ES antigens were fractionated, mainly by SDS–PAGE, and several induced protection in guinea pigs to challenge infection following intraperitoneal immunization. cDNA analysis showed that the proteins were homologues of a human γ-interferon-induced protein (Dopheide *et al*. 1991), human and insect globins (Frenkel *et al*. 1992) and a porcine intestinal peptide, valosin (Savin *et al*. 1990) and recombinant forms of these proteins gave 30–70% protection against homologous parasite challenge (Emery, 1996). A 94 kDa glycoprotein from *T. colubriformis* L_3 induced variable protection of 30–50% in guinea pigs (O'Donnell *et al*. 1989).

Other ES proteins from ruminant gastrointestinal nematodes which are useful immunogens have been identified using antibody probes from immune animals and are discussed in the next section.

Nippostrongylus brasiliensis and *T. spiralis* have long been used as rodent models for studying gastrointestinal nematode infections. When *N. brasiliensis* was maintained in serum from immune rats, precipitates were formed at the mouth, excretory pore and anus of the worm (Sarles, 1938; Taliaferro & Sarles, 1939) and similar precipitates were detected histologically around the orifices of larval parasites in the skin and lungs of immune rats. These precipitates did not appear to damage the worm directly but may have blocked a secretion, such as an enzyme, required for survival in the host. Rats could be partly protected against *N. brasiliensis* challenge by vaccination with ES released by larval parasites into serum or saline during *in vitro* maintenance (Thorson, 1953) and there was evidence that protection could be enhanced by direct delivery of antigen to the gut. Lipolytic activity in larval ES was inhibited by antiserum from immune rats. (Thorson, 1954) and dogs were partially protected against *A. caninum* challenge by vaccination with oesophageal gland extracts from the same parasite (Thorson, 1956). These extracts contained proteolytic and lipolytic enzymes the activities of which could be inhibited with immune dog serum.

Attention again focused on enzymes in nematode secretions in the 1970s when it was shown that a wide range of gastrointestinal nematodes released acetylcholinesterase (AChE) *in vitro* and that immune animals produce antibodies against the enzyme (Ogilvie *et al*. 1973). As immunity develops, the parasite has the ability to secrete an alternative isoenzyme in greater quantities (Edwards, Burt & Ogilvie, 1971; Jones & Ogilvie, 1972). Rothwell & Merritt (1975) vaccinated guinea pigs with soluble fractions from *T. colubriformis* greatly enriched for AChE but also containing an undefined low molecular weight allergen. Only the latter was protective. However, it is interesting to note that cattle vaccinated against the bovine lungworm *D. viviparus* produced antibodies which inhibited AChE released by the parasite *in vitro* and a partially purified preparation of this enzyme induced protective immunity in guinea pigs against subsequent challenge (McKeand *et al*. 1995).

Proteases are released during *in vitro* culture of many parasitic helminths which are required for penetration of the host and survival within it (Tort *et al*. 1999). *In vitro*-released proteases from *Fasciola hepatica* induced high levels of protection in sheep (Wijffels *et al*. 1994) and cattle (Dalton *et al*. 1997) but equivalent proteases from gastrointestinal nematodes have not been extensively tested to date. A 35 kDa cysteine protease isolated from glycerol extracts of *H. contortus* conferred significant protection to lambs against challenge infection with the same parasite (Cox *et al*. 1990; Boisvenue *et al*. 1992). The protease had anticoagulant properties and was capable of degrading fibrinogen (Cox *et al*. 1990) but it was not clearly established if the protease was an ES component or a gut-associated enzyme. Recently, Knox, Smith & Smith (1999) reported several trials where the protective efficacy of cysteine protease-enriched extracts from the adult parasite was analysed, enrichment being achieved by thiol-sepharose affinity chromatography. Water-soluble and membrane-associated cysteine proteases were without effect as immunogens and these extracts may have contained cysteine proteases known to be present in adult parasite *in vitro* ES (Karanu *et al*. 1993). This implies that ES cysteine proteases may not be useful immunogens but this would require confirmation by conducting vaccine trials with proteases purified from ES alone.

Glutathione S-transferases (GSTs) induced significant reductions in parasite numbers when used as immunogens in sheep and cattle challenged with *Fasciola hepatica* (Sexton *et al*. 1990; Morrison *et al*. 1996). Antisera raised against GST purified from adult *H. contortus* inhibited enzyme activity *in vitro* but did not affect survival of the parasite *in vitro* (Sharp *et al*. 1991). In parallel experiments, a specific GST inhibitor reduced parasite survival *in vitro* (Sharp *et al*. 1991).

Early reports (Campbell, 1955; Chipman, 1957) indicated that antigenic material secreted by adult and larval *T. spiralis* contained potentially useful immunogens. Despommier & Muller, (1970) showed that a fraction containing mainly β secretory granules derived from the larval stichosome administered once to mice in Freund's complete adjuvant depressed the number of encysted larvae in the muscle

of recipients by 95% following challenge. Subsequent experiments (Despommier, Campbell & Blair, 1977) confirmed these results and indicated that this method of vaccination mainly affected fecundity of the female worm. In further experiments (Despommier & Laccetti, 1981*a*, *b*), a solubilised extract from the large particle component of the stichocyte (designated S3) and an immunoaffinity purified sub-fraction induced protective immune responses in mice. Despommier (1981) used molecular size chromatography and preparative isoelectric focusing to purify one antigen to homogeneity enabling analysis of amino acid content. Subsequently, monoclonal antibodies were used to isolate 3 antigens, one of which, a 48 kDa protein, induced a high level of protection in mice against challenge infection, protection being comparable to that elicited by exposure to the entire infection (Silberstein & Despommier, 1984).

Swine trichinosis is an important zoonotic infection and it has been established that pigs can expel adult worms from the intestine and do develop resistance to reinfection (Bachman & Molina, 1933; Campbell & Cuckler, 1966). Pigs can be protected by vaccination with radiation-attenuated muscle larvae (Cabrera & Gould, 1964) and with ES antigens from muscle larvae (Vernes, 1976), the latter antigens also being present in S3 stichocyte antigen preparations used by Despommier (1981). Muscle larvae numbers were reduced by about 50% compared to challenge controls in pigs immunized with S3 antigens, immunization only marginally enhancing gut expulsion (Murrell & Despommier, 1984). The 48 kDa stichocyte antigen described above was ineffective in the natural pig host (Gamble, Murrell & Marti, 1986).

Perhaps amongst the most interesting but least defined group of ES antigens are those released during the moulting process. A very early study (Stoll, 1929) suggested that immune responses capable of reducing egg production and resulting in the expulsion of adult worms were initiated by an early parasite stage in sheep challenged with *H. contortus*. The suggestion was supported later (Stewart, 1953) and expulsion occurred when the larvae in the challenge infection were moulting from the third to the fourth larval stage (Soulsby & Stewart, 1960). This 'self cure' response was also observed in pigs infected with *A. suum* (Taffs, 1968). These early experiments led to several protection trials in different host-parasite systems which give equivocal results. Silverman, Poynter & Podger (1962) protected guinea pigs (> 90%) against *T. colubriformis* challenge infection by immunization with lyophilized whole larvae and ES derived from *in vitro* cultures where the larvae were maintained from the second to the fourth larval stage. However, later experiments (Rothwell & Love, 1974) showed that protective antigens were present in similar amounts in fourth-stage larval and adult worms and could be readily obtained from whole worm homogenates. A high molecular weight fraction of antigenic material secreted during the *in vitro* moult from third to fourth larval stage *Haemonchus* protected (70–80%) lambs of undefined age against challenge infection (Ozerol & Silverman, 1970) but Neilson (1975) could not reproduce this effect. However, the antigens used in the two studies were not exactly comparable. In addition, antigen in the former study was administered without adjuvant and protective immune responses initiated may have been due to self adjuvanting and been different from those initiated by injection with Freund's adjuvant used in the latter study. Trials of similar material from *Ascaris suum* also gave some conflicting results when used to vaccinate mice prior to challenge (Guerro & Silverman, 1969). Secretions produced during the moult from third to fourth larval stage *A. suum* induced significant protection against challenge in guinea pigs while soluble proteins produced by L_2, L_3, L_4 or adult worms during culture were without effect (Stromberg, Rhoury & Soulsby, 1977).

The surface of parasitic nematodes is a dynamic structure; surface antigens are shed continuously and are often highly antigenic (Maizels, Blaxter & Selkirk, 1993). Cetylmethylammonium bromide (CTAB)-solubilized surface antigens from *T. spiralis* induced significant protection in mice (Grencis *et al.* 1986). However, surface extracts from *H. contortus* L_3 were ineffective (Turnbull *et al.* 1992) and cuticle collagens from third and fourth larval stages of the same parasite did not induce significant protection against homologus challenge (Boisvenue *et al.* 1992). Recently, Jacobs *et al.* (1999) induced protective immunity in sheep against *H. contortus* using a surface antigen purified from the infective L_3 stage and showed that efficacy was dependent on route of administration and adjuvant used.

Antigen selection using antibodies or cells from immune animals

The use of antibodies derived from infected and/or immune animals has, to date, proved the method of choice to identify antigens associated with protective immunity. However, this is fraught with difficulty because of the complexity of the serum response, heterogeneous antigen expression by the parasite within and between stages within the host and the fact that the antibody response reflects both current and previous infections. Despite these difficulties, some progress has been made. As described above, monoclonal antibodies derived from immune mice were used to identify and purify the major immunogens of *T. spiralis* and one of these proteins (M_r 48 kDa), at very low doses, induced significant infection in mice against infection (Silberstein &

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Despommier, 1984). Sera from sheep which were defined as resistant or susceptible to *T. circumcincta* were used to identify a 31 kDa protein which was recognised preferentially by resistant animals as early as 3 weeks after experimental infection and was present in and secreted by third larval stage parasites (McGillivery *et al*. 1992). Lambs immunized with the protein were significantly protected against challenge infection (McGillivery *et al*. 1992) but this effect could not be reproduced (Morton *et al*. 1995).

Recently, attention has begun to focus on the development of methods which allow the identification of parasite antigens that trigger local cellular and antibody responses such as T cell Westerns (Haig *et al*. 1989), mast cell stimulation assays (Emery, McClure & Wagland, 1993) and antibody probes derived from lymph nodes draining the site of infection (Meeusen & Brandon, 1994*a*; Meeusen, 1996; Newton & Munn, 1999). These techniques should help to identify the parasite antigens which stimulate immune responses specifically responsible for worm rejection.

Antibody secreting B cells (ASCs) are produced by lymph nodes draining the site of infection in *Taenia hydatigena* infected sheep and form distinct foci around the challenge parasites (Meeusen & Brandon, 1994*a*). These ASCs secreted antibodies with specificity for the susceptible larval stages of the parasite. In rats experimentally infected with liver fluke, ASCs are only produced as long as the parasite is present within the tissue drained by the node and different nodes within the same animal can show contrasting antibody specificities dependent on the parasite stage present in the draining tissues (Meeusen & Brandon, 1994*b*). Techniques have now been developed to harvest ASCs from sheep immune to gastrointestinal nematode infection and maintain them *in vivo* (Meeusen, 1996). Animals immunized by repeated infection are given a single large challenge infection after a period of rest from a priming infection to allow the local cellular response to end. The large challenge infection induces the rapid activation of lymphocytes, including ASCs, in the draining lymph nodes with specificity for antigens presented by the challenge infection. These lymph nodes can be harvested at a time which coincides with parasite rejection and the lymphocytes they contain cultured *in vitro* for at least 5 days without further stimulation. The *in vivo*-induced ASCs continue to secrete antibodies which can then be harvested from the culture fluids and used directly on Western blots, for example, to identify parasite antigens associated with the period of parasite rejection (Meeusen, 1996).

ASC antibody probes have now been used to identify protective antigens from *H. contortus* (Jacobs *et al*. 1999) and *T. circumcincta* (Meeusen, 1995). Antibodies produced by ASCs from abomasal lymph nodes of sheep immune to *H. contortus* infection recognized L_3 homogenate antigens in the size ranges 44–48 kDa and 70–83 kDa, the latter being purified using immuno-affinity media prepared using ASC antibodies (Bowles, Brandon & Meeusen, 1995). The antigen, designated Hc-sL3, is glycosylated (Ashman *et al*. 1995), developmentally regulated (Raleigh, Brandon & Meeusen, 1996; Raleigh & Meeusen, 1996), is expressed on the surface of exsheathed L_3 (Bowles *et al*. 1995) and can be purified using size-exclusion chromatography (Ashman *et al*. 1995). Merino sheep vaccinated with Hc-sL3 showed 64–69 % and 45–55 % reductions in faecal egg outputs and adult worm burdens respectively after a single challenge with 10000 L_3 (Jacobs *et al*. 1999). The choice of adjuvant is crucial; protection can be induced with aluminium hydroxide and infection exacerbated with Quil A as adjuvant indicating that protection is dependent on the induction of a T helper 2 (Th_2)-type response (Newton & Munn, 1999). Studies on the mechanism of rejection (Ashman *et al*. 1995; Rainbird, MacMillan & Meeusen, 1998) indicated that protection was unrelated to IgE-dependent immediate hypersensitivity mechanisms thought to be responsible for rapid expulsion (Newton & Munn, 1999).

The same approach has been used to identify equivalent antigens, in the size range 26–36 kDa from *Teladorsagia circumcincta* (Meeusen, 1995; Raleigh *et al*. 1996) and *T. colubriformis* (Meeusen, 1995). The *T. circumcincta* proteins, purified from gels, induced 40–60 % reductions in faecal egg outputs from lambs given the antigen with Quill A as adjuvant (Meeusen, 1995). These proteins included a prominent doublet at 31–33 kDa and a monoclonal antibody to this doublet was used to isolate cDNAs which encoded tandem-repeat-type β-galactoside binding lectins (galectins; Newton *et al*. 1997). *T. colubriformis* galectin cDNA clones have also been analysed and defined (Greenhalgh, Beckham & Newton, 1999).

By comparing serum antibody responses of sheep partly immune to primary and secondary infections with *H. contortus*, Schallig *et al*. (1994) identified 15 and 24 kDa adult ES antigens specifically recognized by immune animals. These antigens have subsequently been evaluated in protection trials in partly purified form (Schallig & van Leeuwen, 1997) and as essentially pure preparations (Schallig, van Leeuwen & Cornellisen, 1997) giving reductions in faecal egg output and final worm burdens of 77 % and 85 %, respectively. Both antigens are exclusively expressed in the L_4 and adult parasite, and cDNAs encoding them have been isolated and expressed in *E. coli*. The cDNA sequences showed some homology to ES components identified from *T. colubriformis* and *A. caninum*. Early data indicate that denatured, then refolded recombinant versions of these proteins do induce high levels of protection in 8-month-old lambs (Vervelde *et al*. 1999*a*). The same workers

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reported that protection levels increased with increasing lamb age and that protection was correlated to ES-specific serum IgE levels and increases in abomasal mast cell and eosinophil numbers (Vervelde *et al*. 1999*b*).

Isolation of specific antigens from parasite organs that may serve as targets for antibody or immune effector cells

Activity of host antibodies against the parasite gut wall is well documented. Structural changes in the nematode intestine have been attributed to the host immune response in the rodent *N. brasiliensis* system (Ogilvie & Hockley, 1968) and in *N. battus* harvested from lambs (Lee & Martin, 1980). In addition, Seesee, Wescott & Graham (1976) demonstrated antibodies of various immunoglobulin classes bound to sections of the parasite gut, particularily the microvilli of the intestinal cells, in *N. brasiliensis* retrieved from mice. Although not an obligate blood feeder, this observation might indicate that adult *N. brasiliensis* does, in part, feed on host blood components, possibly as a result of ingesting plasma proteins available at the intestinal mucosal surface as a result of serous exudation resulting from the host inflammatory response to the parasite. Moreover, other economically important genera such as *Ostertagia* and *Teladorsagia*, again not obligate blood feeders, do take in host immunoglobulin (Murray & Smith, 1994). The late larval and adult stages of *H. contortus* actively ingest host blood and artificially-induced systemic antibody responses to these components are clearly detrimental to the parasite (Munn, Graham & Coadwell, 1987, Newton & Munn, 1999).

This approach to the immunological control of blood-feeding endo- and ecto-parasites has received practical confirmation in a number of host–parasite systems and includes the identification of covert (hidden) antigens. It is exemplified by the pioneering work of Munn and co-workers who have isolated several protective antigens from the intestinal luminal surface of blood-feeding stages of *H. contortus* (Munn, 1977; Munn & Greenwood, 1984; Munn, Graham & Coadwell, 1987; Munn & Smith, 1990). It is worthy of note that the initial identification of these proteins was as a result of electronmicroscopy studies of the structure of the intestine of *Haemonchus* and other strongyles (Munn, 1977; Munn & Greenwood, 1984). Targeting gut membrane proteins was pioneered in ticks and this work led to the launch of a commercial vaccine against *Boophilus microplus*, the Australian cattle tick (Willadsen, 1995).

Lambs have been successfully immunized against haemonchosis using several gut antigens fractionated from adult *H. contortus*. A helical polymeric structure, termed contortin (Munn, 1977), loosely associated with the luminal surface of the plasma membrane of the intestinal epithelium from early in the fourth larval stage, was isolated in relatively pure form from saline extracts of the adult parasites by differential centrifugation (Munn *et al*. 1987). Young lambs immunized with this contortin-enriched preparation (CEP) were substantially protected (78·5 % reduction in worm burden) against challenge with the homologous parasite (Munn *et al*. 1987). Lambs which did not mount a significant antibody response to the preparation died of acute haemonchosis, suggesting that antibody was the prime effector mechanism for protection. This work showed, for the first time, that proteins expressed on the surface of the gut could induce protective immune responses and stimulated the search for other antigens associated with the parasite intestine luminal surface.

A major component of the microvilli of adult *H. contortus* is an integral membrane glycoprotein of $M_r = 110$ kDa, designated H11 and this was found to be a major antigenic contaminant of CEP by Western blot analysis. However, only traces of H11 were detectable on gels stained for protein suggesting it could be a 'strong' protective antigen (Smith & Munn, 1990). Injection of sheep with microgram amounts of esssentially pure H11 stimulated substantial (> 90 % reductions in worm burden) protection to challenge infection (Munn & Smith, 1990) which was closely correlated with specific systemic IgG titres to H11.

H11 is an integral membrane glycoprotein expressed exclusively in the intestinal microvilli of the parasitic stages. It can be purified from detergent extracts of the adult parasite using a combination of lectin-affinity and ion-exchange chromatography (Smith & Smith, 1993; Munn *et al*. 1997) and was defined as a microsomal aminopeptidase by cDNA cloning (Smith *et al*. 1997). The native protein exhibits both aminopeptidase A and M-type activities which are attributable to distinct isoforms (Graham *et al*. 1993; Smith *et al*. 1997). On a more historical note, using aminopeptidase purified from the gut of *Ascaris suum*, Ferguson *et al*. (1969) noted a 50 % reduction in larval counts in guinea pigs immunized with the enzyme prior to homologous challenge.

Addressing the practical requirements for a vaccine in the field, H11 is an effective immunogen in very young lambs (Tavernor *et al*. 1992); it is effective in a range of breeds and against anthelmintic-resistant worms (Newton & Munn, 1999). Protection persists for at least 23 weeks after vaccination (Andrews, Rolph & Munn, 1997) and does not interfere with the development of acquired immunity (Smith & Smith, 1993). Pregnant ewes, challenged in their third trimester, were highly (98 %) protected as judged by faecal egg output and a degree of protective immunity was transferred from vaccinated ewes to lambs challenged 5 weeks

post-partum (Andrews *et al*. 1995). This latter result indicated that protection was antibody-mediated, a conclusion supported by observations that antisera from vaccinated lambs inhibited the microsomal aminopeptidase activities and that the degree of inhibition was highly correlated to the level of protection obtained (Munn *et al*. 1997). Protection is reduced when H11 is progressively denatured by treatment with SDS alone or SDS + dithiothreitol (Munn *et al*. 1997), results which indicate that conformational epitopes are required for the full expression of protective immunity.

As noted above, cDNAs encoding all three isoforms of H11 have been cloned and have been expressed as full-length enzymically-active recombinant proteins in the baculovirus-S*f*9 insect cell system and defined fragments of the extracellular domains have been expressed in *E. coli* (M. Graham, T. S. Smith and E. A. Munn, unpublished) and the resultant recombinant proteins are currently being evaluated in vaccine trials.

Homologues of H11 have been identified in *T. circumcincta* and *O. ostertagi* and are also being evaluated in protection trials (E. A. Munn and W. D. Smith, unpublished).

Smith, Smith & Murray (1994) specifically labelled glycoproteins on the luminal surface of the gut of adult *H. contortus* with a panel of lectins and also with [^{35}S]methionine. Lectins with affinity for *N*-acetylgalactosamine stained the brush-border membrane of the intestinal cells most intensively and this property was susbsequently used to purify candidate surface proteins by affinity chromatography. The protein complex obtained, designated *Haemonchus* galactose-containing protein complex (H-gal-GP), conferred substantial protective immunity (reductions of 93% in egg output and 72% in worm burdens) on lambs 2–12 months old in a series of protection trials (Smith *et al*. 1994; Smith & Smith, 1996). The complex comprised groups of peptides around 35, 45, 170 and 230 kDa as judged by non-reducing SDS–PAGE. Protection was decreased by reduction of the complex with SDS and DTT prior to immunization and subfractions of the complex prepared by SDS–PAGE yielded partial protection suggesting that more than one component is required for effective vaccination (Smith & Smith, 1996). Protection trials with individual peptides prepared by electroelution form SDS- and native-PAGE and by chromatographic fractionation are continuing. ELISA data indicate that protection is antibody mediated (Smith *et al*. 1999).

Initially, little was known about the functional properties of H-gal-GP. Biochemical studies indicated that it contained proteolytic activity with aspartyl protease activity predominating at acidic pH and metallo-protease activity at neutral pH (Smith *et al*. 1993). These initial findings have been confirmed by a combination of N-terminal sequence analysis of the individual peptide components and analysis of cDNAs encoding them (Longbottom *et al*. 1997; Redmond *et al*. 1997; Smith *et al*. 1999) and recent work has shown that the complex also contains galectin (Newlands *et al*. 1999), cystatin and thombospondin homologues (Newlands, Skuce, Smith, Smith & Knox, unpublished). Several cDNAs have been expressed in bacteria and yeast and the resultant recombinant proteins are currently being evaluated in protection trials.

Extracts (Knox, Redmond & Jones, 1993) and ES (Karanu *et al*. 1993) from adult *H. contortus* contain several cysteine proteases. As noted above, cysteine protease-enriched preparations from water soluble and membrane-asociated adult parasite extracts were not protective but lambs vaccinated with equivalent preparations from integral membrane protein extracts showed reduced worm burdens and faecal egg outputs of 47 and 77%, respectively in three trials in lambs 3–10 months old at the start of the experiment (Knox *et al*. 1999). The protective proteins, termed thiol sepharose binding proteins (TSBP), are expressed on the microvillar surface and in the cytosol of the intestinal cells and are not recognized by lambs immunized against infection by repeated challenge. The extract comprises a relatively simple group of peptides with a 60 kDa component particularly prominent. To date, full-length cDNAs encoding 3 distinct cathepsin B-like cysteine proteases (Skuce *et al*. 1999*a*) and a glutamate dehydrogenase homologue (Skuce *et al*. 1999*b*) have been isolated and expressed and, again, are being evaluated in protection trials.

O. ostertagi and *T. circumcincta* both contain close homologues of H-gal-GP and TSBP as judged by SDS–PAGE and enzyme analyses, and both induce variable degrees of protection in homologous vaccination/challenge experiments (Knox *et al*. 1995; W. D. Smith & D. P. Knox, unpublished). In some trials, reduced worm burdens and egg outputs of 50% and 85%, respectively have been observed while no significant effects have been noted in other trials. The reasons for this variation are unclear and may reflect antigen stability, dose or may simply indicate that antigens expressed on the surface of worms which are not obligate blood feeders are not appropriate vaccine candidates. Perhaps the worms simply ingest insufficient antibody!

Jasmer and co-workers (Jasmer *et al*. 1993) prepared monoclonal antibodies (mAbs) to target glycoproteins expressed on the gut surface of adult *Haemonchus*. Two of 49 mAbs were analysed further and both bound to the microvillar surface of freshly isolated gut and had specificity for carbohydrate epitopes. Antigen isolated from detergent extracts of adult worms by immunoaffinity chromatography using both mAbs significantly reduced total worm counts by up to 60% and, unusually, to a lesser extent, faecal egg output (34%) in 14-month-old

goats given a single challenge infection 5000 L_3. The relevant proteins had M_r values of 100, 52, 46 and 30 kDa. One of the carbohydrate epitopes recognized was also detected in larval *A. caninum* and a mixed population of *C. elegans*. Molecular studies revealed that the 52 and 46 kDa proteins are products of the same gene (designated *GA1*) and are expressed initially as a polyprotein (Jasmer, Perryman & McGuire, 1996). The 52 kDa component shows 47% sequence identity to the 46 kDa protein and carries a glycerophosphatidyl inositol anchor. The protein can be detected in abomasal mucus of infected lambs and may, in fact, be an antigen recognized by the host in the course of natural infection. These antigens show some similarities to another complex of proteins designated P1 (Smith *et al*. 1993) which comprised peptides of M_r 45, 49 and 53 kDa. Vaccination of lambs with P1 resulted in a 30% reduction in worm numbers, with the effect being greater in female worms compared to males, and a 69% reduction in faecal egg output.

RECOMBINANT PROTEIN PRODUCTION

Candidate protective antigens have been isolated and characterized from many gastrointestinal nematodes of veterinary importance and the challenge now is to produce these antigens in an immunologically active form using recombinant DNA technology and to show that the recombinant (sub-unit) vaccine is effective in field conditions. These steps are the key in converting laboratory developments into a commercially available vaccine. This has already been achieved, and commercial vaccines produced, for *Taenia ovis* infections in sheep and *Boophilus microplus* infections of cattle (reviewed by Rickard *et al*. 1995; Willadsen, 1995). There are useful lessons to be learned from these developments. Firstly, strong reactivity in the immunoassay chosen for antigen selection does not always correlate with protective efficacy. A weakly immunopositive cDNA (45W) encoding the host protective 47/52 kDa doublet from *T. ovis* isolated from a λgt11 expression library induced much higher levels of protection when administered as a β-galactosidase fusion protein expressed in *E. coli* compared to a strongly immunopositive cDNA (45S) encoding a smaller portion of the protein (Lightowlers, 1994). The cDNAs encoding the protein of interest should be sub-cloned into a variety of bacterial plasmid vectors to determine efficacy. Neither β-galactosidase fusion proteins stimulated protective immunity against *T. ovis* challenge in recipient sheep while 45W, expressed as a GST fusion protein, was effective (Rickard *et al*. 1995). The GST component was cleaved with thrombin following expression in pGEX-2T and the resultant 45W peptide was equally effective as the GST fusion construct. One problem encountered was that yields of the fusion protein were low and it was unstable, but this was overcome by modifying the C-terminal end of the protein (Rickard *et al*. 1995). An additional problem encountered was that the GST-fusion was expressed in *E. coli* mostly as insoluble inclusion bodies but this was overcome by solubilisation in urea and its subsequent removal.

The development of the Bm86 antigen-based vaccine against *B. microplus* has involved similar steps (Willadsen, 1995). The antigen is an 89 kDa glycoprotein with an extracellular location on the digest cells of the tick gut. In developing the vaccine, a variety of recombinant proteins was tested, including an *E. coli* expressed β-galactosidase fusion protein that gave significant protection but was not as effective as the native protein (Rand *et al*. 1989). Alternative constructs, baculovirus expression and downstream processing of the bacterially-expressed protein were all tested and yielded products with vaccine efficacies comparable to the native protein (Tellam *et al*. 1992). In addition, Bm86 has been expressed in *Aspergillus* at low levels (Turnbull *et al*. 1990) and in the yeast *Pichia pastoris* (Rodriguez *et al*. 1994), the later being an effective immunogen (Rodriguez *et al*. 1994).

Bacterially-expressed recombinant versions of several gastrointestinal nematode antigens have been produced which induce protective immunity (Emery, 1996). However, difficulties can be anticipated where protective epitopes are conformational in nature, for example the active site region of an enzyme, where glycosylation contributes to protection or, in the case of complexes such as H-gal-GP, where more than one protein may be required to stimulate the full protective response. These difficulties are being addressed by expressing parasite antigens in baculovirus (e.g. H11), in yeast vectors (e.g. cathepsin Ls from *F. hepatica* (Dowd *et al*. 1997)), in the free-living nematode *C. elegans* (D. L. Redmond & D. P. Knox, unpublished) and by testing combinations of recombinant proteins. DNA vaccination may provide the answer (see below).

Another option being investigated is to identify regions of the target molecule which are likely to be accessible to immune effectors and are, in themselves, immunogenic. This can be achieved by, for example, computer modelling of protein structures from primary amino acid sequence data. Regions exposed on the surface of the protein can then be expressed as linear peptides in bacteria and immunogenicity evaluated. This approach mirrors the evaluation of synthetic peptide vaccines which comprise short regions of the protein target. In the case of the *T. ovis* 45W antigen, synthetic peptides were synthesized corresponding to putative host-protective regions at the N- and C-terminal ends of the protein. The N-terminal peptide was clearly more immunogenic and only antibodies to this bound to oncosphere antigens. (Dadleymoore *et al*. 1999).

Finally, the method by which a host-protective recombinant protein is produced must be amenable to commercial scale-up and the protein readily purified, preferably by a one-step procedure. Production costs, their effects on profitability and vaccine cost per dose to the farmer will, ultimately, dictate whether a vaccine ever reaches the market place.

FUNCTIONAL GENOMICS AND ANTIGEN SELECTION

The combination of protein analysis (proteomics), be it by peptide sequencing or analyses of biochemical/immunological function, and gene sequence analyses (genomics) has proven to be a very powerful combination (functional genomics) in the efforts to develop recombinant protein-based vaccines. The precise definition of the peptide components of antigenic mixtures can be exploited to devise more refined fractionation procedures such as substrate/inhibitor-based affinity chromatography for enzymes or lactose solubilization for galectins (Newlands *et al*. 1999). In addition, knowledge of protein function defines the likely requirements for recombinant protein expression. Functional genomics has an even wider application in identifying potential protein targets for vaccination or novel drug targeting. Genome sequencing, expressed sequence tag (EST) analyses and direct protein sequencing have and will continue to provide a range of potential protein targets for further analysis. From the vaccinologists point of view, the first requirement will be that the chosen target molecule is accessible to an immune response stimulated by vaccination. Immunolocalization and *in situ* hybridization analyses can provide an early indication of accessibility exemplified, to an extent, by fractionation trials conducted with *H. contortus* TSBP (Knox *et al*. 1999; Skuce *et al*. 1999*a*). The prominent component of TSBP is a 60 kDa protein now identified as a GDH by cDNA sequence analysis and subsequent biochemical analyses (Skuce *et al*. 1999*a*). This protein has been purified, almost to homogeneity, and is not host-protective (Skuce, Smith & Knox, unpublished). Immunolocalization studies demonstrated that GDH was expressed in the cytoplasm of the intestinal cells, not on the microvillar surface, and hence would not be accessible to circulating antibody (Skuce *et al*. 1999*a*).

MUCOSAL IMMUNE RESPONSES AND ANTIGEN DELIVERY

In many of the host–parasite systems referred to above, the precise mechanisms of worm rejection remain unclear. What is known has been extensively reviewed in recent years (e.g. Miller, 1984; Rothwell, 1989; McClure & Emery, 1994; Miller, 1996). The hidden antigen approach is successful against blood-feeding nematodes but is ineffective against non-blood feeders such as *N. battus* (Smith, 1993) and *T. colubriformis* (Emery, 1996) and, to date, has had only partial efficacy against *Ostertagia* and *Teladorsagia* genera (W. D. Smith & D. P. Knox, unpublished). These parasites contain equivalent antigens but may not ingest sufficient antibody.

Candidate antigens selected from non-blood feeders may need to be presented to the immune system in a manner which stimulates an appropriate local mucosal response. This response is, essentially, a Th_2-like hypersensitivity reaction resulting in rapid worm rejection and featuring IgE production, mast cell proliferation and worm-specific degranulation. The response can be accelerated by depleting γ-interferon (McClure *et al*. 1995), a cytokine which can suppress Th_2 responses during nematode infections in rodents (Else *et al*. 1994). In addition, local IgA responses have been related to reductions in worm length in lambs infected with *T. circumcincta* (Smith, 1988) and are likely to mediate aspects of immune exclusion (Walker, 1994). Therefore, the meaningful evaluation of the ability of any antigen to stimulate protective immune responses is absolutely dependent on it being presented to the immune system in such a way as to stimulate the correct response.

Antigens undergoing evaluation have, until recently, usually been delivered subcutaneously, intramuscularly or intra-peritoneally in association with an adjuvant, the latter, being included to improve immunogenicity. In the past, Freund's adjuvants have been used extensively but a recent study has shown that Freund's adjuvant actually increases the susceptibility of sheep to *T. colubriformis* infection (Wagland *et al*. 1996). Adjuvants can act in five ways namely (1) immunomodulation, (2) antigen presentation, (3) induction of appropriate cellular response, (4) targeting and (5) depot generation (Cox & Coulter, 1997). Immune responses at mucosal sites are linked through the common mucosal immune system so that oral or intra-nasal antigen delivery can result in the induction of secretory immune responses at all mucosal sites. Again, caution is required before advocating any vaccine delivery system because successful oral vaccination of mice against *Trichuris muris* infection was more dependent on host genetics than on the means of antigen delivery (Robinson, Bellaby & Wakelin, 1995). Recent research has identified a variety strategies to optimise mucosal vaccination. This topic has been reviewed recently (Walker, 1994; Husband *et al*. 1996) and discussions included the variety of adjuvants available, their mode of action, antigen delivery in replicating vectors such as *Salmonella* (Chatfield *et al*. 1995) or *Vaccinia*, non-replicating

systems such as microspheres or antigen conjugated to an adhesive molecule such as cholera toxin. Antigens can now be delivered in association with cytokines and it can be anticipated that cytokines which support the development of Th_2 responses (IL-4) and inhibit Th_1 responses (IL-4 and IL-10) would be of value in helminth vaccine formulations (Lofthouse *et al*. 1996). Finally, protective immune responses can be stimulated by DNA vaccination (see below) and these responses can be manipulated by co-administration with cytokine fusion constructs (e.g. Maecker *et al*. 1997).

DNA VACCINATION

DNA vaccines have been defined as the 'third generation of vaccines' (Waine & McManus, 1995) and recent developments in this field have been the subject of a thoroughly comprehensive review (Alarcon, Waine & McManus, 1999). DNA vaccines are based on plasmid vectors which express the inserted gene or cDNA of interest under the control of a strong promotor. They eliminate the requirement for the expression and purification of recombinant proteins and are stable, not requiring refrigerated storage. DNA can be taken up by host cells during *in vitro* tissue culture to allow testing confirmation of protein expression before *in vivo* administration. *In vivo*, the DNA can persist for long periods of time, allowing prolonged expression of the encoded protein and the protein is more likely to resemble the normal eukaryotic structure than recombinant proteins expressed in bacterial cells (Alarcon *et al*. 1999). It is not clear precisely how DNA vaccination works but the DNA plasmid is delivered directly to the target cells, is taken up and expressed by the host cells and the protein product is recognized as foreign inducing an immune response.

Attempts to apply DNA vaccination to nematode infections have yet to be reported but encouraging results have been obtained in rodents given DNA vaccines encoding antigens from *S. japonicum* (Yang, Waine & McManus, 1995; Waine *et al*. 1997) and *S. mansoni* (Ďupre *et al*. 1997). In both cases, a Th_1-like response was induced and, in the latter, parasite antigen (GST-28) was detectable in the skin of rats at the site of DNA injection. Sera from these rats mediated antibody-dependent cellular cytotoxicity *in vitro* and killing of schistosomula. Parasite challenge induced a rapid increase in the specific IgG antibody response.

The approach has also been applied, with some success, to vaccination of sheep against *T. ovis* with plasmid DNA constructs encoding the 45W antigen (Rothel *et al*. 1997*a*) and recombinant ovine adenovirus constructs (Rothel *et al*. 1997*b*), the latter inducing some protective immunity against challenge. Sheep which received the plasmid vaccine and were then boosted with the adenovirus construct mounted IgG_1 responses 65-fold higher than those in sheep which received either vaccine alone.

Humoral responses in the mice following vaccination with DNA constructs encoding glutathione S-transferase from *Fasciola hepatica* have been evaluated (Smooker *et al*. 1999). The DNA constructs directed cytoplasmic or extracellular expression, and the level of response and isotype differed between the groups with intramuscular injection of the cytoplasmic construct generating a Th_1-type response whilst intradermal injection of the extracellular construct gave a Th_2 type response. In addition, the humoral response was highest after injection of the extracellular construct.

Clearly, DNA vaccination has enormous potential for the development of stable and effective vaccines. As our understanding of how DNA vaccination works and how the responses generated can be manipulated and maximized, it is not beyond the bounds of possibility that DNA vaccination will, in the future, replace antigen testing in vaccine trials. Finally, it may not even be necessary to screen for protective antigens given that direct injection of partial expression libraries from *Mycobacterium pulmonis* induced protection against challenge (Barry, Lai & Johnston, 1995) and mice can be partially protected against *Plasmodium chabaudi* infection by a similar approach (Spithill, Setiady & Smooker, 1999).

CONCLUDING COMMENTS

The last decade has seen potentially ground-breaking advances in vaccine development which include antigen selection but, in particular, the refinement of recombinant DNA technologies which are crucial for the production of antigen cheaply and in quantity. Problems are still being encountered in expressing antigens in an immunogenic form in the vectors currently available. Unfortunately, the process still tends to be the subject of trial and error. However, it will be disappointing if a recombinant protein-based vaccine with the desired efficacy against *H. contortus* is not available within the next decade. Whether such a vaccine becomes available to the farmer will depend on commercial prospects and constraints at the time. In parallel with a need for continued antigen isolation and testing, there is a need to develop easy means for mucosal antigen delivery in ruminants. Then new and existing antigens can be tested in the knowledge that the immune response stimulated by vaccination has been appropriate. DNA vaccination has obvious potential but a note of caution is required because there may be consumer resistance to consuming meat products containing foreign DNA. These concerns can be alleviated by defining the precise

fate of the DNA innoculum. Finally, multivalent vaccine development will be hastened by collaboration between laboratories working on the same and different nematode species. There is considerable commercial interest in vaccination but their interests are not necessarily best served by secrecy agreements. Our commercial partners need to ask themselves how they are going to develop a multivalent vaccine. It is simple really – collaboration!

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