Vaccine development and diagnostics of *Dictyocaulus viviparus*

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SUMMARY

Parasitic bronchitis is a serious disease of cattle and is caused by the nematode, *Dictyocaulus viviparus*. For over 30 years, a radiation-attenuated larval vaccine has been used for prevention of this disease. This vaccine has been used with considerable success in the UK and parts of Western Europe, however, it has several disadvantages. It has a short shelf-life and the vaccine has to be produced annually necessitating the use of donor calves. Following vaccination, calves must receive further boosting from natural challenge to maintain protective immunity. Sales of the irradiated larval vaccine have decreased dramatically since the 1970s. This is thought to be due to increased reliance of farmers on anthelmintic programmes to control lungworm infection. It is possible that, under certain circumstances, these programmes do not allow sufficient parasite exposure to stimulate protective immunity to further *Dictyocaulus* challenge. This is borne out by the recent documented increase in the number of outbreaks of parasitic bronchitis in the UK. A stable vaccine against *D. viviparus* that is capable of stimulating a more prolonged immunity would be beneficial. Recent research has been directed at identification and isolation of components thought to be involved in parasite survival in the host and examination of their potential as vaccine candidates. One of these components is acetylcholinesterase (AChE), an enzyme secreted by adult worms. This review describes the development of the secreted AChE as a vaccine candidate, as well as documenting recent developments in the immunodiagnosis of *D. viviparus*.

Key words: *Dictyocaulus viviparus*, vaccine development, immunodiagnosis, acetylcholinesterase.

INTRODUCTION

Dictyocaulus viviparus is a trichostrongylid nematode whose adult stages inhabit the main stem bronchi and tracheae of cattle. This parasite causes a severe, sometimes fatal, bronchopneumonia, the most common clinical manifestations being coughing, respiratory distress and weight loss. Since the 1950s, a vaccine composed of irradiated larvae (Dictol or Huskvac, Intervet) has been available for the control of parasitic bronchitis (husk). This vaccine stimulates a strong protective immunity and, following its commercial introduction, was very successful in reducing the number of outbreaks of disease. Traditionally, husk was a disease seen in calves during the latter half of the first grazing season, however, reports of lungworm in adult cattle have increased dramatically over the last decade (David, 1993, 1996, 1997; Robinson, Jackson & Sarchet, 1993; Williams, 1996; Veterinary Investigation Diagnosis Analysis (VIDA), 1997, Central Veterinary Laboratory, Weybridge). Figures from VIDA show that between 1992 and 1994 there were a total of 538 recorded outbreaks compared with 232 in the preceding 3 years. Since 1994, there has been a further steady increase in the number of reported annual outbreaks, peaking at 543 in 1997 which represented a 64% increase on the figures quoted for 1996 (see Fig. 1). The numbers that are recorded by VIDA are thought to be a vast underestimate of the actual prevalence as many clinical cases are treated by farmers without veterinary consultation or without involvement of the veterinary investigation services. The increase in recorded diagnoses of parasitic bronchitis is thought to reflect the increasing commercial involvement and awareness of the disease, as well as the availability of an ELISA for detection of specific antibody. Nevertheless, the rise in outbreaks probably reflects the growing tendency of farmers to replace vaccination with strategic treatments using highly effective anthelmintics (Connan, 1993; Mawhinney, 1996). The sales of Huskvac have dropped considerably over the last two decades with approximately 750000 animals being vaccinated per annum in the mid 1970s, falling to 250000 cattle per annum in the 1990s (I. Mawhinney, personal communication). Whilst most modern anthelmintics have excellent efficacy against *D. viviparus*, their repeated use may result in reduced exposure to the parasite antigens causing animals to remain susceptible to disease in later life (Urquhart *et al.* 1981; Vercruysse *et al.* 1987). Even if farmers do vaccinate, but subsequently use repeated anthelmintic treatments, animals will have reduced exposure to further natural challenge and immunity will wane leading to susceptibility to disease. Parasitic bronchitis in adult cattle is potentially serious

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https://doi.org/10.1017/S0031182099005727 Published online by Cambridge University Press

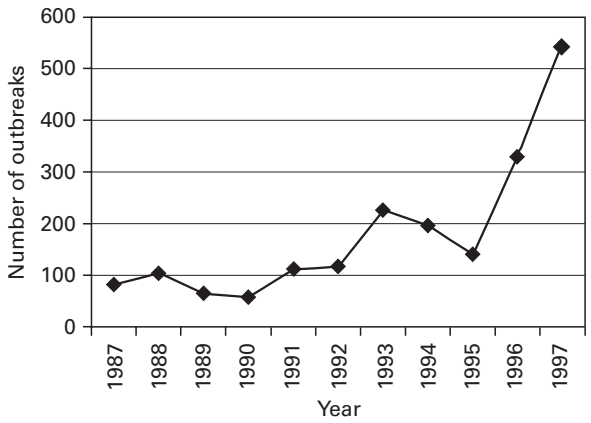

Fig. 1. Recorded outbreaks of *Dictyocaulus viviparus* (1987–1997). Data taken from VIDA analysis III (1998), Central Veterinary Laboratory, Weybridge.

and can have high morbidity with significant economic consequences through costs of treatment and reductions in milk yield, fertility and body weight (Woolley, 1997).

The irradiated larval vaccine has several disadvantages. For example, it is relatively unstable and therefore not suitable for extensive application abroad. Furthermore, the *D. viviparus* larvae must be produced annually, requiring the sacrifice of many donor calves. As the vaccine does not confer a parasitologically-sterile immunity, animals can continue to act as carriers (Hendriks & van Vliet, 1980; Urquhart, 1985). Thus, on farms with a history of parasitic bronchitis, each new batch of calves must be vaccinated. Furthermore, as mentioned above, in the absence of subsequent field challenge (as in the case of animals treated long term with anthelmintics), immunity induced by the vaccine falls dramatically leading to susceptibility to disease (Michel & McKenzie, 1965). A recombinant vaccine against *D. viviparus* that is more stable and would provide longer lasting immunity would be beneficial.

SOURCES OF PROTECTIVE ANTIGENS IN *D. VIVIPARUS*

Several studies have been performed to investigate the sources of putative protective antigens in *D. viviparus*. Many of the earlier studies provided indirect evidence that immune responses mounted against antigens expressed by the later developmental stages such as fourth stage larvae (L_4), fifth stage larvae (L_5) or adult worms are involved in protective immunity. For example, when immune cattle were re-infected, it was observed that larvae penetrated the lungs where further development was terminated from day 11 p.i. onwards (Michel, 1969). Furthermore, in studies with irradiated larvae, it appeared that the worms had to invade the lungs for protection to be induced (Cornwell, 1960; Poynter *et al*. 1960). The precise antigens that stimulate immunity to *D. viviparus* have not been identified but molecules released by adult worms may play an important role. When adult excretory/secretory (ES) products were characterized by immunoprecipitation followed by SDS–PAGE, 18 of the 20 polypeptides present in the ES products were seen to be recognized by infected, immune calves (Britton *et al*. 1993*a*). Subsequently, different antigen fractions from several life cycle stages of *D. viviparus* were compared for their ability to induce protection in the laboratory model host, the guinea pig. In one study where animals were immunized in the context of Freund's complete adjuvant, those groups that received somatic extracts of adult worms or third stage larvae (L_3) were not protected significantly against challenge (McKeand *et al*. 1995*a*). In contrast, animals that received adult ES products were significantly protected against re-infection when compared with challenge controls (Mann–Whitney *U*-test, $P < 0{\cdot}05$). To examine the protective role of antibody, naive guinea pigs were passively immunized with sera from animals that had been immunized previously with adult ES products or from guinea pigs exposed to experimental L_3 infection (McKeand *et al*. 1995*a*). The serum recipients in both groups were challenged with *D. viviparus* L_3 1 hour after passive transfer and their worm burdens examined at day 6 p.i. The numbers of lung larvae in both sera recipient groups were significantly lower than in guinea pigs that had received normal sera (Mann–Whitney *U*-test, $P < 0{\cdot}05$) suggesting that, in the laboratory host at least, antibody-mediated mechanisms contribute to immune protection in both L_3 infection and adult ES immunization. These results corroborated with earlier passive transfer studies in calves where antibody was shown to play a protective role in immunity to L_3 challenge (Jarrett *et al*. 1955). These results argue that measurement of antibody responses to *D. viviparus* is pertinent to the definition of protective antigens.

RATIONAL APPROACH TO VACCINE DESIGN

This involves identification of molecules that are potentially critical to parasite survival in the host. One strategy for reducing nematode survival *in vivo* is to induce immune responses to enzymatic components that may assist the worm in invasion of host tissue, feeding, replication or evasion of host immunity. Often, these components are found on the parasite surface or in their ES products. Antibody responses to the surface antigens of *D. viviparus* have been characterized in detail (Britton *et al*. 1993*b*; McKeand *et al*. 1996; Scott, McKeand & Devaney, 1996), however, the relationship of these responses to protective immunity is unclear. The antibody

https://doi.org/10.1017/S0031182099005727 Published online by Cambridge University Press

responses to the adult surface, for example, may have little relevance to immunity as it has been shown that these stages readily shed surface-bound antibody when maintained at 37 °C (McKeand & Kennedy, 1995).

The ES products of adult *D. viviparus* have been shown to contain enzyme activities of different classes. These include acetylcholinesterases (McKeand *et al.* 1994*a*), proteinases (Britton *et al.* 1992) and superoxide dismutases (Britton, Knox & Kennedy, 1994). Five migratory isoforms of AChE were shown to be present in *D. viviparus* adult ES products by gel electrophoresis followed by specific enzyme staining (McKeand *et al.* 1994*a*). These AChEs were common to adult ES products and adult worm somatic extracts, however, chemical assay showed the AChE activity to be 200 times more abundant (per unit protein) in the ES products. Expression of these AChE isoforms appeared to be regulated in that these enzymes were not detected in somatic extracts of L_3 stages. All five isoforms of the adult AChEs were shown to be immunogenic in that they were recognized by antibody from infected calves, indicating that these enzymes are released *in vivo* (McKeand *et al.* 1994*a*).

Why would a parasitic nematode expend the energy to release AChE? These enzymes have been ascribed a role in immunomodulation via the hydrolysis of host acetylcholine (ACh) which, in addition to its role in neurotransmission, has been found to enhance the functions of several types of host immune effector cell. Lymphocyte activation, mast cell degranulation and neutrophil-mediated antibody-dependent cytotoxicity have all been shown to be enhanced in the presence of ACh (reviewed by Rhoads, 1984). This is of relevance to *D. viviparus* infection because, in infected animals, increased numbers of neutrophils (Jarrett & Sharp, 1963), mast cells (H. R. P. Miller, personal communication) and lymphocytes (Jarrett & Sharp, 1963) are observed in the lungs. Thus, it could be postulated that adult *D. viviparus* release AChE to hydrolyse ACh and ablate its effect on these types of effector cell within the immediate environment of the worm. The theory that AChE secretion by gastrointestinal nematodes leads to a so-called biochemical holdfast by reducing intestinal contractions has not been substantiated. Although many intestinal nematodes release AChE (for example, Pritchard *et al.* 1991; Blackburn & Selkirk, 1992), these enzymes have now been shown to be secreted by non gut-dwelling parasites such as *Brugia malayi* (Rathaur *et al.* 1987) and the bird lungworm, *Syngamus trachea* (Riga *et al.* 1995).

In an attempt to investigate the role of *D. viviparus* AChE in protective immunity, adult ES fractions were enriched for AChE activity by electroelution of protein from the appropriate region of polyacrylamide gels and the fractions used to immunize guinea pigs (McKeand *et al.* 1995*b*). The AChE-enriched fraction produced significant levels of protection after challenge compared with the adjuvant control group (Mann–Whitney U-test, $P < 0{\cdot}05$) and stimulated high levels of AChE-specific antibody. In a separate study, adult ES products were used to immunize two inbred strains of guinea pigs as opposed to the outbred strain used in the experiments described above (McKeand *et al.* 1994*b*). The results showed that the two inbred strains differed in their susceptibility to challenge infection following ES immunization and also in their antibody responses to *D. viviparus* antigens. Interestingly, when antibody binding to *D. viviparus* AChE was compared between the two strains, it was observed that strain 13 guinea pigs, which were significantly protected against re-infection, recognized more isoforms of AChE than strain 2 guinea pigs, which were not protected. It is not yet known, however, whether antibodies specific to *D. viviparus* AChE can passively confer immunity to challenge.

It has proved impossible to purify sufficient native AChE from *D. viviparus* adult ES products to perform meaningful immunization studies in either guinea pigs or calves. Recently, an AChE-encoding cDNA has been isolated from *D. viviparus*. Initially, an AChE-encoding fragment of 365 bp was generated from adult worm RNA by reverse transcriptase polymerase chain reaction. This was used to screen an adult *D. viviparus* cDNA expression library. Several cDNA clones were isolated which sequencing showed to be different lengths of the same gene. The longest cDNA was 1·7 kbp and this showed between 50 and 60 % identity to other AChE genes (J. B. McKeand, unpublished observations). At the protein level, this sequence was 550 amino acids in length and presented 45 % identity with the *Nippostrongylus brasiliensis* acetylcholinesterase B precursor (over 510 amino acids); 50 % identity with the *C. elegans ace-2* product (over 395 amino acids) and 35 % identity with the human cholinesterase precursor (over 506 amino acids). The *D. viviparus* AChE sequence contains conserved positions of the components of the catalytic triad. Part of this cDNA has been subcloned and expressed in a bacterial expression system to produce a polypeptide of approximately 55 kDa. This recombinant molecule is recognized by sera from guinea pigs immunized with the AChE-enriched ES fraction as well as by sera from immune, infected calves (J. B. McKeand, unpublished observations). Moreover, Western blotting experiments have shown that rabbit serum raised against the recombinant AChE recognized a component of approximately 55 kDa in adult ES products. This recombinant protein was recently assessed in an immunization study in calves and, unfortunately, did not induce significant levels of protective immunity when compared with challenge control animals (J. B. McKeand, unpublished data).

Immunization studies using AChE from other parasitic nematodes have also produced equivocal results. In an earlier study, guinea pigs were immunized with AChE-enriched *Trichostrongylus colubriformis* fractions and were not protected against challenge (Rothwell & Merritt, 1975). In a later study, purified secretory AChE from *T. colubriformis* was used to immunize sheep against infections with the homologous parasite, in addition to *Haemonchus contortus* and *Cooperia oncophora* (Griffiths & Pritchard, 1994). A low degree of cross-species protection was achieved with an average reduction in worm burden of all species of 31 % but there were no consistent reductions in faecal egg counts and modest increases in AChE-specific antibody.

As mentioned above, SOD activity has also been detected in *D. viviparus* adult ES products. An ES-specific copper/zinc-dependent SOD was found to be released in large quantities by adult worms (Britton *et al*. 1994). The antigenicity of this SOD isoform was demonstrated by reduction of enzyme activity following incubation of adult ES products with IgG antibody purified from the sera of infected or vaccinated calves (Britton *et al*. 1994). The high level of SOD released by adult *D. viviparus* may be a reflection of the oxygen-rich pulmonary environment of this parasite and antibody inhibition of this SOD may be an important target of protective immunity. Attempts are now under way to clone and express the gene encoding this SOD for use in further immunological studies.

Proteinase activities have also been identified in *D. viviparus* adult ES products (Britton *et al*. 1992). Serine-, cysteine- and metalloproteinases were identified in several stages with secreted materials being more active against protein substrates per unit protein than the somatic extracts. Again, antigenicity of the parasite proteinases was demonstrated by inhibition of enzyme activity with Protein G-purified serum IgG antibody from infected and vaccinated calves. Antibody response to these proteinases may limit parasite-mediated tissue damage thus limiting pathology, as well as reducing worm survival. No further work has been performed on the *D. viviparus* proteinases.

IMMUNODIAGNOSIS OF *D. VIVIPARUS*

Serodiagnosis of parasitic bronchitis by ELISA has been evaluated in naturally infected, experimentally infected and vaccinated animals. These studies have been carried out to aid diagnosis of infection in the field or to provide information on prevalence. Positive ELISA titres appear to be a satisfactory indicator of recent herd exposure, however, they are rather inaccurate in determining the immune status of individual animals (Bos & Beekman, 1985). Furthermore, seroprevalence rates do not always reflect actual outbreaks of clinical disease. For example, in one study where 75 % of herds tested were found to have positive titres to *D. viviparus* only 15 % had clinical husk, although 51 % of the farms had experienced husk in the past (Boon, Kloosterman & Van Der Lende, 1984). Furthermore, all of the current ELISAs used diagnostically incorporate antigen preparations from adult worms (for example, the ELISA used by the UK veterinary investigation service), so that larval invasion following vaccination or during pre-patent infection is not detected. Thus, it is difficult to assess vaccinated animals that have not been exposed to subsequent pasture challenge. For example, in experimentally infected cattle, the response to adult worm antigens was delayed when responses were studied in vaccinated animals which had a slow uptake of infective larvae (Bos, Beekman-Boneschanscher & Boon, 1986). To overcome this, an L_4 stage ELISA has been used in some experimental studies to examine responses in vaccinated calves (Mawhinney, 1997), however, these are not generally available to veterinarians in practice.

Much of the recent work concerned with immunodiagnosis of dictyocaulosis has been performed in the Netherlands and Germany. De Leeuw & Cornelissen (1991) identified a specific 17 kDa antigen for use in diagnosis by comparing somatic extracts of adult worms, ES antigens of adult worms and somatic antigens of L_3 in an indirect ELISA. Species-specificity was examined using sera from calves with mono-infections of heterologous helminth species. When adult worm somatic antigens were analysed by Western blotting, a 17 kDa protein was identified that did not react with the heterologous sera. This was isolated by ultrafiltration and anion chromatography and compared with whole somatic antigen in an indirect ELISA. Extinction values measured in both assays correlated well and the protein has been developed for use in diagnostic ELISA. Subsequently, three different ELISAs were compared for sensitivity, specificity and seroconversion after primary infection (de Leeuw & Cornelissen, 1993). These assays were an indirect ELISA using crude somatic adult antigen; an indirect ELISA containing purified antigens isolated from adult worm somatic antigens and a competition ELISA incorporating purified antigen in combination with *D. viviparus*-specific monoclonal antibodies. The specificity of the competition and the purified antigen ELISA was 97 %, whereas the specificity of the crude antigen ELISA was 67 %. Sensitivities of the purified antigen, the competition and the crude antigen ELISAs were 97, 73 and 99 %, respectively. All three assays detected seroconversion 4–6 weeks p.i. although none detected seroconversion in recently vaccinated calves. In another study, a *D. viviparus*-specific ELISA and IHA were compared for sensitivity, specificity, time of seroconversion and persistence of antibody re-

https://doi.org/10.1017/S0031182099005727 Published online by Cambridge University Press

sponses (Cornelissen, Borgsteede & van Milligen, 1997). Specificity of both tests was very high, however, the sensitivity of the ELISA (100%) was far superior (IHA 78·1%) and detected antibodies earlier in infection, although vaccinates were not seropositive. Seroprevalence using this ELISA was determined in a field study where 48·6% animals were found to be positive and when the ELISA was used in five different laboratories, the repeatability and reproducibility were promising enough to introduce this as the routine test in The Netherlands. The sequence of the purified antigen used in this ELISA has not been characterized but is likely to be a native equivalent to the recombinant major sperm protein (MSP) mentioned below. This ELISA is available commercially as a kit (Ceditest, Institute for Animal Science and Health ID-DLO, The Netherlands) and has now been used widely in The Netherlands, Germany, Belgium, France and Sweden (F. H. M. Borgsteede, personal communication).

In separate studies, an adult worm antigen of diagnostic potential was detected by Western blotting of crude adult worm antigen with sera from infected and vaccinated cattle (Schnieder, 1992). A *D. viviparus*-specific region around 18 kDa was identified, isolated and a lambda ZAP II cDNA library screened with rabbit antiserum to the antigen. Of the clones identified, the one with the highest expression yields was expressed as a glutathione S-transferase protein (DvGST3-14) and also, after cleavage with thrombin, as pure recombinant parasite protein (Dv3-14). An immunoblot dipstick test was developed which provided results within 90 min of blood sampling and was found to detect infections with more than 99% specificity and sensitivity by 30–85 days p.i. (Schnieder, 1993*a*). Subsequently, this antigen was shown to be encoded by a gene fragment with homology to MSP from *Ascaris suum*, *C. elegans* and *Onchocerca volvulus* (Schnieder, 1993*b*). The recombinant antigen had now been subcloned in a *Drosophila* expression system and has been shown to work well in the dipstick format (T. Schnieder, personal communication). This test is offered commercially but the response so far in Germany has been disappointing (T Schnieder, personal communication).

CONCLUSIONS

Thus, despite the presence of the irradiated larval vaccine for nearly 40 years, there is still much research interest in the immunology of *D. viviparus* infection. Partly, this reflects the re-emergence of husk as an important disease in cattle in wet, temperate climates but also because an improved vaccine is sought that would promote a longer-term immunity in the absence of pasture challenge. In addition, *D. viviparus* provides a rather unique system in which to study immune responses to a parasitic nematode, as unlike the situation with most ruminant helminths, exposure to this parasite results in a relatively strong and rapid immunity. Attempts have been made to define the precise protective responses and antigens involved in dictyocaulosis but these still elude us. In terms of the recombinant AChE, it is likely that a molecule more similar to the native enzyme, than the form described here, may induce better protection and, to this end, the AChE clone will be sub-cloned into a baculovirus system. *D. viviparus* serology too has its drawbacks, with a lack of larval specific diagnostic tests available that can identify the immune status of an individual animal.

ACKNOWLEDGEMENTS

The unpublished AChE work cited in this review was supported by the BBSRC. The author is grateful to Fred Borgsteede, Graham David, Ian Mawhinney, Keith Matthews and Thomas Schnieder for their helpful comments in the preparation of this manuscript and Amanda Davidson for much of the technical work relating to the AChE work.

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