Immunodominant glycoproteins of the bovine lungworm *Dictyocaulus viviparus*

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Cover: Tobacco farmer with oxen. Piñar del Rio, Cuba.
background: eggs of *Dictyocaulus viviparus* containing first stage larva.

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Immunodominant glycoproteins of the bovine lungworm *Dictyocaulus viviparus*

Immunodominante glycoproteinen van de runder longworm *Dictyocaulus viviparus*

(met een samenvatting in het Nederlands)

Proefschrift

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Voor mijn ouders

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CHAPTER 1

General intoduction

GENERAL INTRODUCTION

1. Introduction

Parasitic nematode infections are a burden for animal husbandry. In general, the infections do not cause a high mortality but morbidity can be high with concomitant loss of production. Control measures to prevent infestation of the animals are difficult due to the continuous exposure of the animals to contaminated pasture. Anti-helminth drugs are used to combat nematode infections, but resistance of the worms to the drugs is increasing and limits the efficacy of this approach. The annual cost of worm parasites to livestock producers is estimated at more than $100 million in the USA despite the constant use of antihelminthics and pasture management. Furthermore, control costs are high with the market for anthelmintic drugs estimated at $ 3 billion dollars globally. In Europe annual spending approaches 1 billion euro (49). Infected animals eventually develop a protective immune response against the worms, suggesting that development of vaccines may ultimately solve the problem. For the nematode *Dictyocaulus viviparus*, the etiologic agent of bovine parasitic bronchitis (husk) or lungworm disease in cattle, a vaccine consisting of attenuated infectious larvae is commercially available in some countries, but this vaccine provides only short-lived protection. In this Chapter, an overview is presented of the all aspects of *D. viviparus* infection in cattle as a start point to better understand the interaction of the parasite with the host immune system and thus to discover and characterize novel vaccine antigens that may provide long-lasting protection against *D. viviparus* and perhaps also other infectious helminths.

2. The parasite

Dictyocaulus viviparus (Bloch, 1782) is a large parasitic nematode (up to 8 cm in length) that infests cattle, buffalo and some deer species. The parasite can be found

in temperate and tropical regions world-wide. In many parts of the world it is one of the principal nematodes in cattle. In the Netherlands at least 72% of the farms have animals that test positive for *D. viviparus*, as judged from larval excretion in adult cows (21).

2.1 *Life cycle*

D. viviparus is a dioecious nematode (different sexes) with a direct life cycle (no intermediate host). The adult worms live in the upper respiratory tract of cattle. Despite its name, *D. viviparus* is not viviparous, but ovo-viviparous; the female worms produce eggs that contain L1 larvae that are fully developed by the time the egg is released in the lung. Within 24 h after release the eggs hatch to first stage larvae (L1). The eggs and/or L1 larvae are coughed up, swallowed by the host, and passed through the gastrointestinal tract. The L1 larvae in the faeces develop without feeding into the infective, third stage larvae (L3) within a period of seven to ten days when conditions are optimal. Under harsh environmental conditions the L3 larvae remain embodied in an extra protective (L2) sheath. The L3 larvae are rather sluggish and take advantage of the fungus *Pilobolus* (44) to disperse from the faeces to the herbage. The L3 larvae crawl into the sporangiophore of the *Pilobolus*, where the spores are formed. When the spores are maturated, the structure below the sporangiophore bursts and the spores together with L3 larvae are spread to a distance of several meters. Once the infective L3 larvae are ingested by the grazing host, they start the somatic migration by penetrating the intestinal wall and migrating to the mesenteric lymph nodes and the blood circulation to the lungs. During this migration they moult via the L4 to the L5 stage. In the lungs they escape from the capillaries and access the alveoli. Here the L5 stage develops without further moult into adult male and female worms. When conditions are not suitable for reproduction the worms can arrest their development until circumstances have improved. Adult worms are mostly found in the upper bronchi

or even in the trachea. Smaller worms mostly reside in the smaller bronchioles. The pre-patent period (the period between infection and excretion of larvae in the faeces) is 24 days.

2.2 *Classification*

D. viviparus is a nematode that belongs to the family of Dictyocaulidae of the superfamily of Trichostrongyloidea. This superfamily belongs to the order of the Strongylida, all within the phylum Nematoda (2). More recent work on the phylogeny within the phylum based on small subunit sequences identified five clades within the phylum Nematoda (8). In this phylogenetic tree the order Strongylida is placed in clade V together with the suborder Rhabditina (containing *Caenorhabditis elegans*) and minor groups. Currently the genus *Dictyocaulus* consists of six species (28) with possibly a 7th species from cervids in Sweden (35). The species of the genus *Dictyocaulus* infest sheep, goat, deer, equines and camelids. The genus *Dictyocaulus* differs from the other members of the trichostrongyloids in that the adult worms of *Dictyocaulus* reside in the lungs, while other trichostrongyloids are gastrointestinal nematodes. In addition, the adult worms of *Dictyocaulus* are at least twice the length of the largest adults of the other trichostrongyloids. *Dictyocaulus* is also one of the few members of the trichostrongyloids that migrate within the host. Furthermore, *Dictyocaulus* larvae do not feed during development from L1 to L3, are more sluggish and do not withstand harsh conditions in contrast to other trichostrongyloids that feed on bacteria, are very vivid, and (in many cases) can survive in the environment for many months to up to 1-2 years.

2.3 *Anatomy and physiology*

The anatomy of *D. viviparus* follows the general architecture of nematodes. A cross-section of an adult *D. viviparus* is shown in Figure 1. The outer layer of the

worm is the cuticle, a rigid structure that is composed of cross-linked proteins and polysaccharides. The cuticle which can take up nutrients, covers the whole body and is secreted by the underlying hypodermis. It also protects the body against the environment and can actively shed bound immunoglobulins (57). The hypodermis is a layer of cells between the cuticle and the somatic muscles. The hypodermis synthesizes proteins and is particularly active when a new cuticle has to be formed. Below the hypodermis locate the longitudinal somatic muscles. These muscles are responsible for the locomotion of the nematode. The pseudocoel (a partial body cavity) is filled with fluid and gives, together with the cuticle, strength to the organism. The pressure within the pseudocoel is regulated by the excretory canal. In the adult worms much of the pseudocoel space is occupied by the gonads; the uterus (not visible in Figure 1) and ovary in the female and the testis in the male.

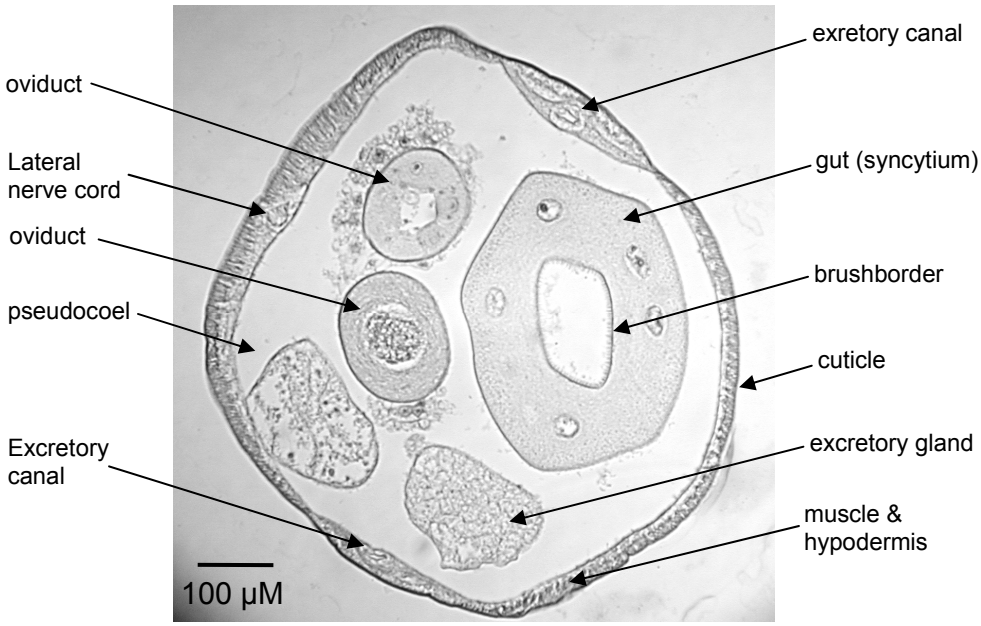

Figure 1. Cross-section of female adult of D. viviparus.

The nervous system of *D. viviparus* consists of a ventral and a dorsal nerve cord that form a ganglion around the oesophagus. The cells from the intestine are in nematodes often fused to a polynucleated cell (syncytium) (3). On the luminal side the intestinal cells contain microvilli (brushborder) for enhanced uptake of nutrients and secretion of digestive enzymes. These enzymes together with molecules from the excretory canal and from the reproductive tract form the excretory/secretory (ES) products. Many of the proteins from the ES are glycoproteins with immunogenic properties (55, 83).

3. Bovine parasitic bronchitis

3.1 The course of D. viviparus infection

The disease caused by *D. viviparus* is primarily related to the penetration of the lung epithelium by the larvae and by the presence of eggs, larvae and adults in the lungs. A sub-clinical catarrhal enteritis may develop when high numbers of larvae penetrate the intestinal wall. The sequence of events following infection was first described in more detail in the late 50's and early 60's of the previous century when animals were experimentally infected with 5,000 L3 (clinically ill, but not-fatal) and 50,000 L3 (fatal before patency) (41, 42). In calves infected with a low dose of larvae, the most prominent lesions in the pre-patent period result from infiltration of eosinophils in response to the larvae entering the bronchioles (about day 9 post-infection). The eosinophil influx may be protective (see below, The cellular immune response), but eosinophilic exudates may also block the bronchioles resulting in atelectasis (collapse of the alveoli). Furthermore, epithelisation (displacement of flat epithelium by cuboidal cells) of (non-collapsed) alveoli may occur. This all contributes to a decreased gas exchange. At a later stage during the patent period (3.5-6 weeks after infection) large adult worms further

block the bronchioles, and recruit massive amounts of eosinophils and, to a lesser extent, neutrophils, and ciliated cells are replaced by undifferentiated cells (74). Additionally, eggs and L1 larvae are aspirated back into the lungs giving rise to a serious eosinophilic granulomatous pneumonia, the development of interstitial emphysema and a further epithelisation of the alveoli. This pathology and the resulting further decrease of gas exchange causes the clinical symptomatology of parasite bronchitis including fast respiration rates due to hypoxia and frequent coughing. In the post-patent period, when no more eggs are produced and secretion of larvae in the faeces has stopped, senile or remnants of dead worms may still give rise to disease due to the epithelisation of the alveoli and peri-bronchial fibrosis which may result in a chronic irreversible decline of lung function. However, usually, the clinical signs usually disappear after about six weeks, although even in the post-patent period coughing occurs and respiration rates can remain higher than before infection.

When calves become infected with a high dose (50,000) of L3 larvae, the above described pathology is more extreme and accompanied by interstitial emphysema and edema. The resulting hypoxia may lead to impaired cardiac functioning which in turn increases pulmonary edema, leading eventually to death of the animal even before the patent period is reached.

3.2 *Diagnosis*

The first indication of the possible existence of a *D. viviparus* infection is an increased respiration frequency, breath through the mouth with the head and neck outstretched and have a deep, harsh cough. Animals in their first grazing season are more prone to acquire the disease, because of lack of immunity. It should be considered that when calves have grazed on pasture fertilized with manure from pigs, respiratory problems can also be caused by migrating larvae of *Ascaris suum*.

Laboratory diagnosis involves the demonstration by the Baermann method of excreted L1 of *D. viviparus* in the faeces. The method is based on the migration of the L1 larvae out of the faeces into a funnel where the larvae are collected and examined. The method is sensitive enough to detect one patent female worm in young cattle (18). Obviously, this method is only suitable to diagnose patent infections. A complementary diagnostic tool is based on serology. Two serodiagnostic tests have been developed that both are based on the recognition of purified or recombinant major sperm protein (MSP) present in adult worms (12, 75). Both tests are specific for *D. viviparus* and have the same drawback as the Baermann-methode in that they detect the presence of adult worms and are therefore not suitable to diagnose pre-patent infections. However, in the post-patent period the serological (ELISA and dipstick) test still give positive results until 5-6 months and 3 months after infection respectively, due to the presence of antibodies in the serum.

3.3 Treatment and environmental control

Several drugs are available for treatment of *D. viviparus* infection, including macrocyclic lactones (ML), levamisole and benzimidazols. The most recently developed drugs are the MLs. These MLs have a residual effect, meaning that the systemic levels after treatment remain high enough to prevent re-infection for a certain time. Depending on the specific type of ML this is 28 to 42 days. A major drawback of the use of anthelmintics is the development of resistance. Drug resistance in helminths is still spreading. Resistance first became apparent for sheep-nematodes, but now resistance against all currently used anthelmintics has been reported for many gastrointestinal nematodes in cattle worldwide (38, 76, 77). Resistance of *D. viviparus* to anthelminths has not been reported yet, although in Brazil decreased drug efficacy of abamectin (which belongs to the ML group) has been demonstrated (61).

An alternative to the use of antihelmintics is strategic grazing. This environmental control method is based on avoiding heavily contaminated pasture. However, as it is difficult to estimate the initial infection levels on the pasture and because the built up of the number of infective L3 on pasture goes fast, there is always the risk that exposure is higher than expected. During summer in the Netherlands, re-infection can occur in about 1 month due to the short cycle of a 24 days pre-patent period and 5 days needed to develop from L1 into L3 larvae and to rapidly disperse to the herbage. Additional problems are that strategic grazing is often combined with anthelmintic treatment and that gastrointestinal helminths with a different epidemiological pattern have to be combated at the same time. The use of anthelminthics in combination with strategic grazing poses a risk as the drugs may limit the exposure of calves to lungworm larvae during their first grazing season. This exposure is needed to build up immunity for the second grazing season. Based on Dutch research, it is current belief that a "vigilance and treat" approach combined with strategic grazing and/or treatment against gastrointestinal helminths is possibly the best strategy to prevent infection (19, 20, 22).

4. Immunity against *D. viviparus*

Natural infection of calves with *D. viviparus* elicits humoral immune responses that provide protection against re-infection. Infection induces two types of immunity, one that is effective against incoming larvae and the other protects against adult stages in the lungs (60). The response against larval stages is short-lived, lasting for less than six months. Protection against adult stages lasts for up to 27 months. Experimental infections demonstrate that protective immunity can be achieved after a single primary infection with administration of even only thirty L3 larvae resulting in protection levels of 70 to 94 % (23).

Analysis of the immune response indicates that the infection-induced antibodies directed against the sheath of L3 larvae are predominantly of the IgG1 isotype, while IgG2 responses are very low (56). This T-helper 2 (Th2) biased response is a typical feature of nematodes and is also apparent from the increase in the local mRNA levels for the Th2 cytokines IL-4, IL-5 and IL-13, although Th1 cytokines levels are elevated as well (43). Transfer of serum from immune calves renders naive calves immune for challenge *D. viviparus* infection (39). This unequivocally demonstrates that antibodies can provide protection against disease. The nature of the antigens that induce protection against *D. viviparus* are still unknown.

5. Vaccination against *D. viviparus*

The generally most cost-effective way to prevent infection is by vaccination. For almost 50 years a vaccine consisting of irradiated L3 is used successfully to prevent *D. viviparus* disease (Huskvac, Intervet). The vaccine consists of 2 doses of 1000 irradiated L3 larvae that are administered with a 4 week interval. The attenuated L3 larvae survive about 2 weeks in the host. This period is long enough to induce an immune response, but too short to generate disease (40). Four weeks after the last dose, the calves are protected against infection. However this protection is short-lived and the irradiated L3 larvae induce a poor memory response (60).

Apart from the undefined composition of the vaccine and its short shelf-life, the requirement of regular boosting for sustained protection and the production method using infected animals are major drawbacks of the current vaccine. In practice, the boosting effect is achieved by natural exposure to the parasite. This is not desirable and is particularly problematic when re-infection is prevented by use of anthelmintics (to treat or prevent infections with other helminths) leading to outbreaks of parasitic bronchitis in adult vaccinated cows (68). Development of a

better defined and more effective vaccine produced by an ethically more acceptable method awaits much more knowledge of the parasite antigens and host responses that provide protection.

The nature of the vaccine antigens that provide protection against infection also still remain to be elucidated. The vaccine induces antibodies of mainly the IgG1 isotype that react with the sheath of the L3 larvae and adult ES components. The reactivity with surface components on adult worms is much weaker (56). Vaccination of animals does not generate antibodies that react with the adult antigen MSP used in serodiagnostics.

6. Biology of nematode-parasite interactions

Comparison of the nature of the host response against different nematode species may reveal common and unique interaction between the parasite and the host, as well as provide important insights in the development of nematode-associated pathobiology and protective immunity that may give direction to design of novel vaccines. The hallmark of virtually all helminth infections is eosinophilia, mastocytosis and increased IgE levels, suggesting a Th2-like response. Th2 cells of mice produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 that stimulate mainly antibody production in B cells (IgE, IgG1), while T-helper 1 (Th1) cells produce IFN-γ and IL-2 that mainly stimulate macrophages and the production of IgG2 (62). IFNγ and IL-4 are often considered as indicators for development of Th1 and Th2 responses, respectively.

In cattle it is also possible to identify Th1- and Th2-like responses, although pure Th2 T cell clones from cattle have thus far not been obtained and T cell clones from helminths infected calves produce besides IL-4 also some IFN-γ (16). Nevertheless, antibody isotype responses in cattle seem similar as in mice; IL-4 induces IgG1 responses, while IFN-γ induces IgG2 in both mice and cattle (9).

Many different cell types and substances of the host and parasites are involved in the generation of these responses.

Although eosinophils, mast cells, IgE and IgG1 levels are all upregulated during nematode infections, this does not imply that they are all related with protection. Different effector mechanisms may provide protection against different helminths species or stages. Only the production of IL-4 and IL-13 seem to be a common denominator in the protective response against gastrointestinal nematodes (reviewed in 24).The most relevant factors that are known to determine a protective host response against nematodes in cattle are given below.

6.1 The humoral immune response

6.1.1. Antibodies can confer protection against helminth infection

Passive immunisation with antibodies provides protection against helminths including *Trichinella spiralis, Strongyloides stercoralis* and *Schistosoma japonicum* in mice (37, 47, 51) and *Ancylostoma ceylanicum* infection in hamsters (10). Antibodies exert their effect by neutralisation of bioactive molecules (toxins, enzymes) produced by the parasite, but also by assisting in antibody-dependent cell-mediated cytotoxicity (ADCC). In that latter case, the antibodies bind to the Fc receptor on activated inflammatory cells such as eosinophils, neutrophils, basophils or mast cells. The Fc receptors are usually isotype specific and that is one of the reasons that different antibody isotypes have different effector functions. Opsonic activity is not considered a prime defence mechanism probably because the helminths are just too big to become phagocytosed.

6.1.2 The function of IgE antibodies

Antibodies of the IgE isotype represent only a small fraction of the total immunoglobulin pool in serum. Yet, IgE levels are the strongest associated with

helminths infections. For *Trichinella spiralis* (81), *Schistosoma mansoni* (51, 48) and *Onchocerca volvulus* (1) IgE has been demonstrated to be essential for protection. Despite the low concentration in serum, IgE is particularly important as it can trigger mast cells and basophils, and activate eosinophils to degranulate *via* the high affinity receptor (FcεRI). Eosinophils also have a low affinity receptor for IgE (FcεRIII). It should be noted that IgE receptors are present on eosinophils from humans and cattle, but not on those from mice (4). Whether differences in IgE receptor expression contribute to the observed differences in susceptibility and course of infection among species awaits future investigation.

Helminth infections are known to induce a polyclonal IgE response. It has been suggested that this non-specific IgE outcompetes parasite specific IgE for Fc binding thus avoiding mast cell and eosinophil degranulation in the vicinity of the parasite (81), although recently it was described that triggering of mast cells was independent of total IgE levels (69). Total IgE induced by helminths might also play a role in the prevention of asthma by outcompeting the allergen specific IgE (81). The mechanism that drives the polyclonal stimulation of IgE induction is not known, although a recombinant polyprotein from *Dirofilaria immitis* has been identified that can stimulate non-specific IgE production, without inducing specific IgE (79).

6.1.3 The role of other immunoglobulin isotypes

IgM is the first isotype that can be detected during primary infection with nematodes. IgM antibodies which are produced without the process of immunoglobulin class switching, are of low affinity, but because of their pentameric structure display a high avidity. IgM is a strong activator of the complement system, but this does not seem to play an important role in acquired immunity against helminths.

IgG antibodies are the most abundant isotype in serum and are therefore the most important neutralising antibodies. In mice and cattle, the IgG1 subtype is considered to reflect a Th2 response, while the IgG2 response indicates Th1 activity. During helminth infection in cattle, IgG1 is the prominent IgG sub-isotype (46). Nevertheless, IgG2 may also play a role in infected cattle as the bovine species are unique in that they have an Fc receptor on neutrophils specific for IgG2 (7), enabling IgG2-dependent ADCC. In the bovine fetus there is no placental transfer of immunoglobulins, but the colostrum is a very rich source of maternal antibodies, especially IgG1.

Antibodies of the IgA isotype are present mainly in the mucus of the gut, the respiratory system, and the lactating breast. In cattle IgA can be induced by IFN and TGF-β and can therefore better be classified as a Th1 than as a Th2-type of immunoglobulin (17). Because of the high concentration of IgA in the mucus, it can act there as a neutralising antibody. Another important function of IgA is the activation of eosinophils via the receptor FcαR. In human eosinophils, sIgA is the most effective stimulator of degranulation (26). In *Cooperia oncophora* infected cattle, IgA levels were higher in intermediate responders compared to low responders (46) and IgA levels in mucus peak at the time of expulsion of the adult worms (45). This suggests that IgA may contribute to protection against helminths.

6.2 *The cellular immune response*

6.2.1 Mast cells

Infection with parasitic nematodes induces a strong increase in the number of mast cells in the infected tissues (reviewed in 67). Mast cells are granule-containing immune effector cells that carry a high affinity receptor for IgE (FcεRI) as well as an IgG receptor (FcγRIII). When antibodies bound by these Fc-receptors bind antigen, the receptors are cross-linked and this results in degranulation of the mast

cells. The mast cell granules contain a range of molecules with biological activity. They contain chymase, tryptase and serine esterases (activating metalloproteinase to breakdown the extracellular matrix), the cytokines IL-4 and IL-13 to maintain the Th2-prone environment, toxic mediators such as heparin, as well as inflammatory mediators like histamine and platelet-activating factor (PAF). Because of the high affinity for IgE, only small amounts of IgE and antigen are needed to trigger degranulation of mast cells.

Mast cells play an important role in allergy. The pathological inflammatory reaction in allergy is partially triggered by mast cells responding to innocuous antigens. Mice with a genetic deficient c-kit (CD117) or treated with c-kit neutralising antibody are impaired in mast cell development. In mice, this impairment results in decreased expulsion of *Strongyloides venezuelensis* (52) and *Trichinella spiralis* (31). Mast cell-deficient mice infected with *Nippostrongylus brasiliensis* or *Trichuris muris* display no difference in expulsion when compared with normal mice (6, 15, 25). Similarly, in cattle infected with *Cooperia oncophora* (45) or *Ostertagia ostertagi* (11) mast cells were counted in the gut and abomasum mucosa, respectively, but no correlations with protection or expulsion were found. These findings may indicate that the role of mast cells in the protection against nematodes may vary between parasite species.

6.2.2 Eosinophils

Helminth infections are also accompanied by an increase in eosinophil granulocytes (eosinophilia) and a local aggregation and degranulation of eosinophils (5). Eosinophils develop in the bone marrow from where they enter the circulation. Small numbers of these cells are normally present in the blood, but most are found in the connective tissues just below the respiratory, gut and urogenital epithelium. During infection, different types of chemokines recruit eosinophils to the site of infection. Here the cells are activated by Th2 cytokines,

such as IL-3 and IL-5. This results in degranulation of the cells with release of inflammatory mediators, cytokines, and compounds that are toxic to both the parasite and the host (73). Toxicity to the tracheal epithelium is conferred by, among others, the major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO) (63).

Eosinophil degranulation and killing of nematode larvae after degranulation of eosinophils has been observed *in vitro* (71), but whether these mechanisms operate *in vivo* is still unknown. As eosinophilopoiesis and eosinophil activation are IL-5 dependent, the role of eosinophils in nematode infections has been investigated in mice treated with anti-IL-5. Neutralisation of IL-5 increased the survival of *Strongyloides stercoralis* and *Onchocerca volvulus* but did not influence the expulsion of the species *Nippostrongylus brasiliensis, Trichinella spiralis, Toxocara canis, Trichuris muris* and *Heligmosomoides polygyrus* (5). However, these data should be interpreted with caution as the course of nematode infection in mice may not be representative for other mammals, because mice lack IgE receptors (4). In general, eosinophils seem to contribute in particular to killing of nematodes species that infect the non-natural host. This is not surprising as the innate inflammatory response is generally stronger in non-permissive hosts (58). Eosiniphils are considered to be most effective against larvae, although overexpression of IL-5 in mice results in increased killing of adult *Litomosoides*, a filarial worm from the pleural cavity (54, 65).

6.3 Parasite molecules that modulate the host response

The nature of the molecules of the parasite that affect the host immune response are beginning to be elucidated. Recent work indicates that especially glycoconjugates may play a prominent role. The awareness of the importance of protein glycosylation of nematodes was triggered by observations that glycan-containing ES products and membrane extracts of the gut of nematodes contain modulators of

the immune response and may have vaccine potential. For *Haemonchus contortus* an experimental vaccine consisting of a membrane glycoprotein extract (H11) has been reported to provide > 75% protection against challenge infection (64). Administered as recombinant proteins, the 3 most abundant proteins of H11 induced no protection (50). This lack of protection may be explained by the presence of glycans in the native extract. Similarly, the ability of extracts of the filarial nematode *Brugia malayi*, but also of the free-living nematode *C. elegans* to induce IL-4 production is strongly reduced after treatment with sodium periodate which oxidizes the carbohydrate moieties (78). Nematodes display many different carbohydrate structures. As two groups of glycoconjugates are outstanding for their immunogenic and immunomodulating properties (13) (see also figure 2), they are described in more detail below.

6.3.1 α(1,3) Fucosylated carbohydrates

N-linked glycans with α(1,6) fucosylation are common in mammals in contrast to glycans with α(1,3) fucosylation which are common in invertebrates and plants. Fuc(1,3)-GlcNAc oligosaccharides are known to be very antigenic and allergenic epitope in insects (70), plants (72, 82) and snails. Keyhole limpet hemocyanin (KLH) from the marine gastropode *Megathura crenulata* is also well known and widely used for its immunostimulatory properties. This property is at least partly based on fucosylated N-linked glycans. These structures are also the major cross-reacting glycans with antisera directed against the parasitic trematode *Schistosoma mansoni* (27). For the Fuc(1-3)-GlcNAc moiety on LNFPIII it has been demonstrated that it acts as an adjuvant that stimulates Th2 responses. It induces IgE and IgG1 responses, although LNFPIII itself is not the epitope. A very similar structure without the fucose (LNnt) does not have adjuvant activity (66).

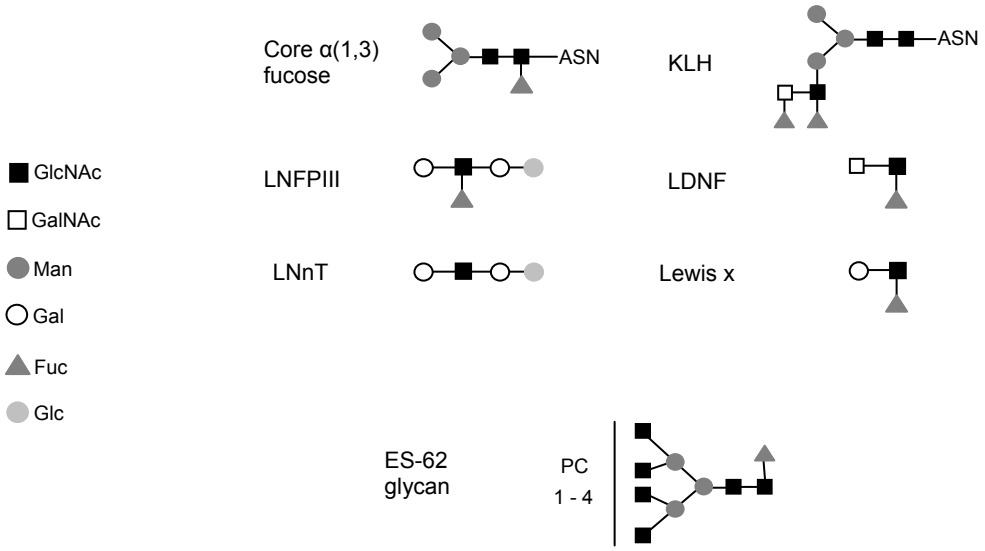

Figure 2. Examples of fucosylated and PC-substituted N-glycans from helminths. ASN indicates the amino acid asparagine, to which the N-glycan is attached. α(1,3) and α(1,6) fucosylation is indicated as fucose directed down or up, respectively. The given epitopes can be part of a larger N-glycan.

Fuc(1-3)-GlcNAc containing oligosaccharides are also part of the Lewis X epitope which is a very common structure in *Schistosoma*. Monoclonal antibodies directed against the Lewis X antigen protect mice against *Schistosoma* infection (32). *D. viviparus* is the only nematode in which the Lewis X antigen has been found (34).

In *Haemonchus contortus*, a gastrointestinal nematode of sheep, a Fuc(1-3)-GlcNAc moiety is present in two different *N*-glycans, core α(1,3) fucose and LDNF. It has been demonstrated that the core α(1,3)fucose structure is able to induce specific IgE (14) and that the presence of antibodies directed against the glycan moiety LDNF correlates with protection against infection (80). The potential contribution of fucosylated *N*-linked glycans to the pathogenicity of *D. viviparus* is totally unknown.

6.3.2 *Phosphorylcholine substituted carbohydrates*

Phosphorylcholine (PC) conjugates are common structures and present in virtually all animals. In nematodes, PC can be attached to glycolipids or to the N-glycan moiety of glycoproteins (53, 13). This presence of PC on N-linked glycans has not been reported for other eukaryotes. This unique decoration of N-glycans of nematodes may explain why this structure is so immunogenic to mammals. Although there are strong antibody responses against PC, no correlation with protection against nematodes has been found. By far the best studied PC containing glycoprotein is ES-62 of the filarial worm *Acathocheilonema viteae* (reviewed in 33). This glycoprotein induces a strong Th2 response. This effect is lost after deglycosylation but can be mimicked by PC attached to ovalbumin, suggesting that PC contributes to the immunomodulatory effect of ES-62 (30). ES-62 influences the function of DC's, macrophages and T-cells. In B-cells the PC containing carbohydrate moiety of ES-62 appears to be required for downregulation of the IgG2 responses in an IL-10 dependent manner. This was demonstrated in mice, where the removal of the N-glycans from ES-62 induces increased IgG2 production (36). Recently, ES-62 has been shown to inhibit the FcεRI-mediated mast cell response (59). These effects indicate that ES-62 has strong immunomodulatory activity but whether all these effects can be attributed to PC is still elusive. The presence of PC-containing N-glycans on *D. viviparus* has thus far not been demonstrated, although PC epitopes were described on the surface of L3, where they were considered as low immunogenic epitopes (29).

7. Scope of the thesis

Parasitic bronchitis in cattle caused by the lungworm *D. viviparus* remains an important problem in many countries. The available vaccine is effective but its composition is undefined and the vaccine provides only transient protection due to a lack of induction of immunological memory. The **aim** of this thesis is to further

unravel the interaction of *D. viviparus* with the host immune system to pave the way for the development of a successful synthetic and/or recombinant vaccine.

As IgE responses are a hallmark of helminth infections and antibodies of the IgE isotype likely play an important role in the establishment of *D. viviparus*-associated pathology as well as in protection against *D. viviparus* as described in this **Chapter 1**, we first developed assays to measure total and parasite specific bovine IgE levels. These unique tools were subsequently applied to measure bovine IgE levels in *D. viviparus* infected calves (**Chapter 2**).

Next we followed the development of the humoral immune response and the correlation of this response with protection against infection in experimentally *D. viviparus* infected and vaccinated calves. IgA, IgE, IgG1, IgG2 and IgM levels were measured against extracts of both L3 larvae and adult worms. The immunogenicity of N-glycans was determined using deglycosylated antigens (**Chapter 3**).

The discovered major immunodominant glycoprotein with vaccine potential described in Chapter 3 was selected for further study. The glycoprotein was purified and the nature, function and immunogenicity of the attached N-glycan was determined (**Chapter 4**).

To further explore the function and provide the basis for recombinant production of the novel candidate vaccine antigen, we determined the nature of the protein backbone of the glycoprotein by mass spectrometry and DNA sequencing. In addition, the localization of the antigen and its immunological conservation among nematodes were investigated **(Chapter 5**). The conclusions of the work described in this thesis and their impact on the understanding of the biology of *D. viviparus* disease and vaccine devlopment are discussed in **Chapter 6**.

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CHAPTER 2

Serum immunoglobulin E response in calves infected with the lungworm *Dictyocaulus viviparus* and its correlation with protection

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Serum immunoglobulin E response in calves infected with the lungworm *Dictyocaulus viviparus* and its correlation with protection

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SUMMARY

Protection of a primary Dictyocaulus viviparus *infection was measured against a homologueous challenge infection in two independent experiments and this was correlated with serum immunoglobulin (Ig)E responses. A primary infection of 30 third stage larvae (L3) of* D. viviparus *on day 0 protects calves for 70% against a challenge infection of 2000 L3 on day 35 compared to calves with no primary infection. The variation in post mortem worm counts within this group (*n = *6) was very large with mean worm counts of 145 (range 3–446) lungworms. Parasite specific IgA, IgE, IgG1 and IgG2 and total IgE levels in serum were measured by ELISA. Parasite specific IgA, IgG1 and IgG2 were elevated after infection, but correlation with protection was only found with IgG1 levels on day 42 and with IgG2 levels on day 70. IgE was measured in a sandwich ELISA using antisheep IgE that cross-reacts with cattle IgE. No parasite specific IgE could be detected. However, total serum IgE was elevated after infection and total serum IgE levels before and on the day of challenge correlated with protection (*P < *0·05). Total serum IgE also correlates with peripheral eosinophil counts between days 14 and 28 after primary infection. Western blots with three different parasite antigen preparations, L1, excretory/secretory products and crude worm adult antigens, were used to detect parasite specific IgE in sera depleted of IgG and IgM. These depleted sera from protected calves contained parasite specific IgE, while sera from nonprotected calves were negative. A band of approximately 100 kDa was recognized in all three antigens. In a second experiment, primary doses of 30, 60, 120, 240, 480 and 960 L3 of* D. viviparus *were used and necropsy was 11 days after challenge. This experiment confirmed the correlation between protection and total IgE levels before and on the day of challenge. The rapid and strong IgE responses in protected animals after such a low infection might be caused by the specific characteristics of the lungworm antigens or by the somatic migration of the worm and might be involved in the rapid development of protection against lungworm reinfections in cattle.*

Keywords *IgE*, Dictyocaulus viviparus*, cattle, ELISA, Western blot*

INTRODUCTION

Host immunoglobulin (Ig)E responses have been associated with helminth infections for a long time (1) Studies on IgE responses in ruminants were restricted to sheep and cattle. Sheep IgE levels in serum or lymph increased after infection with gastrointestinal nematodes (2–6). In one of these studies (4), a correlation between total serum IgE and protection against *Haemonchus contortus* infection was found (4). In cattle serum, IgE levels following natural infections with gastrointestinal nematodes were measured in a series of studies (7–10). The results of these studies were not consistent but, in general, there was an increase in serum IgE following mild helminth infections. To our knowledge, there are no data on the IgE response following infections with the lungworm *Dictyocaulus viviparus* in cattle, but lungworm specific IgA, IgG1, IgG2 and IgM in serum and bronchoalveolar lavage fluid in cattle have been measured (11). No correlation between levels of these isotypes and protection was found. IgE responses and eosinophilia are both hallmarks of Th2 response and lungworm infections result in a pronounced eosinophilia (12). Eosinophils possibly play a role in host defence by binding to lungworm larvae as demonstrated by *in vitro* assay (13). Therefore, because of the rapid development of protection against challenge infections, lungworm infections may provide a suitable model to study the relationship between protection, IgE response and eosinophilia. The present study reports the results obtained

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from primary and challenge lungworm infection in young calves and its correlation with IgE and eosinophils.

MATERIALS AND METHODS

Animals and experiments

This study comprised two independent experiments. Experiment 1 was part of a larger experiment (14). Eighteen Holstein-Friesian heifer calves, aged 3 months, were randomly divided into four groups. Group 1 ($n = 6$) was infected at day 0 with 30 L3 of *D. viviparus* and was challenged at day 35 with 2000 L3. Group 2 ($n = 6$) was the challenge control group and received only 2000 L3 on day 35. Group 3 ($n = 3$) only received the primary dose at day 0 of 30 L3 and group 4 ($n = 3$) was the noninfected control group. All calves were housed indoors. At day 70 the calves from group 1 and group 2 were necropsied.

Experiment 2 was part of a larger study (15). This experiment was only used to confirm the correlation between total IgE levels and protection found in Experiment 1. Sixteen Holstein-Friesian heifer calves, aged 3 months, were all housed indoors. Twelve of them were randomly divided into pairs and infected pairwise at day 0 with 30, 60, 120, 240, 480 or 960 L3 of *D. viviparus*. The four remaining calves served as challenge control group. All 16 calves were challenged with 2000 L3 at day 35 and necropsied at day 46.

Worm counts for both experiments were carried as described previously (16). The worms from the challenge infection of Experiment 2 were differentiated from those of the primary infection by their much smaller size.

Faecal larval counts

Rectal faecal samples of all animals were collected twice a week. Examination for lungworm larvae was carried out with the Baermann method using 30 g of faeces. The results were expressed as larvae/g faeces (LPG).

Blood sampling

Twice a week, blood was collected from the jugular vein. For eosinophil counts, blood was collected in tubes with ethylenediaminetetraacetic acid and, for serology, it was collected in tubes without additives.

Eosinophil counts

In Experiment 1, peripheral blood eosinophils were counted in Bürker counting chambers (0·9 mm^3) in eosinophil staining solution according to pilot (50% propylene glycol, 0·1% eosin, 0·01% Na_2CO_3, 50 U of heparin in H_2O).

Parasite extracts

Adult lungworms were collected 35 days after infection as described (16). Excretory/secretory (ES) products of these worms were obtained as described for *H. contortus* (17). L1 lungworm larvae were collected by the Baermann method from faeces of lungworm infected calves. Crude extracts from L1 and adult worms were prepared as described for *H. contortus* (18).

Determining specificity of mAb anti-IgE (IE7) and parasite specific IgE

Pooled sera from Experiment 1

Sera from day 35–70 from the two most protected animals (calves 753 and 757), and from the two least protected animals (calves 770 and 779) from group 1 of Experiment 1 were pooled separately and were used as high and low IgE references, respectively.

Affinity chromatography

One mg of protein A/G purified mAb antisheep IgE (IE7) was coupled to 1 ml of drained cyanogen bromide activated sepharose 4B (sepharose-IE7) and blocked with Tris according to the manufacturers protocol (Pharmacia Biotech, Uppsala, Sweden). For estimating the nonspecific binding to the column, a control column was prepared with phosphate-biffered saline (PBS) instead of IE7 (sepharose-PBS). Two ml of pooled serum was heat treated (1 h, 56°C) to increase binding (4), passed through a 0·45-µm filter and applied to the column. This was performed for high IgE and low IgE pooled sera on both columns. The columns were washed with 10 ml of PBS and eluted with $5 \times 0{\cdot}5$ ml of 0·2 M glycine (pH 2·8). The eluted fractions were neutralized immediately with 50 µl 0·7 M Tris per 0·5 ml fraction. These eluted fractions were dot blotted to demonstrate specificity of IE7 for IgE and they were used to probe Western blots to demonstrate parasite specific IgE. For the latter application, the amount of total IgE in sepharose-IE7 eluted fractions was quantified by ELISA as described below. Fractions containing IgE were pooled and diluted with PBS to the same total IgE concentration as in the serum from which it was derived and, subsequently, it was used to probe Western blots.

SDS-PAGE and blotting

To demonstrate specificity of IE7 for bovine IgE, dot blots were made of 2 µl samples on a dry nitrocellulose membrane. Samples contained IgA, IgG1, IgG2, IgM and a variable amount of IgE. Blocking was as for Western blots. All serum and antiserum dilutions for dot and Western blots were made in PBS with 0·1% gelatine and 0·05% Tween-20

(PBS-GT). Dot blots were incubated with 1 : 100 mouse anti-IgA (Serotec, Oxford, UK), 1 : 1000 mouse anti-IgG1, 1 : 1000 mouse anti-IgG2 (Sigma-Aldrich Chemie BV, the Netherlands), 1 : 3000 diluted mouse anti-IgM (Sigma-Aldrich Chemie BV) or IE7(10 μg/ml) both followed by 1 : 3000 diluted rabbit anti-mouse IgG/HPO (Dako, Dlostrup, Denmark). Substrate for all isotypes was 3,3′-diaminobenzidine tetrachloride with chloro-naphtol (19). SDS-PAGE and Western blots were made as described (4). Ten μg/lane parasite antigens were electrophorized under reduced conditions, blotted and incubated with pooled sera or with sepharose-IE7 eluted fractions. Parasite specific IgE was demonstrated in undiluted serum or sepharose-IE7 eluted fraction by incubation with IE7 (10 μg/ml), 1 : 1000 diluted goat anti-mouse Ig/AP (Dako) and BCIP/NTB substrate. Parasite specific Ig was demonstrated with 1 : 3000 diluted rabbit anti-Ig (mainly IgG)/HPO (Dako) and 3,3′-diaminobenzidine tetrachloride with chloro-naphtol (19).

Serology

Polyclonal antibovine IgE

Part of the cDNA coding for the ε chain of bovine IgE (BigE1-2) was amplified and expressed and the recombinant (recBIgE1-2) was used to produce polyclonal antibovine IgE. This was carried out with the same primers and methods as described for sheep (4).

ELISAs

For all ELISAs, Greiner plates (high binding) were coated overnight in 0·06 M Na_2CO_3, pH 9·6. Plates were washed in between every incubation step with 0·05% Tween-20 in distilled water. All incubations, unless indicated otherwise, were for 1 h at 37°C with 100 μl per well and all dilutions of sera and antisera were made in PBS-GT. All the given values are means of duplicates.

Total IgE ELISA

All collected sera were tested in total IgE ELISA. Plates were coated with IE7 (7 μg/ml). Test sera were heat treated (1 h 56°C) and applied to the plates in a 1 : 5 dilution. Polyclonal rabbit antibovine IgE diluted 1 : 1000 was applied, followed by incubation with 1 : 4000 conjugate goat anti-rabbit Ig/AP (Dako). p-Nitrophenyl phosphate (PNPP, Pierce, IL, USA) was used as the substrate and after overnight incubation at 4°C the plates were read at 405 nm. The OD values of a serial dilution of a high positive standard serum were transformed by four parameter logistic fit ($y = (a - d)/(1 + (x/c)^b) + d$) to arbitrary units IgE (U) on a linear scale. The value of the blank (PBS-GT instead of serum) was set at 0 mU and the OD-blank value of the undiluted standard serum was set at 1000 mU IgE. Using the standard sera, the OD-blank values of the test sera were transformed to mU IgE.

Parasite specific IgE ELISA

All collected sera were tested in a parasite specific IgE ELISA. Plates were coated with 5 μg/ml crude adult worm antigen or ES of lungworm. Heat treated test sera were applied undiluted to the plates followed by IE7 (3 μg/ml). Goat anti-mouse Ig/AP conjugate AP (Dako) was used in a 1 : 2000 dilution. PNPP (Pierce) was used as substrate and plates were read at 405 nm after overnight incubation at 4°C.

Parasite specific IgA, IgG1 and IgG2 ELISA

Sera from days 7, 14, 28, 42, 56 and 70 were tested. Plates (Greiner Labortechnik, Frickenhausen, Germany; High binding) were coated with 5 μg/ml ES of lungworm. Sera were applied to the plates in a 1 : 100 dilution followed by the appropriate mAb; 1 : 100 anti-IgA, 1 : 1000 anti-IgG1 or anti-IgG2. Goat anti-mouse Ig/AP conjugate AP was used in a 1 : 2000 dilution. PNPP was used as substrate and plates were read at 405 nm after overnight incubation at 4°C.

Statistical analysis

Protection, as used here, is defined as: resistance against reinfection compared to an age-matched control group based on post mortem worm counts and is calculated by dividing the differences in worm counts between the control and primary infected calves by the mean of the worm counts of the control calves. Statistical analysis was carried out using the Number Cruncher Statistical System (NCSS, Dr J.L. Hintze, Kaysville, UT, USA). Significance of correlation was calculated by the nonparametric Spearman's rank correlation test. Significance of differences in means between groups was calculated with the Kruskal–Wallis nonparametric test. Correlation's and differences were considered significant when $P < 0{\cdot}05$.

RESULTS

Specificity of IE7 for bovine IgE

Figure 1 shows the specificity of IE7 as tested after affinity chromatography by dot blots and Western blots. Figure 1(a) shows dot blots spotted with pooled sera from protected and nonprotected calves were positive for IgA, IgG1, IgG2 and IgM, but only the pooled serum from the protected calves reacted with IE7. Eluted fractions from pooled sera from protected calves from both columns, sepharose-IE7 and sepharose-PBS, were also positive for IgA, IgG1, IgG2

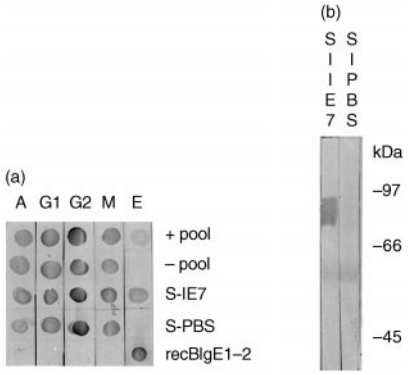

Figure 1 Blots that determine the specificity of the mouse anti-sheep IgE (IE7) against bovine IgE are shown. (a) Dot blots 1 : 40 diluted pooled sera of protected calves (+ pool) and nonprotected calves (– pool), 1 µg eluted fraction of pool serum of protected calves from sepharose-IE7 (S-IE7) or sepharose-PBS (S-PBS), 1 µg recBIgE1-2. All probed with anti-IgA, IgG1, IgG2, IgM and IE7. (b) Western blots with 5 µg eluted fraction of pool serum of protected calves from sepharose-IE7 (S-IE7) or sepharose-PBS (S-PBS) were probed with IE7. The molecular weight standard is indicated.

and IgM. This indicates that IgA, IgG and IgM bind to some extent to the sepharose. Negative control blots (everything except isotype specific mAb) were all negative. RecBIgE1-2 was recognized only by IE7, not by any antisera against one of the other isotypes. The eluted fraction from the sepharose-IE7 column binds with IE7 and, on Western blot, it was demonstrated that the molecular size of the protein that was recognized by IE7 in this fraction was 80 kDa, the expected size of the ε chain of IgE (Figure 1b).

Experiment 1

Lungworm infection

Clinical lungworm disease was observed in group 1 and group 2 from day 50 onwards. One calf of group 2 died at day 66 due to pneumonia. Faecal larval counts remained negative in all infected groups until 21 or 25 days after infection and remained positive thereafter. Challenge infection with 2000 L3 at day 35 increased larval excretion in group 1, but this excretion remained much lower than that observed in the challenge control group group 2. Faecal larval counts of group 3, which only received the primary infection of 30 L3, remained low.

The worm counts of group 1 and group 2 are given in Table 1. Priming with 30 L3 resulted, on average, in 70% protection in the animals of group 1.

Table 1 Total *D. viviparus* counts of the individual calves of group 1 and group 2 at day 70 from Experiment 1

Group 1	Worm counts	Group 2	Worm counts
753	3	752	338
770	289	754	700
771	56	756	385
773	62	765	451
757	12	781	573
779	446		
Mean	145		489
SD	181		147

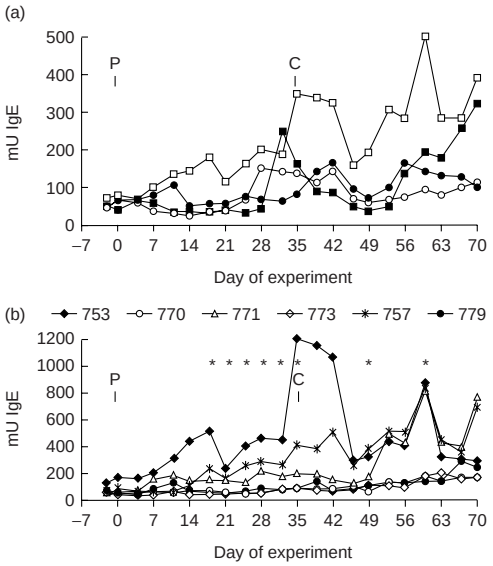

Figure 2 Total IgE levels in serum expressed as mU IgE from Experiment 1. P indicates primary infection of group 1 and group 3 with 30 L3, C indicates challenge infection of group 1and group 2 with 2000 L3. (a) Mean values of group 1 (□), group 2 (■), group 3 (○) and grup 4 (●). (b) Values of the individual calves of group 1 illustrating the within group variability. *Indicates days when the IgE levels correlate significantly with protection.

Serology

Serum total IgE levels: The results of the total IgE ELISA in serum expressed as mU IgE are given in Figure 2. The levels of total IgE in group 4 remained low during the experiment with a tendency to increase slightly towards the end of the trial. The mean levels of group 3 did not differ significantly from group 4. The levels in group 2 increased from day 57 onwards until the end of the experiment (significantly higher than group 4 at days 67 and 70). The increase

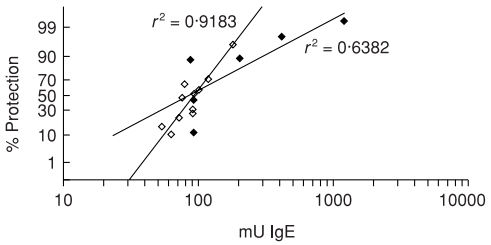

Figure 3 Plot showing the approximation of the calculated correlation between protection on a probit scale and total IgE levels at the day that challenge infection is given (day 35) of the individual calves from Experiment 1 (■) and Experiment 2 (□). The linear correlation is given.

observed in group 2 at days 32 and 35 was due to high levels in one calf (calf 781) and was not caused by a known helminth infection. The mean levels of total IgE of group 1 were significantly increased from day 11 onwards. The difference between group 1 and group 2 was significant at days 11–28 and days 39–56. Without the data of day 32 and 35 from calf 781, group 1 was significantly higher than group 2 between days 11 and 56. There was no significant difference between the IgE response in group 1 after infection with 30 L3 and the response of group 2 after infection with 2000 L3. The variation in total IgE levels within group 1 was very high as shown in Figure 2(b). A significant negative rank correlation was observed in group 1 between worm counts and total IgE levels from day 18–35 and at day 49 and 60 ($r = -0{\cdot}8857$ up to $-1{\cdot}000$). An approximation of the calculated correlation is illustrated in Figure 3 for the IgE values at the day of challenge, day 35. The two calves (753 and 757) with by far the highest IgE levels before and around the day of challenge, were > 97% protected against challenge infection. The two least protected calves (770 and 779) with worm counts comparable to those in group 2 did not show any IgE response at all. The remaining two calves (771 and 773) had moderate worm counts and moderate or low total IgE levels.

Parasite specific IgE

All the sera tested with ES or crude worm adult (CWA) antigens were negative by ELISA. The Western blots shown in Figure 4 were negative for parasite specific IgE with both pooled sera for all three antigens when whole serum was used. However, the sepharose-IE7 eluted fraction of the protected, but not of the unprotected calves gave some clear bands with all three antigens used. The same antigens were recognized by Ig from pooled sera from protected and nonprotected calves, with the serum of the protected calves showing a slightly stronger reaction. All bands recognized by IgE were also recognized by Ig and no antigens were recognized by Ig present in the sepharose-IE7 eluted fraction.

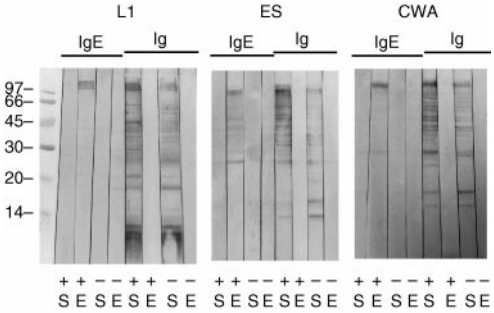

Figure 4 Western blots of L1, ES and CWA probed with serum (S) or with sepharose-IE7 affinity purified IgE (E) from calves of Experiment 1. Pooled serum was used from protected (+) or non-protected (–) calves and detection was specific for IgE or Ig. The molecular weight of standards is indicated in kDa.

Parasite specific IgA, IgG1 and IgG2

The parasite specific IgA, IgG1 and IgG2 levels in serum of calves from group 1 are shown in Figure 5. Mean serum IgG1 levels already increased significantly before challenge infection and increase was continued until the end of the experiment. IgA and IgG2 levels only started to increase significantly after challenge infection and continued to increase until the end of the experiment, although some animals reached their highest IgA levels already at day 56. Because the calves with low protection had also elevated IgA, IgG1 and IgG2 levels, significant positive correlation with protection was restricted to IgG1 levels at day 42 ($r = 0{\cdot}8857$, $P < 0{\cdot}05$) and IgG2 levels at day 70 ($r = 0{\cdot}8857$, $P < 0{\cdot}05$).

Eosinophils

The eosinophil counts were peformed for group 1 and group 2 (Figure 6). No counts were performed for group 3 and group 4 but, before the challenge at day 35, group 2 served as the control for group 1. In group 1, there was a peak around day 21 and, both in group 1 and group 2, there was a peak around day 49. A significant rank correlation with total IgE was only found in group 1 between days 14 and 28 ($r = 0{\cdot}90$, $0{\cdot}90$, $0{\cdot}60$, $0{\cdot}30$ and $1{\cdot}00$, respectively). No significant correlations were found between eosinophil counts and protection.

Experiment 2

Lungworm infection

All primary infected calves started to shed larvae from day 25 onwards. The LPG was positively correlated with the primary infection dose. The control calves remained negative throughout the experiment. Table 2 gives the worm counts

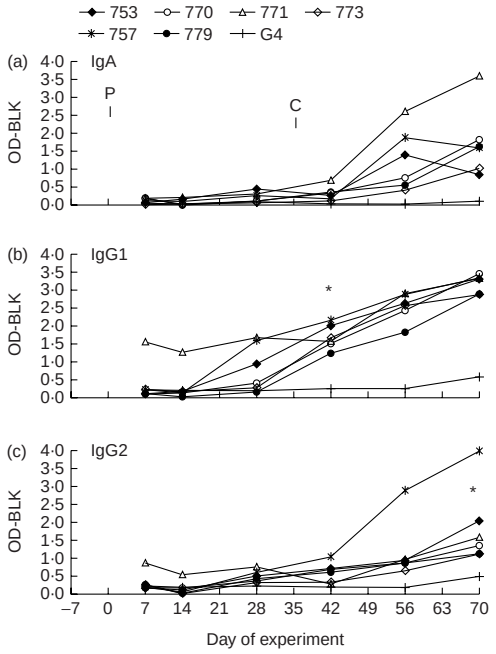

Figure 5 Parasite specific IgA, IgG1 or IgG2 serum levels against ES of the individual calves of group 1 from Experiment 1. P Indicates primary infection and C indicates challenge infection of all calves. The control group 4 is included in all panels. *Indicates days when the levels correlate significantly with protection.

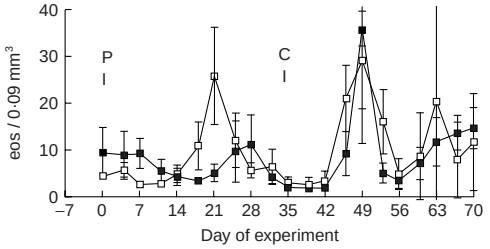

Figure 6 Mean number of peripheral blood eosinophil counts ± SE of group 1 (□) and group 2 (■) from Experiment 1. P indicates primary infection of group 1 with 30 L3, C indicates challenge infection of group 1 and group 2 with 2000 L3.

Table 2 Total *D. viviparus* counts of the individual calves at day 46 from Experiment 2

Primary infected	Worm counts	Control	Worm counts
30a	364	0a	369
30b	386	0b	627
60a	422	0c	422
60b	163	0d	510
120a	577		
120b	245		
240a	342		
240b	220		
480a	157		
480b	272		
960a	20		
960b	445		
Mean	–		482
SD	–		112

Calves were numbered (a or b) according the primary dose to indicate the individual calf within a pair. Only the worms from the challenge infection are given.

of the challenge infection of all calves. With increasing primary infection dose, there was a tendency that the worm counts from the challenge infection decreased.

Serology

The individual values of serum total IgE measured by ELISA is given in mU in Figure 7. The IgE levels in the primary infected calves were significantly higher than in the noninfected controls from day 14 onwards. Worm counts were significantly negatively correlated with IgE levels from day 32 onwards. An approximation of the calculated correlation is given in Figure 3 for day 35, the day that the challenge infection was given. Only one primary infected calf (120a) had lower total IgE levels than the control calves, this calf was also the only one that had a protection of less than 0% and could therefore not be indicated in Figure 3. Noteworthy is also Calf 960b. This calf received the highest primary dose, but this resulted only in very low IgE levels and virtually no protection against challenge infection. No significant correlation was found between primary dose and total IgE levels. No parasite specific IgE was detected in whole serum with ELISA.

Parasite specific IgE

Figure 8 shows Western blots of ES incubated with pooled serum from day 35–45 of the two most protected calves (480a and 960a) and of the two least protected calves (120a and 960b). The blots were negative for parasite specific IgE with both pooled sera when whole serum was used. However, the affinity purified IgE enriched fraction of the protected, but not of the unprotected calves recognized some bands. These same bands were recognized by Ig in pooled serum of protected and unprotected calves. Blots with L1 and CWA antigen and incubated with the same panel of

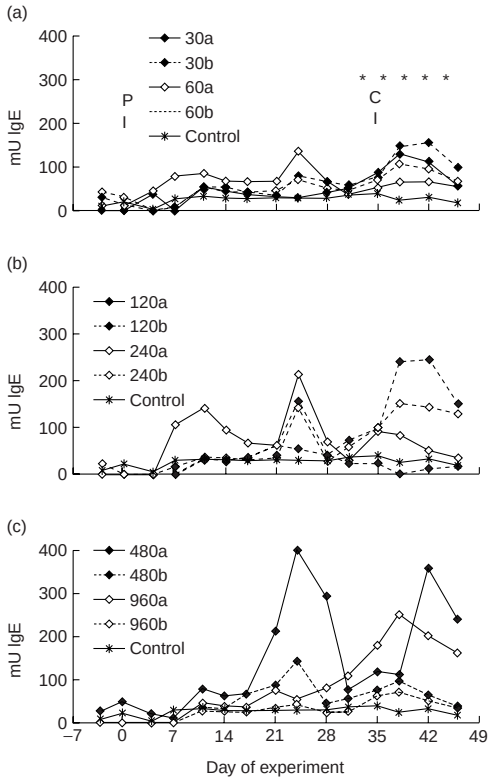

Figure 7 Total IgE levels in serum expressed as mU IgE from Experiment 2. P indicates primary infection and C indicates challenge infection of all calves. Calf numbers indicate primary dose and a or b indicate individual calves within a pair with the same dose. Low (a), middle (b) and high (c) doses are given in different panels for reasons of clarity. The controls are included in all panels. *Indicates days when the IgE levels correlate significantly with protection.

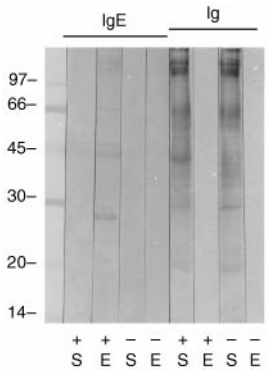

Figure 8 Western blots with ES probed with serum (S) or with sepharose-IE7 affinity purified IgE (E) from calves of Experiment 2. Pooled serum was used from protected (+) or nonprotected (–) calves and detection was specific for IgE or Ig. The molecular weight of standards is indicated in kDa.

sera gave only a very faint reaction with parasite specific IgE from the protected calves but strong reaction with Ig from both pooled sera, protected and unprotected (not shown).

DISCUSSION

Infection of mammals with parasitic nematodes induces elevated levels of IgE (1). Of significant interest is that much of this IgE is not specific to parasite antigens and that total IgE and antigen specific IgE are regulated differently, at least in humans (20) and rodents (21). In mice and man, increased IgE levels and eosinophilia are the hallmarks of Th2 response. These same types of responses can also be found in ruminants (22,23). In ruminants, studies were carried out on IgE responses following gastrointestinal nematode infections. In sheep, serum and lymph IgE levels increased after gastrointestinal nematode infections (2,5,6) and a correlation between total IgE and protection in *H. contortus* infected sheep was found (4). Data found in cattle have been conflicting (7–10). A significant increase in serum total IgE after natural infections with gastrointestinal nematodes was present, but the authors did not mention a within group correlation between IgE and worm burden. Between the groups, an inverse relation between total and parasite specific IgE levels and nematode infections in calves was observed. The mixed field infections and seasonal variation made it difficult to interpret these data from an immunological point of view. Furthermore, a polyclonal antibody was used to measure IgE in one study (7) while, in the other studies, a monoclonal antibody was used. The mAb used in these studies was not available to us to use as a reference for our mAb. This might have been interesting, since we used a mAb raised against sheep IgE (IE7). However, IE7 also proved to be specific for bovine IgE. Its specificity for IgE in the bovine system is shown in Figure 1. The very low readings of many calves in the total IgE ELISA and the negative Western blots with whole serum also indicate that there is no cross-reactivity with the much more abundant IgA, IgG or IgM. To date, no studies have been performed on IgE responses in lungworm infections in cattle, but increased levels of parasite specific IgA, IgG1, IgG2 and IgM in serum and of parasite specific IgA, IgG1 and IgG2 in the

bronchoalveolar lavage fluid in calves after lungworm infection were reported (11). However, no significant correlation between protection and antibody levels in serum or in lavage fluid was observed. In our study (Experiment 1), a positive correlation between protection and IgG1 levels at day 42 and IgG2 levels at day 70 was found. This correlation was present only on one out of six sampling data after primary infection. Furthermore, the differences in IgA, IgG1 and IgG2 levels between the protected and nonprotected animals were small, while the differences in protection were large. The published data (11) and the data of our study did not give evidence for involvement of IgA, IgG1, IgG2 or IgM in protection against lungworm. In contrast, in the two experiments presented here, we found increased total IgE levels in sera after infection with lungworm and the levels at and around the day of challenge were positively correlated with protection in both experiments. These correlations do not necessarily reflect a direct effector role for total IgE, rather the total IgE level might reflect the level of Th2 responses and these responses are considered to be protective against worm infections. One of the effective Th2 responses can be increased parasite specific IgE, which was found to be restricted to the protected calves in both experiments. The weaker reaction in Experiment 2 can be explained by the lower protection that was achieved in that experiment (mean protection of the two best protected calves was 98·4% for Experiment 1 and 81·6% for Experiment 2). Parasite specific IgE was detected on Western blots after depletion of the serum of IgA, IgG and IgM, but could not be detected in whole serum. This apparent inhibition was most likely caused by the competition of parasite specific IgE with the much more abundant IgA, IgG and IgM (24,25). The fact that all the bands recognized by purified IgE were also recognized by the other isotypes from the same serum further emphasizes this assumption. Serum depleted of IgA, IgG and IgM from protected calves, but not from nonprotected calves, were found positive for parasite specific IgE. A band of approximately 100 kDa was recognized in all three antigens used: L1, ES and CWA antigen, especially in the protected animals of Experiment 1. Based on the molecular weight, it was concluded that this band was not the immunodominant acetylcholine esterase of 67 kDa (26), superoxide dismutase of 15–20 kDa (27) or nematode polyprotein antigen/allergen (NPA) of 15 kDa (28). Although only four pooled sera were tested, parasite specific IgE seemed to be correlated with protection and this 100 kDa band might consist of another interesting immunodominant protein. In neither of the two experiments was a correlation between the height of the primary dose and total IgE levels found. In rats infected with *Nippostrongylus brasiliensis*, a clear correlation was found between primary dose and total IgE (29). As the total IgE response is largely determined by genetic factors (20), this difference might be explained by the fact that we used outbred animals. If we had used more animals per group, we might have found a correlation between primary infection dose and total IgE response.

The eosinophilia 21 days after the primary infection and 14 days after challenge infection in Experiment 1 was in agreement with earlier studies (12). We also found a positive correlation between serum IgE and number of eosinophils between day 14 and 28 in group 1. We were not able to find a significant correlation between protection and eosinophilia. A reason for this might be that eosinophils, although associated with helminth infection, do not play a role in actually killing the parasite (30). Another possibility is that we should have measured eosinophils locally rather than in peripheral blood.

Protection in cattle against reinfection 10 days after primary infection of lungworm was demonstrated (31). Can the IgE response be fast enough to play a role in this protection? There is a delay in production of IgE at the site of sensitization and entering into the serum. The IgE measured in the serum was most likely produced in the gut and/or gut associated lymph nodes or in alveolar lymph nodes. In intrapulmonary infected calves, anti-*Pasteurella haemolytica* IgG and IgM could be detected 5 days earlier in lung lavage fluid than in the serum (32) and in *Trichinella spiralis* infected rats the increase in serum IgE lags the increase in IgE in the gut by 3 days (33). We found increased serum IgE levels in protected calves as soon as 10 days after primary infection. Considering the delay in entering the serum, the IgE production might have started 5–7 days after infection and hence was fast enough to contribute to the protection found at day 10. Lungworm infections truncated by the use of doramectin were demonstrated to be capable of inducing a protective response, although the incoming larvae were killed by the endectocide just after penetrating the gut (34). This means that the stage that penetrates the gut can already induce an immune response in the host. In rats infected with *T. spiralis*, *Heligmosomoides polygyrus* or *N. brasiliensis*, there is an increase in intestinal IgE in infections with only those species (*T. spiralis* and *H. polygyrus*) that penetrate the epithelial cells of the gut at some point during their cycle (35). In the case of *T. spiralis* infections, the highest intestinal IgE levels were found at the time of worm expulsion. In this infection, more than 99% of the total IgE produced is not circulating in the serum, but is transported to the lumen of the small intestine (33). It may be that, in the present study, the penetration of the gut by the lungworm larvae on their way to the lungs causes the strong IgE response. It would be interesting to see if the low primary dose of only 30 L3 larvae can induce high intestinal IgE levels. Another possible reason for the strong IgE response can

be the presence of the allergenic carbohydrate structure Lewis (x) in lungworm, but not in other nematodes (36). In further research, we aim to assess the local IgE responses in the lungs and small intestine in cattle with lungworm infections and further identify the immunodominant 100 kDa protein and the specificity of the IgE in these infections.

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CHAPTER 3

Differential *N*-glycan- and protein-directed immune responses in *Dictyocaulus viviparus*-infected and vaccinated calves

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Differential *N*-glycan- and protein-directed immune responses in *Dictyocaulus viviparus*-infected and vaccinated calves

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SUMMARY

Calves with naturally acquired *Dictyocaulus viviparus* infection mount an effective immune response. In the search for protection-inducing antigens, we found that several *D. viviparus* third-stage larval (L3) and adult ES products carry N-glycans. Deglycosylation of the worm antigens using PNGase F resulted in reduced IgA, IgE, IgG1 and IgG2 (but not IgM) reactivities in sera of primary infected animals, suggesting that the carbohydrate moieties contained immunodominant epitopes. Challenge infection resulted in increased specific serum antibody levels against ES and L3 in the re-infected and challenge control groups. Testing of sera by enzyme-linked immunosorbent assay (ELISA) demonstrated a significant increase in IgG1 and IgE (but not IgA or IgG2) reactivity against the deglycosylated antigens in the re-infected group compared with the challenge control group. Sera from calves vaccinated with irradiated larvae showed a strong anti-N-glycan response, but no booster response against the protein backbone after challenge infection, consistent with the absence of a memory response. Together, our results suggest that *D. viviparus* proteins carry immunodominant N-glycan moieties that elicit a strong but short-lived immune response during infection and after vaccination, whereas the protein backbones effectively induce a memory response which results in a long-lasting, potentially protective immune response in re-infected, but not in vaccinated calves.

Key words: *Dictyocaulus viviparus*, N-glycans, PNGase F, antibody isotype, memory response.

INTRODUCTION

The bovine lungworm *Dictyocaulus viviparus* causes severe parasitic bronchitis in cattle. The infection commences with the oral ingestion of herbage contaminated with third-stage larvae (L3). The L3s penetrate the intestinal wall and moult to the fourth- and fifth-stage larvae (L4 and L5, respectively) during the migration *via* the mesenteric lymph nodes to the lung. In the lungs, the worms reach the adult stage and the females start producing eggs. The eggs are re-ingested and shed as first-stage larvae (L1) into the environment where they develop into L3, completing the life-cycle. The clinical signs are caused by inflammatory reactions in the lung that block the airways and cause a collapse of the alveoli, resulting in emphysema and oedema which manifest themselves as coughing and dyspnea. A vaccine consisting of irradiated, attenuated L3 (Huskvac, Intervet) is available and provides short-lived immunity.

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During natural infection, the host generates a protective immune response against *D. viviparus*. Protection can be mimicked *via* the passive transfer of serum from immune to susceptible cattle (Jarrett *et al*. 1955), indicating that humoral immunity is sufficient to confer protection. It has been suggested that, during a naturally acquired infection, 2 types of immunity arise, one of which is effective against larvae which have not yet entered the lungs and another which protects against the adult stages developing in the lungs (Michel and Mackenzie, 1965). The protective response against the larval stages evolves within 11 days post-infection (Michel, 1962) and is short-lived, lasting for less than 6 months. The development of the protective response against lung stages takes up to 3 months and lasts for at least 27 months. Consistent with these observations, vaccination with irradiated L3 induces protection only against the larval stage, whereas experimental infection with non-irradiated L3 (which develop into adults) induces both types of immunity (Michel and Mackenzie, 1965). This information suggests important differences in the antigen composition and/or elicited immune response between the L3 and adult stages of *D. viviparus*.

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Studies aimed at identifying the molecular basis of the short-term or long-term protection against *D. viviparus* have long been focused on finding correlations between protection and cytokine levels (Johnson *et al*. 2005) or local and/or systemic parasite-specific IgA, IgE, IgG1, IgG2 and IgM responses (McKeand *et al*. 1996; Scott *et al*. 1996; Kooyman *et al*. 2002). Except for good correlations with total IgE levels, this approach has not yielded the desired outcome, probably because of the use of variable, crude antigen preparations, differences in infection protocols between experiments and/or heterogeneity in the immune response of cattle.

Certain proteins of *D. viviparus* are known to induce a strong antibody response. The secreted enzyme, acetylcholinesterase (AChE), has been demonstrated to be a target for circulating antibody in infected calves (McKeand *et al*. 1994) and the immunization with AChE resulted in protection in guinea pigs (McKeand *et al*. 1995) but not in cattle (Matthews *et al*. 2001). Infected calves also recognize the native and recombinant polyprotein DvA-1 (Britton *et al*. 1995), but its involvement in the protective response is not known. Thus, the nature of the protection-inducing larval and/or adult antigens during infection still remains to be defined.

In the present study, we compared the protein composition of 2 different extracts from *D. viviparus*, a total protein extract of L3 and excretory/secretory (ES) product of adult worms, focusing on possible variation in protein glycosylation, as glycosylated proteins are increasingly recognized as major antigens and/or important modulators of host immune responses (Khoo and Dell, 2001; Thomas and Harn, 2004; Hein and Harrison, 2005).

MATERIALS AND METHODS

Parasite extracts

Adult *D. viviparus* were collected at 35 days of infection, as described previously (Eysker *et al*. 1990). Worms were washed several times in phosphate-buffered saline (pH 7·2; PBS) to minimize contamination with host components. ES products were prepared as described for *Haemonchus contortus* (see Schallig *et al*. 1994), except that ES was precipitated with 10% trichloroacetic acid, washed twice with acetone and then dissolved in 8 M urea and 10 mM Tris (pH 7·4). L3 were harvested from faecal cultures kept for 1 week at 15 °C, cleared from debris by centrifugation on a sugar cushion (Eysker and Kooyman, 1993), washed with water, and then stored at −80 °C until needed. An extract of L3 was prepared by homogenizing larvae in a small, electric glass mortar in extraction buffer (8 M urea, complete protease inhibitors (Roche, Germany), 10 mM Tris-HCl, pH 7·4). The suspension was centrifuged for 20 min at 4 °C, the pellet was discarded and the protein content of the supernatant determined using a standard assay (Bradford, 1976).

Deglycosylation of glycoproteins

Protein deglycosylation in solution was carried out by diluting 200 μg of ES or L3 extract kept in 8 M urea and 10 mM Tris, pH 7·4 in 4 volumes of 50 mM K_2HPO_4, 25 mM EDTA (pH 7·0) in distilled water. Sodium dodecyl-sulphate (SDS) was added to a final concentration of 0·1%. Samples were boiled for 5 min, and 10% Triton X-100 was added to a final concentration of 1%. After cooling the samples to room temperature, (22–24 °C), 1 μl (=1 U) of recombinant peptide-N-glycosidase F (PNGase F, Roche, Germany) was added per 10 μg of protein. Deglycosylation was allowed to proceed overnight at 37 °C and terminated by freezing at −20 °C. Mock-treated samples were incubated simultaneously in all cases. An isolate enriched for the immunogenic and non-glycosylated major sperm protein (MSP) of *D. viviparus* (see Schnieder, 1993) was also PNGase F-, and mock-treated to verify that PNGase F treatment did not change peptides or peptide epitopes.

Protein deglycosylation was also carried out on proteins which were blotted to nitrocellulose after electrophoresis (see below). Deglycosylation was performed as described, except that the boiling step was omitted and that 10 times more PNGase F was used (1 μl per 1 μg protein). The treatment was terminated by washing the blot several times with PBS. Mock-treated samples were incubated simultaneously in all cases. Staining of the proteins on the blots (before and after treatment) was performed using Direct Blue 71 (DB71, Sigma-Aldrich, USA), to verify that there was no loss of protein binding by the PNGase F treatment.

Detection of glycosylation

Before or after deglycosylation, antigen extracts were separated on 10% SDS-PAGE gels (7·5 μg protein/lane). Glycoproteins were stained employing the Pro-Q Emerald 300 glycoprotein gel and blot stain kit (Molecular Probes, USA) according to the manufacturer's instructions. Ovalbumin was used as a positive control. After staining, the same gel was stained with silver to detect proteins.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA using ES or L3 extracts as antigens was performed as described previously (Kooyman *et al*. 2002). Deglycosylated and glycosylated ES or L3 antigens were coated at 2 μg/ml. In order to test parasite-specific IgE levels, it was necessary to purify IgE from serum using immobilized anti-IgE. Eluted IgE was collected in PBS with 0·1% gelatine and

0·05% Tween 20 (PBS-GT). The volume of PBS-GT was the same as that of the serum from which the IgE was purified and the elution was used undiluted in the ELISA. The recovery of IgE was verified using the ELISA and the yield was consistently greater than 90%. After testing several serum dilutions, 1/1000 was shown to be optimal for testing the other immunoglobulin isotypes. Anti-IgG1 (mca 627, Serotec, 1/1000 dilution), anti-IgG2 (mca 626, Serotec, 1/1000 dilution), IgA (mca 628, Serotec, 1/1000 dilution) and rabbit anti-IgM (ICN, 1/2000 dilution) were used as isotype-specific antibodies. Conjugates (goat anti-mouse/alkaline phosphatase and goat anti-rabbit/alkaline phosphatase, DAKO) were used at a dilution of 1/2000. The substrate p-nitrophenyl phosphate (pNPP, Pierce, USA) was used. Reactions were allowed to develop for 2 h (IgG1, IgG2 and IgM) or overnight (IgE and IgA). All samples were tested in duplicate, and positive and negative controls were included on each plate. Absorbance values were measured at a wavelength of 405 nm and expressed as optical density (OD_{405}). Plate-to-plate variation was consistently less than 10%.

SDS-PAGE and Western blotting

Electrophoresis (10% polyacrylamide gels) and immunoblotting were carried out as described previously (Kooyman *et al*. 2002) with some minor modifications. For all gels, 7·5 μg antigen was loaded into each lane. Molecular weight markers (Amersham Pharmacia Biotech Inc, USA) used were 94 kDa (phosphorylase b), 67 kDa (albumin), 43 kDa (ovalbumin), 30 kDa (carbonic anhydrase) and 20 kDa (trypsin inhibitor). Following the transfer, proteins were stained with Direct Blue 71. After destaining, blots were incubated successively with cattle sera (1/1000 dilution), anti-IgG1 (1/1000 dilution) and alkaline phosphate-conjugated goat anti-mouse immunoglobulin (1/2000 dilution). Immune-reactive bands were detected with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (both from Sigma-Aldrich, USA).

Sera from infected calves

Serum from blood from infected calves was prepared as described previously (Höglund *et al*. 2003). For the experimental infection experiment, helminth-free (Swedish Red and White Breed) calves were allocated to a primary infection group ($n=5$) and a challenge control group ($n=6$). The primary infection group received 100 *D. viviparus* L3s for 5 consecutive days, commencing at day 0. At day 73, all calves were challenged with 1500 L3s. At day 101, all cattle were euthanized and worms were recovered from the lungs and counted. Worm counts after the challenge infection were significantly greater in the challenged control group (mean: 355, range: 168–520) than in the group previously infected (mean: 13, range: 9–23) ($P<0·0001$), indicating that the primary infection induced protection against the succeeding challenge infection.

Sera from vaccinated calves

Serum from blood from vaccinated calves was prepared as described previously by Johnson *et al*. (2003). For the vaccination experiment, parasite-free raised Friesian-cross dairy calves were allocated to 2 groups. One group ($n=6$) was vaccinated with Huskvac® (Intervet, UK) at days 0 and 28, according to the manufacturer's recommendation; the other group ($n=6$) was not. At day 42, both groups were challenged with 700 L3 and slaughtered at day 77. No adult worms were found in the vaccinated calves, whereas a mean of 22 worms was recovered from the non-vaccinated calves, indicating that vaccination induced protection against a succeeding challenge infection.

Statistical analysis

Wilcoxon Signed Ranks test was used for paired samples (ELISA results before and after PNGase F treatment of the same group) and Mann-Whitney test was used for unpaired observations (ELISA results of re-infected and challenged control groups using the same antigen). For all analyses, the SPSS (version 10.01.0) software package was used.

RESULTS

Glycosylation of ES products from adult D. viviparus

To assess the extent of protein glycosylation in *D. viviparus*, ES products from adult worms were subjected to SDS-PAGE and Emerald 300 staining. This analysis yielded 2 major doublets of 57 and 43 kDa, 3 bands of about 90 kDa and 1 band with a molecular mass of ~200 kDa (Fig. 1B). Subsequent silver staining of the same gel to detect all proteins in the ES revealed 2 additional bands below 30 kDa not detected by Emerald glycan staining (Fig. 1A). Comparison of the Emerald and silver staining of glycosylated ovalbumin and the non-glycosylated molecular weight markers confirmed the validity of the glycoprotein staining procedure (Fig. 1A and B).

To learn more about the nature of the protein glycosylation, ES extracts were treated with PNGase F prior to electrophoresis. This glycosidase enables the discrimination between O-linked and N-linked glycosylation by specific cleavage of N-linked glycans which lack a core $\alpha 1 \rightarrow 3$ linked

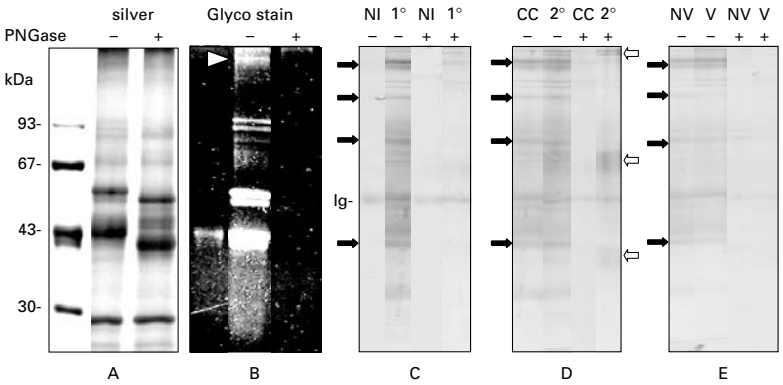

Fig. 1. SDS-PAGE of mock (–) or PNGase F treated (+) ES (7.5 μg/lane) stained with (A) silver, (B) Emerald 300 glycoprotein stain, or (C, D, E,) subjected to immunoblotting. (A, B) Note that only the glycosylated molecular weight marker (ovalbumin, 43 kDa, left lanes) reacted positively with the glycan stain. A white arrowhead indicates the ~200 kDa glycoprotein. (C) Reactivity of IgG1 from pooled serum from non-infected controls (NI) or primary infected (53-days) calves (1°). Black arrows indicate most predominant putative glycoproteins carrying immunodominant N-glycans, not detected after PNGase F treatment (+). Ig indicates possible contamination with host immunoglobulins. (D) Reactivity of IgG1 from pooled serum from challenge control (CC) and re-infected calves (2°) obtained at 28 days after challenge. Open arrows point to protein bands in ES/PNGase that are recognized by IgG1 from re-infected animals after challenge. (E) Reactivity of IgG1 from pooled serum from non-vaccinated control (NV) and vaccinated animals (V) obtained at 28 days after challenge infection.

fucosyl group. After PNGase F treatment, electrophoresed ES components were not stained with Emerald 300 (Fig. 1B), indicating that the proteins carried only N-linked glycans. When stained with silver (Fig. 1A), an apparent shift of most of the deglycosylated proteins to a lower molecular weight was evident. The components of 90, 57 and 43 kDa migrated in the deglycosylated forms as single bands of 83, 55 and 42 kDa, respectively, whereas the glycan-negative components of less than 30 kDa did not change in molecular weight after PNGase F treatment. The ~200 kDa glycoform was not detectable after deglycosylation.

Glycosylation of L3 protein extract

Emerald 300 staining of L3 extracts yielded only one major, rather diffuse glycoprotein band of ~200 kDa (Fig. 2B). Subsequent silver staining revealed numerous, non-glycosylated proteins (Fig. 2A). Based on the relative intensities of the silver staining, the glycoprotein detected was of a relatively low abundance. PNGase F treatment of L3 extract resulted only in a slightly less intense Emerald 300 staining and a small decrease in the weight of the high molecular weight glycoprotein (Fig. 2B). This finding may indicate that not all attached carbohydrates are N-linked and/or that part of the N-linked oligosaccharide carries a core $\alpha 1 \rightarrow 3$ linked fucose. The specificity of the effect was demonstrated by the unaltered migration of the Emerald 300-negative L3 proteins after PNGase F treatment (Fig. 2).

Immunogenicity of the ES and L3 glycoproteins in calves with primary D. viviparus *infection*

The ELISA was used to investigate the contribution of the glycosylation to the antibody responses against *D. viviparus* ES and L3 during a primary infection. Testing of the IgG1 response, which is the most prominent antibody isotype in lungworm infections (Scott *et al*. 1996; Kooyman *et al*. 2002), revealed strong reactivity to both ES and L3 antigens. This reactivity was reduced significantly after PNGase F treatment (Fig. 3).

To identify the glycoproteins which contributed to the immune response, the reactivity of IgG1 in the sera from infected calves were analysed by Western blotting. As shown in Fig. 1C, multiple ES bands reacted with the IgG1 fraction. Some immune-reactive (glyco)proteins stained poorly with silver or Emerald 300, indicating that they were of low abundance but were immunogenic. Sera from naive animals reacted exclusively with a 55 kDa band. Control experiments showed that this 55 kDa protein also reacted with rabbit anti-bovine IgG (data not shown) and, therefore, most likely represented the heavy chain of host immunoglobulin which had remained bound to ES during isolation. To investigate the contribution of N-glycans to the observed ES immune-reactivity, the blots were treated with

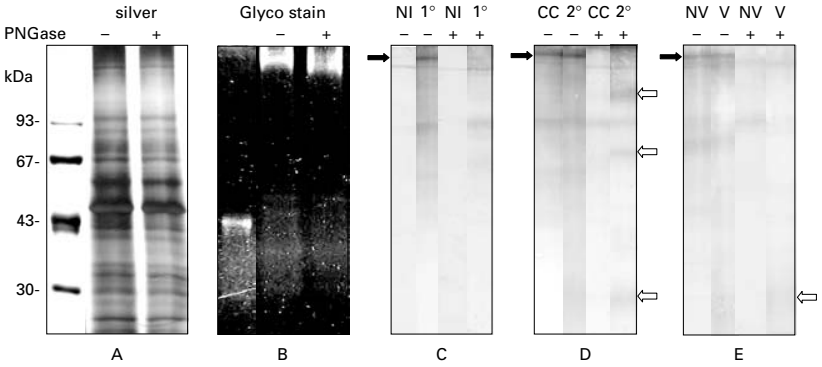

Fig. 2. SDS-PAGE of mock (–) or PNGase F treated (+) L3 total protein extract (7.5 μg/lane) stained with (A) silver, (B) Emerald 300 glycoprotein stain, or (C, D, E) subjected to immunoblotting. (A, B) Note that only the glycosylated molecular weight marker (ovalbumin, 43 kDa, left lanes) reacted positively with the glycan stain. (C) Reactivity of IgG1 from pooled serum from non-infected controls (NI) or primary infected (53-days) calves (1°). The black arrow indicates a putative glycoprotein carrying immunodominant N-glycans, not detected after PNGase F treatment (+). (D) Reactivity of IgG1 from pooled serum from challenge control (CC) and re-infected calves (2°) obtained at 28 days after challenge. Open arrows point to protein bands that are recognized by IgG1 from re-infected animals after challenge after L3/PNGase treatment. (E) Reactivity of IgG1 from pooled serum from non-vaccinated control (NV) and vaccinated animals (V) obtained at 28 days after challenge infection. The open arrow indicates immune reactivity noted for vaccinated animals only.

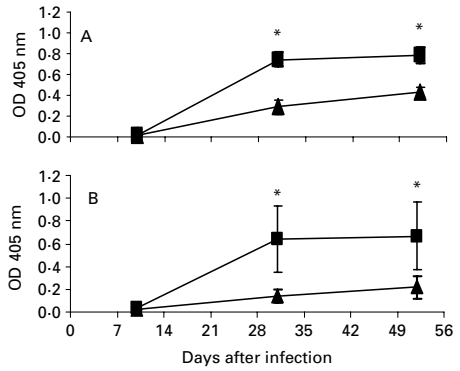

Fig. 3. ELISA showing the immunoreactivity of serum IgG1 from primary infected animals with (A) ES or ES/PNGase and (B) L3 or L3/PNGase antigen. Note that the signal for ES and L3 (■) was higher than for ES/PNGase and L3/PNGase (▲), respectively. On days marked with * this difference was significant ($P<0.05$). Non-infected control animals remained negative and are not shown. Results are given as mean OD_{405} +/− S.E.M.

PNGase F. This treatment resulted in a strong reduction in IgG1 reactivity with most of the immune-reactive proteins, indicating that carbohydrate moieties were the major antigenic components recognized by the immune sera (Fig. 1C).

Similar Western blot analysis of the L3 fraction showed strong reactivity of the immune sera with the high molecular weight glycoprotein. This reactivity was not present after PNGase F treatment (Fig. 2C). The reduced immune-reactivity against PNGase F treated ES and L3 was not caused by a loss of bound protein from the blot during treatment, as protein staining with DB 71 after PNGase F- or mock-treatment yielded similar results (not shown). Together, the results indicate that the parasite derived N-glycan moieties are immunodominant antigens during primary infection.

Effect of re-infection of calves with D. viviparus *on the immune response*

To further establish whether the N-glycans elicited a short-term or long-term memory response, we compared the IgG1 responses of re-infected calves (primary infection followed by challenge) with those of calves with a primary infection (challenge control). ELISA testing revealed that challenge infection elicited an antibody response, against both ES and L3 antigens (Fig. 4). Seven days after challenge, the IgG1 levels for the re-infected animals were higher than for the challenge controls due to 'residual antibodies' elicited by the first infection. Twenty eight days after challenge, the sera from both groups showed comparable immune-reactivity. Assessment of the immune response to PNGase F-treated ES or L3 antigen revealed a reduced response for both groups of calves, indicating the presence of N-glycan specific IgG1 both after primary infection and re-infection. Also, these experiments showed

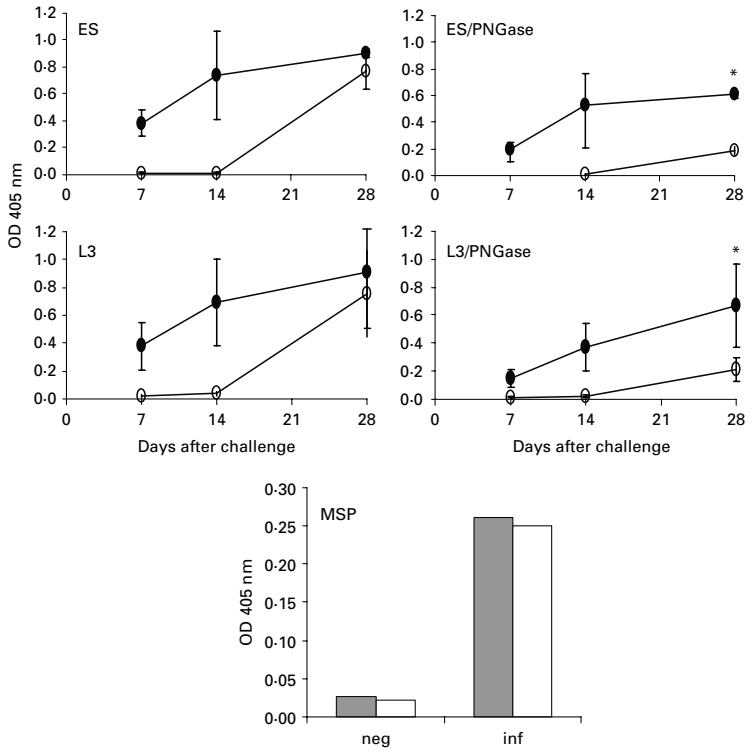

Fig. 4. ELISA showing the reactivity of serum IgG1 from re-infected (●) and challenge control calves (○) after challenge infection with ES, L3 and the respective deglycosylated antigens (ES/PNGase and L3/PNGase, respectively). Note the lower signal for both ES and L3 in both groups after PNGase F treatment. * At day 28 re-infected animals showed a significantly ($P<0.01$) better response to deglycosylated antigens than the challenge control animals. Results are given as mean OD_{405} +/− S.E.M. Reactivity of serum IgG1 from negative and re-infected calves towards a non-glycosylated protein (MSP) was the same before (filled bars) and after PNGase F treatment (open bars), indicating that PNGase F treatment did not change peptide epitopes.

a significantly ($P<0{\cdot}01$) higher response to the deglycosylated proteins in the re-infected group compared with the challenge control group, suggesting that the protein backbone rather than the attached N-glycans elicits a booster response. Control experiments with MSP as an antigen showed that PNGase F treatment did not affect its reactivity with IgG1 from re-infected calves (Fig. 4). Thus, PNGase F did not destroy the peptide epitopes.

Western blotting confirmed the ELISA results. Similar responses to ES for both groups at day 28 post-challenge as well as the booster response against the peptide backbone in the re-infected group were found on blots (Fig. 1D). For L3, the sera from the re-infected animals yielded additional immunereactive bands compared with the challenge controls. This booster effect was particularly evident after PNGase F treatment (Fig. 2D), indicating the existence of a booster response to the protein backbone of distinct L3 antigens. These data suggest that N-glycans elicit an IgG1 response both during the primary infection and during re-infection, whereas only the protein backbone elicits a memory response.

Isotype specificity of the immune response

Since the effector's function of an antibody is isotype-dependent, we also compared the N-glycan-specific IgA, IgE, IgG2 and IgM antibody reactivities in sera from re-infected calves with those of calves with primary infection (Fig. 5). All immunoglobulin isotypes tested for both groups of calves reacted with ES antigens. PNGase F treatment of the antigens resulted in a much lower response for all isotypes, except for IgM, suggesting that the N-glycan response was not restricted to a distinct isotype.

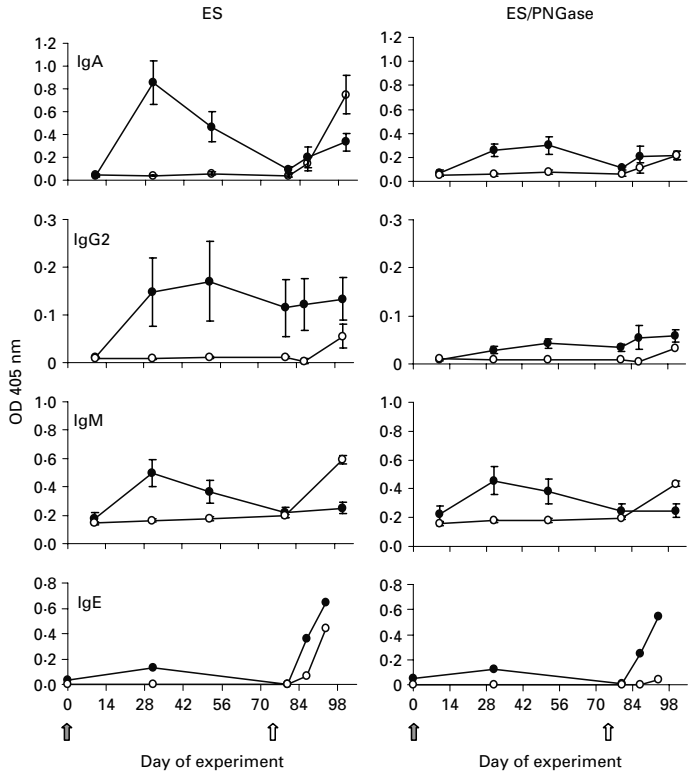

Fig. 5. ELISA showing the IgA, IgG2, IgM and IgE isotype response of re-infected (●) and challenge control animals (○) towards ES (left column) and deglycosylated ES (ES/PNGase, right column). Arrows indicate the time of the primary (filled arrows) and challenge infection (open arrows). Results are given as mean OD_{405} +/− S.E.M., except for IgE for which serum was pooled per group per date.

Analysis of the response for isotype specificity (other than IgG1) showed that deglycosylated ES only boosted the IgE response. For deglycosylated L3, none of the (non-IgG1) isotypes showed an enhanced response towards the protein backbone (not shown). Thus, N-glycans appear to elicit a broad isotype response, whereas only the protein-specific IgG1 and IgE responses are boosted after re-infection.

Effect of vaccination on the N-glycan and protein-specific immune response

The apparent rapid induction of a short-lived memory response against N-glycans but of a long-term response against protein epitopes upon re-infection with *D. viviparus* led us to investigate the effect of vaccination on the immune response against these antigens. ELISA testing demonstrated that vaccination induced a specific serum IgA, IgG1 and IgG2 responses against ES, whereas IgM and IgE levels remained low (Fig. 6). The IgA response after vaccination was similar to the IgA response in infected calves, whereas the IgG1 and IgG2 responses were much lower in the vaccinated calves.

After the treatment of the antigens with PNGase F, IgA, IgG1 and IgG2 reactivities towards the deglycosylated antigens was much lower than to the non-deglycosylated ES antigens. Also, challenge infection after vaccination did not boost ES-specific IgG1 or IgE (Fig. 6). Western blot analysis of ES (Fig. 1E) and L3 (Fig. 2E) with IgG1 from sera from vaccinated calves (28 days after challenge) confirmed that the vaccine-induced immune response was mainly directed against the N-glycans and not against the peptide backbone, as the bands were not detected after PGNase F treatment (except for a diffuse band of 20–25 kDa, Fig. 2E). These results may explain the short-lived protection which typifies the protective effect using the current vaccine.

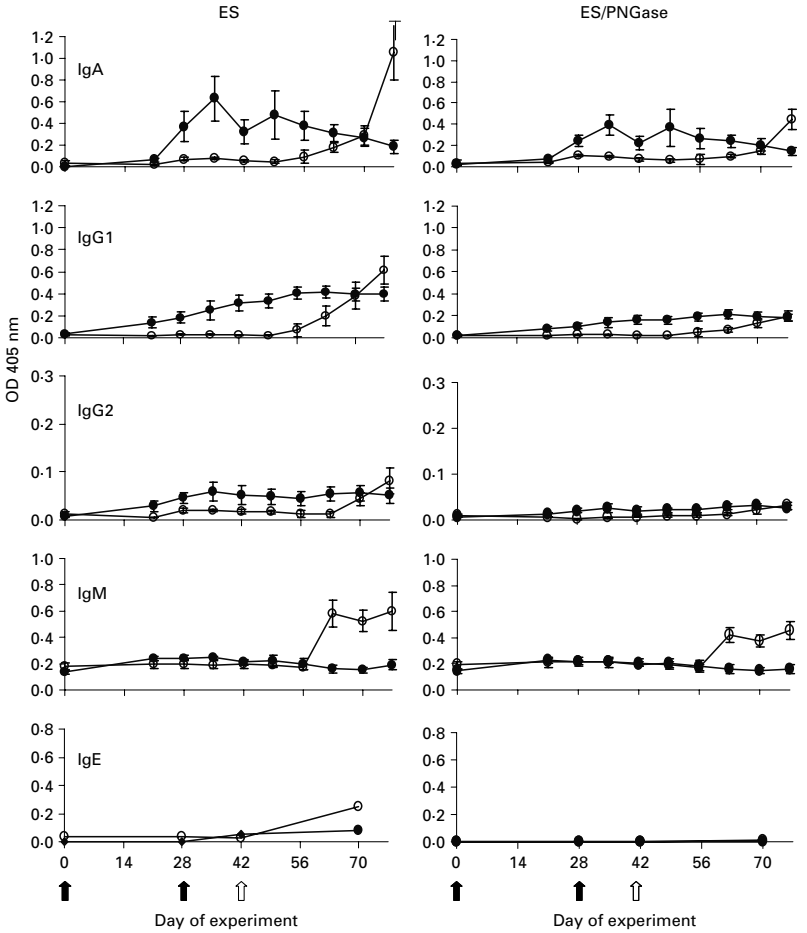

Fig. 6. ELISA demonstrating the IgA, IgG1, IgG2, IgM and IgE Isotype response of vaccinated (●) and challenge control animals (○) towards ES (left column) and deglycosylated ES (ES/PNGase, right column). Arrows indicate the time of vaccination (black arrow) and challenge infection (open arrow). Results are given as mean OD_{405} +/− S.E.M., except for IgE for which serum was pooled per group per date.

DISCUSSION

In this study, evidence is presented that multiple *D. viviparus* proteins are 'decorated' with N-glycan moieties and that the glycosylation is a crucial determinant of the nature of the immune response in cattle after natural infection with and vaccination against *D. viviparus*. The N-glycan moieties carried immunodominant epitopes which failed to elicit a memory response, in contrast to the protein backbone. This finding may explain the short-lived memory induced by the natural infection or vaccination.

Thus far, glycosylation in *D. viviparus* has not gained much attention. More than a decade ago, Britton *et al.* (1993) used several techniques to study glycosylation in adult ES products and successfully identified a 42 kDa glycoprotein, while a structural study (Haslam *et al.* 2000) reported the presence of complex glycans with Lewis^x antennae in detergent extracts of homogenized worms without further identification of the protein backbone. The conclusion from the present study that *D. viviparus* carries a 'protein glycosylation machinery' was based on both the specific staining of multiple proteins for glycans and the altered electrophoretic mobility of these proteins after treatment with the glycosidase PNGase F. The finding that treatment of adult ES with PNGase F resulted in complete deglycosylation of most of the glycoproteins

indicates that most ES proteins carry N-glycans without a core $\alpha 1 \rightarrow 3$ fucosylation and lack O-linked carbohydrate moieties. In the total protein extract from L3, a single glycoprotein was identified that was incompletely deglycosylated upon PNGase F treatment. The differences in glycoprotein content between ES and L3 extracts may reflect differences between the developmental stages, but may also result from the use of different fractions (soluble ES products of the adult stage *versus* total protein extract for L3). The immune-reactivity of sera from infected calves against *D. viviparus* extracts suggested that all glycoproteins except one (~55 kDa protein present in the ES fraction) were newly identified glycoproteins of parasite origin and not contaminating host glycoproteins. At this point, it should be noted that, due to the 10% polyacrylamide gel used, glycosylation of additional proteins of <20 kDa cannot be excluded.

The present findings indicate that the glycosylation of *D. viviparus* proteins has a significant effect on the host immune response against the parasite. Comparison of the immunoreactivity of antigens before and after deglycosylation using sera from calves with primary infection clearly demonstrated a strong reduction in the immunoreactivity of all but one of the immunoglobulin isotypes (i.e. IgA, IgE, IgG1, IgG2 and IgM) tested against the deglycosylated antigens, whereas no effect was detected using the MSP control protein. These data suggest that the glycans largely contribute to the immunogenicity of the parasite and that they contain immunodominant epitopes. These findings seem to deviate from the data published by Britton *et al*. (1993), who showed that the presence of N-glycans on the 42 kDa glycoprotein (identified by them) did not influence the immunoreactivity. This difference may be explained by their use of lower serum dilutions (1/20) and/or a different assay (immunoprecipitation) in which the antigen rather than the antibodies may become limiting. The use of low serum dilutions has also been suggested as a limitation in the detection of carbohydrate-specific antibodies in mice infected with the blood-fluke *Schistosoma mansoni* (see Richter *et al*. 1996). Another difference between the 2 methods is the use of proteins in the native state in the study of Britton *et al*. (1993) compared with denatured proteins in the present study, although we consider it unlikely that the denaturation procedure would change the recognition of the N-glycans.

Interestingly, the binding of IgM was hardly affected by deglycosylation of ES or L3 extract. In *Schistosoma*-infected mice (Richter *et al*. 1996) and chimpanzees (Eberl *et al*. 2001) as well as after infection with other nematodes, IgM is mainly directed against carbohydrates. *H. contortus* infected sheep, for example, elicit a strong IgM response against periodate-sensitive carbohydrate epitopes, such as fucosylated LacdiNAc (LDNF) (Vervelde *et al*. 2003). Only later in infection, the IgM levels decrease and class-switching to IgG1 occurs. This IgM response may be directed against the core fucosylated groups present in many schistosome glycans and LDNF (Nyame *et al*. 2000; Eberl *et al*. 2001). Treatment of lungworm ES products with PNGase F removed N-linked glycans, demonstrating that this fraction does not contain core $1 \rightarrow 3$ fucosylated N-glycans.

Perhaps the most striking finding in the present study was the presence of a booster response against the protein backbone but not against the attached glycans after re-infection. This finding strongly suggests that the glycans, although immunodominant, are unable to induce a memory response. This response against deglycosylated ES antigen was observed for both IgG1 and IgE, but not for IgA or IgG2. Since the re-infected group, but not the challenge control group, was protected against challenge infection, this finding may indicate that IgG1 and/or IgE, but not the other isotypes, play a role in achieving long-lasting protection. The precise nature of the antigens which induce the protective immune response remains to be defined. The primary immune-reactive component recognized by IgG1 from re-infected calves, but not from primary infected or vaccinated calves, was a 67 kDa protein in deglycosylated ES. A *D. viviparus* protein of this size has been identified previously as an acetylcholine esterase (AChE) (McKeand *et al*. 1995). Interestingly, this protein elicits a strong immune response in infected, but not in vaccinated calves (McKeand *et al*. 1994). Also, AChE of *D. viviparus* has at least 2 potential N-glycosylation sites (Lazari *et al*. 2004), but immune sera from re-infected cattle recognize recombinant (non-glycosylated) AChE, indicating that the protein backbone is immunogenic (Matthews *et al*. 2001). As the immunization with a fraction enriched for AChE confers protection to guinea pigs (McKeand *et al*. 1995), it is tempting to speculate that the immunodominant 67 kDa protein identified in the present study is AChE. For L3, several (glyco)-protein bands reacted strongly and exclusively with the immune sera from the re-infected calves. These signals were enhanced after PNGase F treatment, indicating that the booster response was primarily directed against the peptide backbone. The identity of these proteins remains to be defined.

Vaccination against *D. viviparus* is known to provide only short-term protection (Michel and Mackenzie, 1965). In the present study, vaccination resulted in ES and L3 N-glycan-specific IgA, IgG1 and IgG2 antibody responses. Challenge infection of vaccinated calves did not boost the IgG1 or IgE immune-reactivity towards the deglycosylated antigens as was observed for calves with a primary infection, probably because the calves are not

exposed to adult ES in the lungs due to the presence of circulating N-glycan specific antibodies. The difference in booster response between the infected and vaccinated calves may explain the parasitological observations that vaccination yielded a short-lived (< 6 months) protection against larvae arriving in the lung, whereas a long-term protection (for at least 27 months) was only obtained after infection (Michel and Mackenzie, 1965).

Based on the present results and previous observations, a scenario for the development of immunity against *D. viviparus* can be envisioned in which long-lasting immunity against the lung stages is caused by a memory response (Th2) involving specific IgG1 and/or IgE antibodies directed to the peptide backbone of certain parasite products. The short-lived immunity against re-infection can be explained by the presence of mainly anti-N-glycan specific antibodies of different isotypes (elicited *via* a primary infection or vaccination) which are still circulating at the time of challenge. The fact that this short-lived immunity can be demonstrated already during a challenge infection (11 days after primary infection) (Michel, 1962) is typical for a carbohydrate (thymus-independent) antigen response not requiring priming and clonal expansion of T-cells but lacking memory (Janeway *et al*. 2005). The hypothesis that the inclusion of deglycosylated ES antigen into a vaccine may result in long-lasting immunity awaits further testing.

The infection and vaccination experiments were approved by the ethical committees of Sweden and New Zealand. Dr Marion Johnson from AgResearch, Invermay Agricultural Centre, New Zealand kindly provided the sera from the vaccination experiment.

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CHAPTER 4

Antibodies elicited by the bovine lungworm, *Dictyocaulus viviparus*, cross-react with platelet-activating factor

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Antibodies Elicited by the Bovine Lungworm, *Dictyocaulus viviparus*, Cross-React with Platelet-Activating Factor[▽]

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Parasite N-glycans may play an important role in helminth infections. As antibodies from *Dictyocaulus viviparus*-infected calves strongly react with N-glycans, we investigated the characteristics of the major immunodominant glycoprotein (GP300) of this parasite. Probing of worm extracts with various lectins demonstrated unique binding of GP300 to wheat germ agglutinin. Analysis of lectin-purified GP300 revealed that the glycan was substituted with phosphorylcholine and reacted with the phosphorylcholine-specific antibody TEPC-15. Competitive enzyme-linked immunosorbent assay with GP300-coated plates and GP300-specific immunoglobulin G (IgG) in conjunction with free phosphorylcholine or TEPC-15 demonstrated that antibodies from infected calves recognized phosphorylcholine on GP300. Additional assays showed that these antibodies cross-reacted with the phosphorylcholine moiety present on platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), a proinflammatory mediator of the host. Heavily infected calves contained high levels of serum GP300-specific IgG1 but low levels of IgA and IgG2 and showed a reduced influx of eosinophils in the lungs, all consistent with a neutralization of PAF activity. In conclusion, we demonstrated that *D. viviparus* infection elicits GP300-specific antibodies that cross-react with PAF and may neutralize PAF function, thus limiting the development of a protective response as well as parasite-induced host pathology.

Helminths are well adapted to their host and have evolved sophisticated strategies to avoid or modulate the host immune defense. Although the exact mechanisms via which helminths subvert the host responses are not completely understood, they typically induce an anti-inflammatory environment (22). At higher worm burdens, however, this may not be sufficient to prevent disease, as exemplified by the nematode *Dictyocaulus viviparus*, the etiologic agent of parasitic bronchitis in cattle. This parasite is ingested as larvae that, after penetration of the intestinal wall, migrate via the lymph nodes and the blood circulation to the lungs, where they mature into adult worms. Eggs produced by theses adults are coughed up, swallowed, and excreted in the feces as first-stage larvae. In the lungs, pathology develops due to the influx and activation of eosinophils and mast cells that cause restriction of the airways and a collapse of the alveoli, resulting in edema and emphysema (16).

Since during natural *D. viviparus* infection the host ultimately generates a protective immune response (18), prevention against this nematode infection through vaccination may be feasible. Serum transfer experiments in calves indicate that protection is accomplished via immunoglobulins (Igs) (17). Antibody responses against nematodes are often directed against glycoconjugates (5, 28). Immunologically important typical features of nematode glycans include core α(1,3) fucosylation, instead of core α(1,6) fucosylation as in mammals, and the decoration of complex type N-glycans with phosphorylcholine (PC). The presence of both core α(1,3) fucose and PC cause the glycans to be highly immunogenic (1, 6, 9, 31), while core α(1,3) fucose may also give rise to strong allergic reactions.

In search for protection-inducing antigens of *D. viviparus*, we recently identified several glycoproteins that were highly immunogenic. The antibody response was almost exclusively directed against the N-glycan moieties (19). The most immunodominant antigen in adult excretory-secretory (ES) products was a high-molecular-weight glycoprotein that appeared on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels as a double band of ~200 to 300 kDa (GP300). The nature of the immunodominant glycan, however, was not determined. In the present study, we purified GP300 by use of lectin chromatography and identified the nature of the major immunodominant epitope. In addition, we demonstrate that GP300 shares immunoreactivity with the mammalian inflammatory mediator platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine). It is hypothesized that this shared immunoreactivity results in down-regulation of the inflammatory responses against *D. viviparus*.

MATERIALS AND METHODS

Parasites. Adult lungworms were collected from lung washings at day 35 of infection as described previously (7). ES products from these adults were obtained after overnight incubation (19). To obtain adult soluble and water-insoluble extracts, 1 volume of adult worms was homogenized in 2 volumes of phosphate-buffered saline (PBS) containing complete protease inhibitor (Roche, Germany) in a Dounce homogenizer. After centrifugation (3,000 × *g*, 20 min, 4°C) the pellet was resuspended in another 2 volumes of PBS with protease inhibitor and again collected by centrifugation. Both supernatants were combined and stored as soluble extract at −80°C. The pellet was resuspended in 1 volume of PBS, and whole extract was precipitated with trichloroacetic acid-acetone (19). Dry pellet was extracted with 1 volume of urea buffer (8 M urea in 10 mM Tris, pH 7.4; 20°C, 30 min) while shaking. After centrifugation (3,000 × *g*, 20 min, 4°C), the supernatant was collected as the water-insoluble fraction and stored at −80°C.

Calves. Nineteen worm-free Holstein-Friesian female calves of 4 months of age were used. Sixteen calves were infected at day 0 with 30 ($n = 8$) or 500 ($n = 8$) L3 larvae. The remaining calves served as the challenge control group ($n = 3$)

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and remained worm free until infected at day 35, when all animals received a challenge infection of 1,000 L3 larvae. After slaughter at day 56, worm counts from the lungs were performed as described previously (7), and protection against challenge infection was calculated based on worm counts. Blood samples were taken throughout the experiment, and the sera were stored at −20°C until use. Day 35 sera from the eight animals infected with 500 L3 larvae were pooled and used as the positive sera throughout the study. Pooled control (negative) sera were obtained from the same animals at day 0. All experimental procedures were approved by the ethical committee on animal experimentation of Utrecht University.

Collection of BALF. Bronchoalveolar lavage fluid (BALF) was collected as described previously (26). After centrifugation (2,000 × *g*, 10 min, 4°C) and measurement of the protein concentration, BALF supernatants were stored at −20°C until use. The BALF pellet was resuspended in PBS, and the number of cells was counted (microcell counter CC-108; Sysmex). Giemsa staining was performed on cytospins to determine the number of eosinophils, alveolar macrophages, neutrophils, and lymphocytes; 200 cells were scored. Cell numbers were expressed per mg of recovered BALF protein.

SDS-PAGE and Western blotting. Electrophoresis and Western blotting were performed as described previously (19). Blots were incubated with pooled positive or negative bovine sera (1:1,000 dilution) and subsequently with anti-IgG1 monoclonal antibody (mAb) (mca 627, 1:1,000 dilution; Serotec) and alkaline phosphatase (AP)-conjugated goat anti-mouse Ig (1:2,000 dilution; DAKO). The mAb TEPC-15 (1:1,000 dilution; Sigma) served as a positive control for the detection of PC-containing glycoproteins (21) and was used in combination with goat anti-mouse Ig-AP (1:2,000 dilution; DAKO). AP activity was determined using 5-bromo-4-chloro-3-indolylphosphate (BCIP)–NBT (Sigma) as a substrate. For detection of lectin binding glycoproteins, blots were incubated with biotinylated wheat germ agglutinin (WGA), *Lens culinaris* agglutinin, and concanavalin A (ConA) (all from Pierce) at a concentration of 1 μg/ml in Tris-buffered saline (TBS) containing 0.1% gelatin and 0.05% Tween 20. ConA binding was performed in the presence of 1 mM $CaCl_2$ and 1 mM $MnCl_2$. Biotinylated lectins were detected with streptavidin-horseradish peroxidase conjugate (1 μg/ml; Pierce). Reactivity was visualized with DAB (3,3′-diaminobenzidine tetrahydrochloride) as the substrate.

WGA chromatography. Ten milliliters of the water-insoluble protein fraction stored in 8 M urea, 10 mM Tris was diluted with 30 ml of TBS to 2 M urea. After the removal of precipitates (3,000 × *g*, 5 min, 20°C), the supernatant was added to 2 ml of drained WGA-agarose (Sigma) and preequilibrated with TBS containing 2 M urea in a 50-ml tube. After 2 h of end-over-end rotation (20°C) and the removal of the supernatant (unbound fraction), the WGA-agarose with bound glycoproteins was transferred to a disposable polystyrene column (Pierce) and washed with 40 ml of TBS containing 2 M urea. Elution was performed with 10 ml of TBS containing 6 M urea and 1 M *N*-acetylglucosamine (GlcNAc), and 1-ml fractions were collected. Eluted fractions that contained protein (Bradford protein assay) were analyzed on SDS-PAGE gels, pooled, concentrated on a YM10 Centriprep (Amicon) to 1 μg/μl of protein, and stored at −20°C until use.

Affinity purification of anti-GP300. WGA-purified GP300 (100 μg) was coupled to 0.33 g of CNBr-activated Sepharose 4B according to the protocol of the manufacturer (Amersham). Five-milliliter portions of negative or positive pooled sera were diluted with equal volumes of PBS, and 100 μl of 0.5 M EDTA was added to inhibit calcium-dependent binding of C-reactive protein. The sera were incubated (4°C for 16 h or 20°C for 3 h) with the immobilized GP300 in a 50-ml tube by use of an end-over-end rotator. The supernatant (unbound fraction) was removed, and the GP300-Sepharose with bound antibodies was transferred to a disposable polystyrene column (Pierce). After being extensively washed with PBS to remove all unbound material, the bound fraction was eluted with 8 ml of 0.2 M glycin, pH 2.8. Fractions (1 ml) were directly neutralized with 100 μl of 1 M Tris (not pH adjusted). Protein-containing fractions (fractions 1 to 3) were pooled and concentrated on a Centricon YM30 (Amicon) to a final volume of 0.5 ml. Enzyme-linked immunosorbent assay (ELISA) using GP300-coated plates indicated that >90% of the anti-GP300 reactivity of the positive serum was recovered in the bound fraction. The reactivity of the negative pooled sera towards GP300 was too low to determine the percentage of recovery. Part of the purified anti-GP300 (0.5 mg/ml in PBS) was biotinylated with EZ-Link sulfo-*N*-hydroxysuccinimide–biotin (Pierce) according to the instructions of the manufacturer.

Deglycosylation of glycoproteins. Protein deglycosylation was carried out by diluting 200 μg of the water-insoluble protein fraction or 10 μg of GP300 in Tris-urea buffer in four volumes of 50 mM K_2HPO_4, 25 mM EDTA (pH 7.0). SDS and 2-mercaptoethanol were added to final concentrations of 0.2% and 0.5%, respectively. Samples were boiled for 5 min, and 10% Triton X-100 was applied to a final concentration of 2%. After cooling of the samples to room temperature, 1 μl (1 μl = 1 U) of recombinant peptide-*N*-glycosidase F (PNGase F; Roche) was added per 100 μg of protein. Deglycosylation was allowed overnight at 37°C and terminated by boiling for 3 min. Mock-treated samples (all components except PNGase F) were incubated simultaneously in all cases.

Detection of glycosylation. Mock- and PNGase F-treated extracts were run on SDS-PAGE gels. Staining of glycoproteins was done with a Pro-Q emerald 300 glycoprotein gel and blot stain kit (Molecular Probes) according to the manufacturer's instructions. After being stained for glycoproteins, the same gel was stained with silver to visualize total protein contents.

GP300 competitive ELISA. Affinity-purified and biotinylated anti-GP300 (0.05 μg Ig/ml) was mixed with different concentrations of potential inhibitory Igs (100 to 0.003 μg/ml), incubated for 1 h at 37°C, and transferred to ELISA plates coated with 0.05 μg/ml of GP300 as described previously (19). Binding of anti-GP300-biotin was detected with 1 μg/ml of streptavidin-horseradish peroxidase (Pierce) and 3,3′,5,5′-tetramethylbenzidine (15 min). Color development was stopped with H_2SO_4, and optical density at 450 nm (OD_{450}) was measured in a Ceres UV 900C plate reader.

PAF ELISA. Antibody binding to PAF was carried out as described previously (27) but with modifications for the bovine system. Purified PAF (Sigma) was stored in ethanol (EtOH; 20 mg/ml) at −20°C. Maxisorp plates (Nunc) were coated (16 h, 4°C) with 25 μl/well of PAF in EtOH (200 μg/ml). After evaporation of the EtOH, the plates were washed once with TBS, blocked with dilution buffer (TBS containing 0.1% gelatin), and incubated with serial dilutions of anti-GP300. Ig isotype-specific detection was performed using anti-IgG1 (mca 627, 1:200 dilution; Serotec) or anti-IgA (mca 628, 1:200 dilution; Serotec) in dilution buffer and goat anti-mouse Ig-AP (D0486, 1:1,000 dilution; DAKO). The PC-specific antibody TEPC-15 (Sigma) (21) and the negative control antibody IE7 (20) served as controls. All incubation steps were performed for 1 h at 20°C. Antibody binding was determined with a pNPP kit (Pierce), and OD_{405} was measured in a Ceres UV 900C plate reader.

GP300 and ES-specific ELISA. Antibodies in bovine sera (1:1,000 dilution) directed against GP300 were detected with the GP300-specific ELISA. ELISA plates were coated with WGA-purified GP300 that received PNGase F or mock treatment (0.2 μg/ml). Binding of Igs was determined using one of the following isotype-specific mAbs in combination with goat anti-mouse Ig-AP (D0486, 1:2,000 dilution; DAKO) and the pNPP kit: anti-IgG1 (mca 627; Serotec), anti-IgA (mca 628; Serotec), and anti-IgG2 (mca 626; Serotec) (all at 1:1,000 dilution). Reactivity was quantified using a Ceres UV 900C plate reader. The above-described procedure was also used to detect ES-specific antibodies, except that in these assays, plates were coated with 2 μg/ml ES and BALF was diluted to a concentration of 50 μg/ml of protein.

Statistical analysis. Correlations were calculated with Spearman's nonparametric correlation test from the SPSS software package (version 10.01.0).

RESULTS

Extraction of GP300 from adult worms. GP300 has previously been identified as an immunodominant antigen of the ES product of adult lungworm (19). In order to obtain an amount of material sufficient for purification and further analysis of GP300, adult worms were fractionated into water-soluble and -insoluble extracts and tested for the presence of GP300. SDS-PAGE and Western blotting followed by probing with IgG1 from sera from infected animals showed that the water-insoluble fraction contained a double, high-molecular-weight band, typical for GP300 (Fig. 1A).

Lectin binding properties of GP300. Purification of GP300 may be achieved using lectin affinity chromatography. To investigate this, the reactivity of the glycoprotein with different types of lectins was determined using Western blotting. Blots containing the insoluble proteins from adult worms and probed with biotinylated lectins *Lens culinaris* agglutinin and ConA yielded several positive bands, but neither lectin reacted exclusively with GP300 (data not shown). In contrast, biotinylated WGA specifically recognized the GP300 protein doublet (Fig. 1B), indicating that WGA affinity chromatography may enable one-step purification of the antigen.

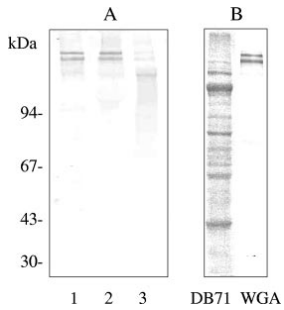

FIG. 1. Extraction of GP300. (A) Western blot demonstrating the reactivities of ES (lane 1), water-insoluble extract (lane 2), and water-soluble extract (lane 3) of adult worms (7.5 μg protein/lane) with IgG1 from *D. viviparus*-infected calves. Sera from noninfected animals did not react (not shown). (B) Total protein profile (DB71) and WGA reactivity of the water-insoluble extract.

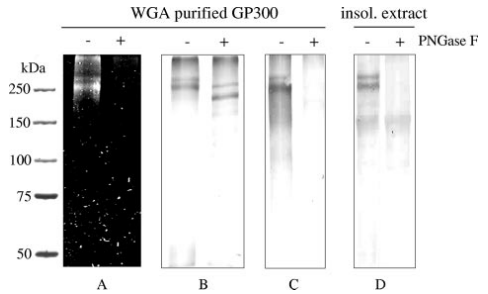

FIG. 3. Posttranslational modifications on GP300. For glycan detection, purified GP300 was PNGase F (+) or mock (−) treated, run on SDS-PAGE gels (0.5 μg protein/lane), and stained with emerald glycan stain (A) and then with silver (B). For detection of PC, WGA-purified GP300 (0.5 μg protein/lane) (C) and water-insoluble (insol.) extract (7.5 μg protein/lane) (D) were separated by SDS-PAGE, blotted, and probed with the anti-PC mAb TEPC-15.

Purification of GP300 using WGA affinity chromatography. To purify GP300, adult insoluble extract was applied to WGA-agarose. After extensive washing to remove unbound materials, bound glycoproteins were eluted with TBS containing 6 M of urea and 1 M of GlcNAc. Analysis of all fractions by SDS-PAGE and silver staining (Fig. 2A) demonstrated that, as expected, the eluted fractions contained only the double band characteristic of GP300. Western blotting with sera from infected animals (Fig. 2B) confirmed that the eluted fractions contained the immunodominant glycoprotein, while virtually no GP300 was detected in the unbound fraction. Based on the amount of recovered protein, it was estimated that 100 mg of water-insoluble protein applied to the WGA affinity column yielded ~90 μg of purified GP300.

To further confirm the identity of the purified material, the purified glycoprotein was submitted to treatment with PNGase F. This enzyme removes the N-linked glycan moieties of the proteins that lack core α(1,3) fucosylation (29). Emerald 300 glycan staining showed that the enzyme treatment completely removed the carbohydrate moiety from the protein, demonstrating that the glycan was N linked without core α(1,3) fucosylation (Fig. 3A). Silver staining showed that deglycosylation reduced the size of the purified protein from ~390 and ~310 kDa to ~320 and ~240 kDa, respectively (Fig. 3B), indicating that the protein is heavily glycosylated.

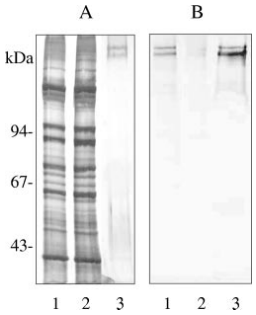

FIG. 2. Purification of GP300. GP300 was purified from water-insoluble extract by use of WGA affinity chromatography and analyzed by SDS-PAGE and silver staining (A) and Western blotting with IgG1 from sera of infected animals (B). Materials loaded onto the gel: starting material (lanes 1), WGA-unbound fraction (lanes 2), and WGA-bound proteins (lanes 3).

The N-glycans of GP300 contain PC. Carbohydrate analysis on GP300 by use of mass spectrometry did not yield a resolution sufficient to determine the structure of the glycan moiety (data not shown), possibly due to the presence of highly charged posttranslational modifications. One charged component previously identified on glycans of nematodes is PC. To investigate the possible presence of PC on GP300, blots with GP300 were probed with the PC-specific mAb TEPC-15. The antibody clearly recognized GP300 and deglycosylation resulted in the loss of reactivity, indicating that the PC was attached to the glycan moiety (Fig. 3C). Probing of blots containing the complete adult insoluble fraction with TEPC-15 showed exclusive reactivity with GP300 (Fig. 3D), suggesting that the substitution with PC is unique to GP300.

Immunogenicity of the PC attached to GP300. To investigate whether antibodies elicited by lungworm infection also recognized the PC moiety on GP300, a competitive ELISA was developed. For this purpose, GP300-specific antibodies were affinity purified from sera from infected calves and biotinylated. These antibodies specifically recognized GP300 (Fig. 4, insert). In the competitive ELISA with plates coated with purified GP300, the binding of biotinylated GP300-specific antibodies to the antigen was measured in the presence of various concentrations of the PC-specific antibody TEPC-15. As shown in Fig. 4, TEPC-15 caused >90% inhibition of binding of GP300-specific antibodies, while no inhibition of binding was observed for the control mAb IE7 (20). Inhibition was also obtained with the pooled sera from which the GP300-specific antibodies were derived but not with pooled sera obtained before infection from the same animals (Fig. 4). Furthermore,

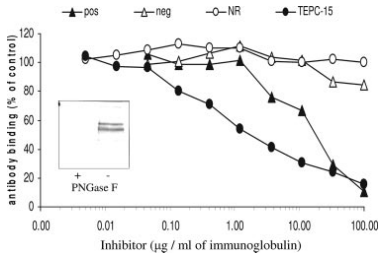

FIG. 4. Competition for GP300 binding between anti-GP300 and TEPC-15. Binding of biotinylated GP300-specific antibodies to GP300-coated ELISA plates was measured in the presence of the anti-PC mAb (TEPC-15) or sera from infected calves (pos). Sera from noninfected animals (neg) and a nonrelevant mAb (NR) served as controls. The inhibition of anti-GP300 binding to the antigen was expressed as the percentage of binding in the absence of inhibitors. Data are means of two independent assays with each measurement performed in duplicate. The insert shows the reactivity of biotinylated GP300-specific antibodies with PNGase F-treated (+) or mock-treated (−) GP300, as determined by Western blotting.

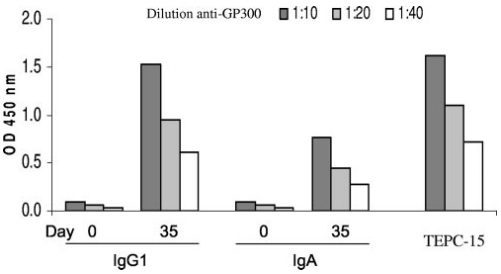

FIG. 5. Cross-reactivity of anti-GP300 with PAF. PAF-coated ELISA plates were incubated with serial dilutions of affinity-purified anti-GP300 derived from noninfected (day 0) and infected (day 35) calves. The PC-specific TEPC-15 antibody served as a positive control. Both IgA and IgG1 isotype reactivities were determined. Data represent the absorbances (mean OD_{405} values for duplicate wells) from one experiment representative of three.

binding of both TEPC-15 and the biotinylated GP300-specific antibodies was inhibited in the presence of 1 mM of free PC but not with 1 to 10 mM of phosphorylethanolamine or phospho-L-serine (not shown). Together, the data indicate PC as the major immunodominant epitope on GP300 in infected calves.

Cross-reactivity of GP300-specific antibodies with PAF. PC-substituted structures from many pathogens are known to exert immunomodulatory effects (10), but mammals themselves also produce PC-containing molecules such as the inflammatory mediator PAF. The finding that lungworm infection elicits PC-specific antibodies led us to investigate whether these antibodies cross-react with PAF and thus perhaps neutralize PAF function. Direct binding of GP300-specific antibodies to PAF was investigated by ELISA with PAF-coated plates. This showed that affinity-purified anti-GP300 derived from sera from infected calves cross-reacted with PAF in a dose-dependent fashion (Fig. 5). The sera contained anti-PAF antibodies of both the IgG1 and IgA isotypes. Importantly, sera from the same calves obtained prior to infection did not contain anti-PAF antibodies (Fig. 5). These results clearly demonstrate that GP300 and PAF share PC epitopes and that lungworm infection elicits cross-reactive antibodies.

Correlation of anti-GP300 with parasite-specific responses. Among many other biological effects, PAF is known to stimulate IgG2 and IgA production and to attract eosinophils. Thus, it is plausible that the generation of GP300-specific antibodies that cross-react with PAF may result in reduced IgG2 and IgA (but not IgG1) responses and a reduced influx of eosinophils in the infected lung. We analyzed the anti-GP300 levels in sera of calves that received either no dose, a low dose, or a high dose of L3 larvae (parasitological data are shown in Table 1). For all three groups of animals, infection resulted in the generation of GP300-specific antibodies of all tested isotypes (IgA, IgG1, and IgG2) (Fig. 6). These antibodies were directed mainly against the glycan moiety, as the reactivity against the protein backbone of GP300 was generally low after primary infection. Even after subtraction of the reactivity against the protein backbone, which (as expected) increased upon challenge of the animals (19), IgG1 was the most reactive isotype against the glycan moiety, and its level increased during the entire duration of infection in all groups. Anti-GP300 IgG1 levels were highest for the group that initially received the highest dose, while this group showed the lowest GP300-specific IgA and IgG2 levels at the end of the experiment (Fig. 6). Similarly, ES-specific IgA, but not IgG1, levels in BALF were lower in the high-dose group at the end of the experiment (ES-specific IgG2 levels in BALF were below the detection limit) (Fig. 7). Comparison of the eosinophil influxes in BALF samples of both groups revealed in addition that the influx of eosinophils, but not of macrophages, in BALF was lower in the high-dose than in the low-dose group of animals (Fig. 7). When both groups were combined, we found a significant negative correlation of GP300-specific IgG1 levels at day of challenge (day 35) with ES-specific IgA levels ($P < 0.01$) and the number of eosinophils ($P < 0.05$) after challenge (day 54). Such correlations were found neither for alveolar macrophage numbers nor for ES-specific IgG1 levels. All these observations are consistent with the hypothesis that anti-GP300 IgG antibodies neutralize PAF activity, resulting in reduced IgG2 and IgA titers and a reduced influx of eosinophils into the lung. A mechanism that down-regulates IgG2 but not IgG1 is of particular interest,

TABLE 1. Parasitological data from infection experiment

Primary dose of L3 larvae (no. of animals)	LPG[a] (mean ± SEM)	Worm count[b] (mean ± SEM)	% Protection[c] (mean ± SEM)
0 (3)	0	507 ± 88	0
30 (8)	1.41 ± 0.73	299 ± 45	41.0 ± 8.9
500 (8)	33.7 ± 13.3	157 ± 38	68.9 ± 7.5

[a] Excretion of larvae per gram of feces (LPG) produced by adult worms from the primary infection. Mean value over all days from day 0 to day 54.
[b] Only young adults, resulting from the challenge infection, were counted.
[c] Percent protection is based on worm counts from infected animals compared to those from the challenge control group.

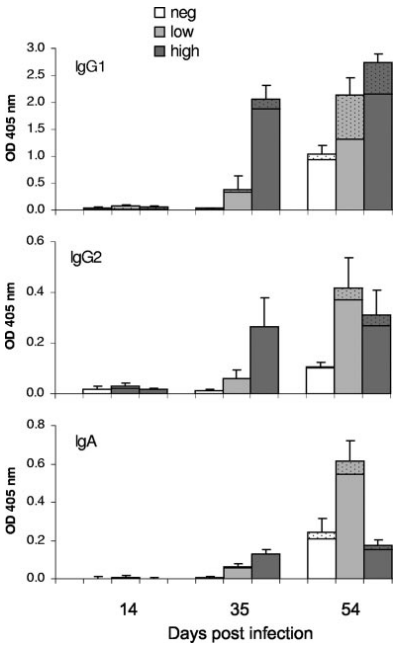

FIG. 6. Isotype-specific recognition of GP300 by sera from lungworm-infected calves. ELISA plates coated with PNGase F-treated (dotted part of the bar) or mock-treated (total bar) purified GP300 were incubated with sera from individual calves collected at days 14, 35, and 54 postinfection and probed for binding of IgG1, IgG2, and IgA. All measurements were performed in duplicate. Results represent means + standard errors of the means (SEM) of the OD_{405} values for each group.

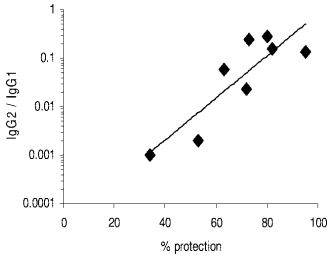

FIG. 8. Correlation between protection against infection and the GP300-specific IgG2/IgG1 ratio at day 54. The ratio of the GP300-specific IgG2 and IgG1 levels was calculated from the data presented in Fig. 6 (mock-treated GP300). The correlation between the ratio and protection ($r^2 = 0.762$) was statistically significant ($P < 0.05$) for the group receiving the high primary infection dose. There was no correlation between the IgG2/IgG1 ratio and protection for the group that received the low primary dose (not shown).

as the IgG2/IgG1 ratio is correlated with protection against infection (Fig. 8).

DISCUSSION

In this study, we purified and characterized the major immunodominant glycoprotein (GP300) of the nematode *D. viviparus*. GP300 binds to the lectin WGA, is substituted with PC, and lacks core α(1,3) fucosylation. Interestingly, the PC group elicits anti-GP300 antibodies in infected calves that cross-react with the PC moiety of the proinflammatory mediator PAF. Neutralization of PAF by anti-GP300 antibodies is proposed as a possible mechanism for the observed down-

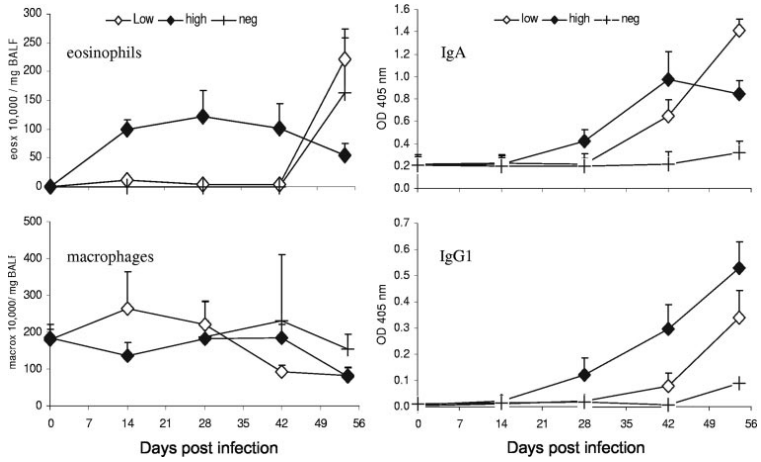

FIG. 7. Analysis of BALF of infected animals. (Left) The numbers of eosinophils (top) and alveolar macrophages (bottom) present in BALF at various times during *D. viviparus* infection were determined. Cell numbers are expressed per mg BALF protein (means + SEM per group). (Right) ES-specific IgA (top) and IgG1 (bottom) levels in BALF (50 μg/ml of protein) collected at the indicated days postinfection were determined by ELISA. Values are the means + SEM per group. neg, negative.

regulation of IgG2 and IgA levels and the reduced influx of eosinophils during lungworm infection.

Western blotting indicated that GP300 was the only WGA-reactive molecule of *D. viviparus*. This allowed the successful purification of GP300 by lectin affinity chromatography. Purification required, besides 1 M of GlcNAc, the presence of 6 M of urea in the WGA elution buffer, possibly due to the high affinity of GP300 for WGA or the formation of larger protein complexes (not shown). The urea requirement during purification may explain why previous attempts to purify glycoproteins of *D. viviparus* with WGA affinity chromatography have not been successful (3). The binding of GP300 to the lectin indicates the involvement of GlcNAc and/or NeuNAc residues. As NeuNAc residues have thus far never been identified on N-linked glycans of nematodes, we expect that the WGA reactivity of GP300 is mediated via GlcNAc residues.

GP300 is also unique in that it was the only glycoprotein of *D. viviparus* that carried PC. This constituent was identified through its reactivity with the PC-specific mAb TEPC-15. Haslam et al. (12) characterized by mass spectrometry N-glycans released from detergent extracts of whole adult *D. viviparus*. Many complex type structures were found, but PC-containing structures were not reported. For other nematodes, PC-linked N-glycans have been demonstrated and found to be all of the complex type with or without core α(1,6) fucosylation and carrying between two and six GlcNAc residues. None of these structures were core α(1,3) fucosylated. For distantly related nematodes like the filaria *Acanthocheilonema viteae* (13), *Trichinella spiralis* (23), and the free-living nematode *Caenorhabditis elegans* (4), PC has been reported to be linked to an *N*-acetylhexosamine, most likely GlcNAc. Because of the reactivity of GP300 with WGA, it seems plausible that in *D. viviparus* PC is also attached to a GlcNAc residue. This suggests that WGA purification may be a good tool to purify PC-substituted N-glycans from other nematodes as well.

Our results clearly indicate that *D. viviparus* infection in calves elicits PC-specific antibodies, and, interestingly, that these antibodies cross-react with the proinflammatory mediator PAF. PAF is a pleiotropic mediator influencing a broad range of cells. In the lungs, it induces vascular permeability, bronchoconstriction, and airway hyperreactivity; patients with asthma, edema, and sepsis show increased levels of PAF (30). In human peripheral blood mononuclear cells, PAF stimulates IgG2 but not IgG1 production, and the effect is neutralized by the PC-specific antibody TEPC-15 (15). In the present study, TEPC-15 served as a positive control in all our experiments and exhibited binding characteristics towards GP300 and PAF similar to those of the bovine GP300-specific antiserum. This suggests that anti-GP300 may also be able to neutralize PAF activity. Indeed, in experimental infections the groups of animals with the highest levels of the putative neutralizing antibody (GP300-specific IgG1) at the day of challenge had the lowest levels of IgG2 (and IgA) after challenge. Furthermore, we found a negative correlation between GP300-specific IgG1 levels at the day of challenge and local parameters such as the influx of eosinophils and ES-specific IgA in BALF after challenge. This was not the case for the influx of alveolar macrophages or ES-specific IgG1 levels in BALF. These observations are consistent with the notion that anti-GP300 may neutralize PAF activity, as both IgA production (14, 25) and eosinophil influx (32) are PAF dependent.

What might be the biological function of the parasite-induced anti-PC antibodies? Our findings indicate that the IgG2/IgG1 ratio (which may reflect the Th1/Th2 ratio) correlates with protection against *D. viviparus* infection (Fig. 8). Furthermore, lungworm disease manifests with clinical signs typically associated with PAF, such as edema, bronchoconstriction, and influx of eosinophils. Thus, it can be imagined that parasite-induced neutralization of PAF limits the development of protection (by limiting the IgG2 response) as well as the development of harmful inflammatory responses (2). The overall effect might be favorable to both the host and the parasite. Such a mutually beneficial effect of inhibition of the PAF response resembles observations with PAF receptor-deficient mice showing enhanced worm survival and decreased inflammation upon infection with the nematode *Strongyloides venezuelensis* (24). Finally, it should be noted that in addition to the development of PAF-reactive antibodies, PC-containing structures such as GP300 may also exert direct immunomodulating effects, as observed for ES-62, a filarial glycoprotein containing PC substituted to N-glycans (11). ES-62 interacts with B and T cells, dendritic cells, and macrophages, skewing the response to a Th2/anti-inflammatory phenotype. Recently, it was demonstrated that most but not all of these effects could be attributed to the PC moiety (8). Neutralization of PAF activity might be another previously unrecognized strategy of parasites to modulate the host response.

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CHAPTER 5

GP300, a thrombospondin-like phosphorylcholine-containing immunodominant glycoprotein of *Dictyocaulus viviparus* and related nematodes

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In Preparation

Summary

GP300 is a high molecular weight glycoprotein of the bovine lungworm *Dictyocaulus viviparus* that carries *N*-linked glycans substituted with phosphoryl-choline (PC). The protein is strongly immunogenic, has immunomodulating properties, and its recognition by IgE antibodies is correlated with protection against infection. Here we identified and characterized the protein backbone of GP300. Mass spectrometry on purified GP300 and DNA sequencing of the corresponding gene indicated that GP300 is a thrombospondin-like protein with 7 thrombospondin domains, 6 kunitz domains and 15 putative *N*-glycosylation sites. The protein was located in the brushborder of the gut, but also in muscles, hypodermis and the lining of the uterus. Related proteins in *H. contortus* and *Cooperia oncophora* were also found to contain *N*-glycans with PC substitution and showed immunological cross-reactive responses. These data suggest the existence in nematodes of a GP300 protein family that is characterized by PC-substituted *N*-linked glycans attached to a thrombospondin-like protein backbone. This finding is of particular interest considering the immunomodulatory function and vaccine potential of members of the GP300 family.

Introduction

Parasitic helminths are well adapted to their host and have evolved smart strategies to modulate the immune system. One class of immunogenic and immune-modulating structures found in nematodes is *N*-linked glycans. These molecules often show core α(1,3) fucosylation (3, 7) or are substituted with phosphoryl-choline (PC) (16, 30). The decoration of *N*-glycans with PC seems exclusive for nematodes and occurs in many, may be even all nematodes. These type of molecules are absent in the host and aid the worms to create their optimal niche (6, 39). To date, the best studied PC containing glycoprotein is ES-62 from the filarial worm *Acantocheilonema viteae*. ES-62 interacts with B and T cells, dendritic cells and macrophages, resulting in an anti-inflammatory response (11, 31). To our knowledge, ES-62 is also the only glycoprotein with a PC substituted *N*-glycan of a parasitic nematode of which the protein backbone has been characterized and cloned. It is an aminopeptidase, produced and excreted in the gut (12, 13).

The nematode *Dictyocaulus viviparus* (superfamily *Trichostrongyloidea*) causes parasitic bronchitis in cattle. The third-stage larvae (L3) of this nematode are taken up orally and the larvae penetrate the intestinal wall. From there they migrate via the mesenteric lymph nodes and the blood circulation to the lungs, penetrate the lung epithelium, and mature to their adult stage in the bronchioles and bronchi. The eggs they produce are coughed up, swallowed, and excreted as first-stage larvae that develop into L3 on the pasture. The clinical symptoms associated with the infection are caused by the larvae, adults worms and/or eggs in the lungs where they induce local influx and activation of eosinophils and mast cells. This may restrict the airways and can result in edema and emphysema and, in severe cases, death of the host.

Infection with *D. viviparus* ultimately generates a protective immune response (18). Short-lived protection can also be achieved by vaccination with irradiated L3

larvae (20) or by transfer of serum from infected animals, indicating that protection is achieved *via* immunoglobulins (19). The antibody responses both in primary infected and vaccinated animals are predominantly directed against *N*-glycans (22). The immunodominant antigen is GP300. This glycoprotein contains *N*-linked carbohydrates that are substituted with PC (23). Antibodies elicited by primary *D. viviparus* infection or by vaccination are mainly directed against the PC molecule. Furthermore, antibodies of the IgG1 and IgA isotype directed against PC-containing *N*-linked glycan of GP300 cross-react with and possibly neutralize platelet-activating factor (PAF) which may limit inflammation and aid the bias towards the Th2 immune response (23). Interestingly, re-infection of the animals boost an IgE (and IgG1) response that is predominantly directed against the protein backbone. This response is correlated with long-lasting immunity (22) and protection (24). The apparent major role of GP300 in both the initial down-regulation of the immune and inflammatory response via its PC substitution and its vaccine potential led us to further characterize GP300.

Here we present mass spectrometry and cDNA sequence results that indicate that the protein backbone of GP300 is a thrombospondin-like molecule. In addition, evidence is provided that homologues of GP300 are present in other trichostrongyloids, that these glycoproteins are also decorated with PC, and that antibodies directed against the PC moiety and the peptide backbone are cross-reactive between nematodes.

Materials & Methods

Parasites

Adult lungworms and L3 larvae of *D. viviparus* were collected from lung washings of infected calves at day 35 of infection as described previously (8, 22). For immunohistochemistry, fresh adult worms were fixed overnight in 4% formaldehyde in phosphate buffered saline (PBS) and then transferred to 70% ethanol. Adult *H. contortus* from abomasum of sheep and adult *Cooperia oncophora* parasites were collected from small intestine of cattle as described (21). *Toxocara canis* and *Taenia taeniaeformis* adults were obtained from a dog and a cat respectively, and generously provided by the department of Pathobiology (Faculty of Veterinary Medicine, Utrecht, The Netherlands). Water-insoluble extract from all parasites were prepared as described previously (23).

Purification of GP300

GP300 was purified from adult worms strored at -80°C using Wheat Germ Agglutinin (WGA) lectin affinity chromatography as described (23).

Deglycosylation of glycoproteins

Deglycosylation of glycoproteins kept in solution was achieved by treatment with peptide-*N*-glycosidase F (PNGase F) as described (23). In some experiments, extracts were separated on SDS-PAGE and transferred onto nitrocellulose prior to deglycosylation to allow direct comparison of protein bands with and without glycosylation. For deglycosylation of blotted glycoproteins the following adjustments to the blotting and above described deglycosylation procedures were made: 1) Blots were not boiled prior to deglycosylation 2) One blot (8x6 cm) was deglycosylated in 20 ml buffer containing 40 µl PNGaseF, 3) Incubation was stopped by extensive washing with Tris-buffered saline containing 0.05% Tween-

20 (TBS-T), pH 7.4, and 4) Blots were quenched with TBS-T containg 0.1% gelatine after deglycosylation. A mock treated blot (in which the PNGase F was omitted from the buffer) was always simultaneously incubated to correct for possible loss of proteins during the deglycosylation step.

Production, affinity purification, and biotinylation of antibodies

The (sheep) anti-rHc-TSP antibody, directed against thrombospondin of *H. contortus* (36), was generously provided by Dr. P. Skuce from Moredun Research Institute, UK. GP300-specific antibodies were affinity-purified from serum of primary infected calves using immobilized GP300 as a matrix, as previously described (23). Western blots and competitive ELISA demonstrated that the GP300-specific antibodies were directed against PC. The PC-specific monclonal antibody TEPC-15 (Sigma) served as positive control for detection of PC containing glycoproteins. Anti-GP300 and TEPC-15 antibodies were biotinylated with EZ-link sulfo-*N*-hydroxysuccinimide-biotin (Pierce) according to the instructions of the manufacturer.

SDS-PAGE and Western blotting

SDS-PAGE and Western blotting were carried out as described (22). Blots were incubated with antibodies or lectins diluted in TBS-T. The following incubation steps were used: 1) Biotinylated-WGA (Pierce, dilution: 1/100,000) followed by streptavidin-HRP (Pierce, dilution: 1/2000), and staining with 3,3'-diaminobenzidine tetrahydrochloride (DAB) in the presence of nickel (38), 2) anti-GP300 (0.5 µg/ml) followed by mouse anti-bovine IgG1 (mca627, Serotec, dilution: 1/1000), 3) TEPC-15 (Sigma, dilution: 1/1000), or 4) Sheep anti-rHc-TSP (dilution: 1/1000), followed by mouse anti-ovine IgG (mca 893, Serotec, diution: 1/500). Probes 2-4 were detected with alkaline phosphatase conjugated goat anti-mouse Ig (DAKO, dilution 1/2000) using BCIP and NBT (Sigma) as a substrate.

Mass spectrometry

For mass spectrometry analysis, WGA-purified GP300 was diluted in SDS-PAGE sample buffer, separated by SDS-PAGE, and stained with Coomassie brilliant blue R250 (0.1% w/v) staining solution. The two protein bands were excised from the gel, rinsed with distilled water, and subjected to in-gel tryptic digestion as described previously (2) with one minor modification, namely that gel pieces were submerged in 50 µl of ammoniumbicarbonate buffer during overnight trypsin digestion (37°C). After digestion, 10 µl of the supernatant was analyzed by nanoflow-LC tandem mass spectrometry using an Agilent 1100 HPLC (Agilent Technologies) coupled to an LTQ ion trap mass spectrometer (Thermo Electron, Bremen, Germany) as described previously (27). For all protein band analysis *.dta files were created using Bioworks 3.1 software (Thermo Electron, Bremen, Germany) which were converted to a single peak list in Mascot generic format using in-house developed software. For protein identification, an in-house Mascot server (35) (Mascot version 2.0 search software; Matrix Science, London, UK). was used to search the MSDB (version 20050701) database (http://csc-fserve.hh.med.ic.ac.uk/msdb.html), allowing a peptide mass tolerance of 0.6 Da, fragment mass tolerance of 0.6 Da, and allowing for 2 missed cleavages. Modifications included in the search were carbamidomethyl at cysteine residues (as a fixed modification), oxidation at methionine at residues (variable) and a +1.0 amu mass change at asparagine residues (variable).

Immunohistochemistry

Adult worms were recovered from the lungs of infected calves and fixed (see parasites). Males and females were separated and embedded in paraffin. After sectioning, paraffin was removed with xylene and the sections (3 µm) incubated (30 min) in methanol and 1% H_2O_2 to inhibit endogenous peroxidase activity and to remove glycolipids that may contain PC (10). Thrombospondin was

demonstrated by co-localization of two probes that recognize different parts of the thrombospondin molecule (23): biotinylated WGA (dilution: 1/1000) recognizing N-glycans, and biotinylated TEPC-15 (dilution: 1/1000) recognizing PC. Binding of the probes was visualized with streptavidin/HRP (dilution: 1/2000) and DAB. Sections incubated with probe-free buffer and streptavidin-HRP served as a negative control.

cDNA synthesis and sequencing

Adult lungworms (stored at -80 °C) were homogenized in liquid nitrogen with a pestle and mortar. Fifty mg of homogenized tissue was used to isolate total RNA with the nucleospin RNA II kit (Macherey-Nagel, Germany). The GeneRacer Kit (Invitrogen) was used to obtain full-length 5' and 3' ends. With this method only full-length capped mRNA is protected from dephosphorylation by calf intestinal phosphatase (CIP). After decapping with tobacco acid pyrophosphatase (TAP) full-length transcripts were being ligated to GeneRacer 5' RNA oligo (5' CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA 3') and reverse transcripted using GeneRacer oligodT primer (5' GCTGTCAACGATACGCTACGTAACGGCATGACAGTG (T)24 3') to obtain first-strand cDNA.

Table 1. *PCR-primers used to amplify D. viviparus GP300.*

Primers		Nucleotide sequence
Degenerate	fwd	5' TT(CT) GCI TG(CT) CCI GA(AG) TGG CA(AG) 3'
	rev	5'(AG)CA ICC ICC (AG)TA CCA (AG)AA (CT)TG 3'
GP300-spec	fwd	5' GGT TGC TCC CTC AAA AAT CTC TT 3'
		5' GTG TGA TGA AGC TAA TAT G 3'
	rev	5' GCA TCT CCA CAT TTT T GTG AAC 3'
		5' GAA CAG CAA CCA TAT TGT 3'
GeneRacer 5'	nested	5' GGA CAC TGA CAT GGA CTG AAG GAG TA 3'
GeneRacer 3'	nested	5' CGC TAC GTA ACG GCA TGA CAG TG 3'

Degenerate sense and antisense PCR primers (see Table 1) based on the sequence of thrombospondin of *H. contortus* were used to amplify by PCR a fragment of *D. viviparus* GP300 cDNA. This fragment showed 85% identity with thrombospondin of *H. contortus*. Based on the DNA sequence (Baseclear, The Netherlands) of a part of the PCR product, novel primers were designed and used in combination with GeneRacer 5' nested and GeneRacer 3' nested primers (see Table 1). This resulted in amplification of the complete GP300 coding sequence. The PCR products were cloned in pCR4-TOPO cloning vector which was transformed into TOP10 *E. coli* (Invitrogen). The plasmid was isolated with the GFX microplasmid Kit (Amersham Biosciences, NJ, USA) and both strands of the insert were sequenced. The sequence of GP300 is available at GenBank (accession number pending).

Computer analysis of the GP300 sequence

Basic Local Alignment Search Tools (BLAST) from NCBI (www.ncbi.nlm.nih.gov) was used to searching for nucleotide and amino acid sequence similarity. DNAstar was used for assembly of the DNA sequences. Signal peptide were predicted with the program signalP 3.0 (4). Alignment of the predicted amino acid sequences was performed with Clustal W (28). Potential *N*-glycosylation sites were predicted with the software available at www.cbs.dtu.dk/services/NetNGlyc. Distinct proteins domains were identified with the Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de).

Results

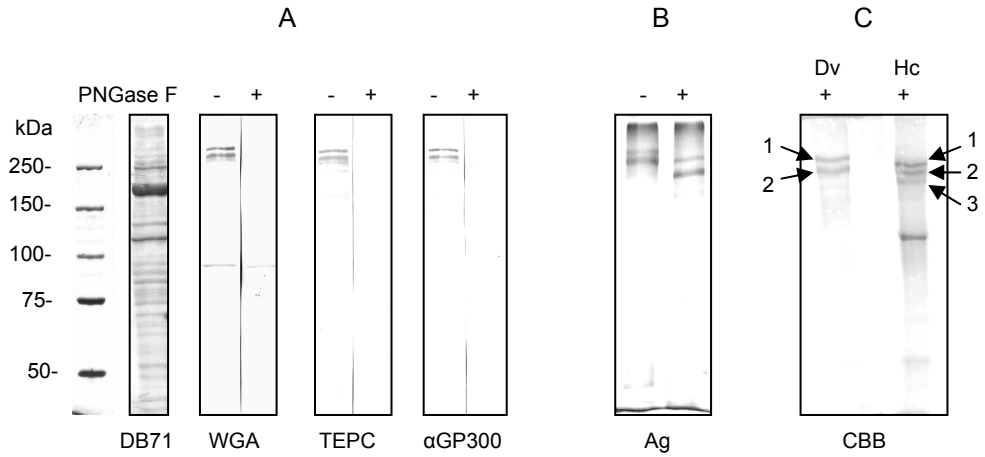

Fig. 1. Purifation of GP300 from water-insoluble extract. *(A) Western blots of water-insoluble extract of D. viviparus were stained with direct blue 71 (DB71) or treated with PNGaseF (+) or buffer only (-) and probed with the lectin WGA, the PC-specific monoclonal antibody TEPC-15, and αGP300 derived from the sera of infected animals. (B) Silverstaining of WGA purified extracts of PNGaseF (+) or buffer (-) treated D. viviparus separated by SDS-PAGE. (C) SDS-PAGE of WGA purified and PNGase F-treated extracts of D. viviparus (Dv) and H. contortus (Hc), stained with CBB. The indicated bands (numbered 1-3) were excised and subjected to mass spectrometry. Molecular weight markers are indicated on the left (all samples were run on 7.5 % SDS-PAGE gels).*

Biochemical characterization of GP300

GP300 was purified from water-insoluble extracts of adult *D. viviparus* via lectin affinity chromotography (23). Western-blotting demonstrated that the purified protein reacted with the lectin wheat germ agglutinin (WGA), the phosphorylcholine-specific antibody TEPC-15, as well as with GP300-specific antibodies affinity purified from sera of infected animals (Fig. 1A). Deglycosylation of GP300 with PNGase F abolished the reactivity with all three probes (Fig. 1A), consistent with the presence of the PC-substituted N-glycan moiety of GP300 (23). Deglycosylation also resulted in increased mobility of GP300 on SDS-PAGE but did not alter its migration as a double band (Fig. 1B).

For mass spectrometry analysis, purified deglycosylated GP300 was separated by SDS-PAGE and excised from the gels (Fig. 1C, left lane). Both bands yielded peptide profiles typical and unique for a protein of the parasitic nematode *Haemonchus contortus*, known as thrombospondin (36). For the highest (band 1) and the lowest molecular weight band (band 2), three and five peptide sequences were obtained that matched this protein (Table 2).

Table 2. *Peptide sequences from D. viviparus GP300 (band 1 and 2) that match H. contortus thrombospondin.*

Band 1	delta	Band 2	delta
EQCETICVEPPGIGR	-0.089	EQCETICVEPPGIGR	0.5308
YWYDYNTK	0.3004	YWYDYNTK	0.3004
WYYNK	0.3883	WYYNK	-0.2417
		CGDAFQYR	0.2146
		YGCCPDGETTALGPR	-0.0095

Table 3. *Peptide sequences from D. viviparus GP300 (band 1 and 2) that match the D. viviparus GP300 sequence.*

Band 1	delta	Band 2	delta
NLSPGYHDVLR	-0.0039	NLSPGYHDVLR	0.0061
IPYCIDTK	0.1878	IPYCIDTK	0.0477
TIAFGPHYSGCDR	0.4362	TIAFGPHYSGCDR	0.3013
EQCETICVEPPGIGR	-0.0893	EQCETICVEPPGIGR	0.5307
YWYDYNTK	0.3004	YWYDYNTK	0.1504
FYYNAYK	0.1188	FYYNAYK	0.0888
WYYNK	0.3883	WYYNK	-0.2417
FVYGGCLGNTNR	0.3682	FVYGGCLGNTNR	0.5882
LVWYYDTSEGR	0.0619	TGQSESGPWGPWVPEK	0.0020
HEYYYFNSNTGVEK	0.0086	YVSCNLDPCAEGTDF	0.0851
		CELLCKPEGGK	0.1719
		LGSALKVDK	0.4182
		TIEGTFDER	0.0996
		IPSGATSIK	0.2260
		IEEARPSSNNLALK	-0.0981
		CGDAFQYR	0.2146
		FYTSEWK	0.2739
		CLTDNEKEIPPDTR	0.2544
		YGCCPDGETTALGPR	-0.0096
		QRPQMPTIEEVCR	-0.1992
		FSSREECEVTCVR	0.5839
		ETGPCTNFVTK	0.0469
		FATLDECQAR	0.0121

Amplification and nucleotide sequence analysis of GP300

Sense and antisense degenerated primers were deduced from the nucleotide sequence of *H. contortus* thrombospondin and used to amplify a 1.2 kb cDNA fragment of *D. viviparus*. cDNA was obtained by reverse transcription of mRNA isolated from adult worms. Sequencing of the obtained PCR product revealed 85% identity (90% similarity) of this fragment with thrombospondin of *H. contortus.* From this sequence, GP300-specific PCR primers were designed and used in combination with GeneRacer nested primers to amplify the 5' and 3' end of GP300 with cDNA as template. Deduced sequences from overlapping PCR fragments displayed a single open reading frame with a start and a stop codon, coding for 1540 amino acids. Comparison of the observed peptides profiles obtained for band 1 and band 2 of the WGA purified *D. viviparus* extracts with the deduced amino acid sequence from *D. viviparus* GP300, yielded 10 and 23 peptides matches, respectively (Table 3).

Computer-assisted analysis of the complete deduced amino acid sequence of GP300 using BlastP revealed 78% overall identity (86% similarity) with thrombospondin of *H. contortus* (AF043121) (36). The sequence similarity extended over the full length of both sequences. GP300 also showed homology with proteins in other nematodes including the papilin of *C. elegans* (NM_072616, 66% identity and 78% similarity) and with insect *D. melanogaster* papilin (AF205357)(25) and the papilin homologue of Manduca sexta, called lacunin (33). The similarity with the latter proteins was lower due to the presence of a large Ser/Thr rich region. All these proteins are considered multidomain, extracellular matrix (ECM) proteins. An alignment of the similarities of GP300 with proteins in other nematodes is shown in figure 2.

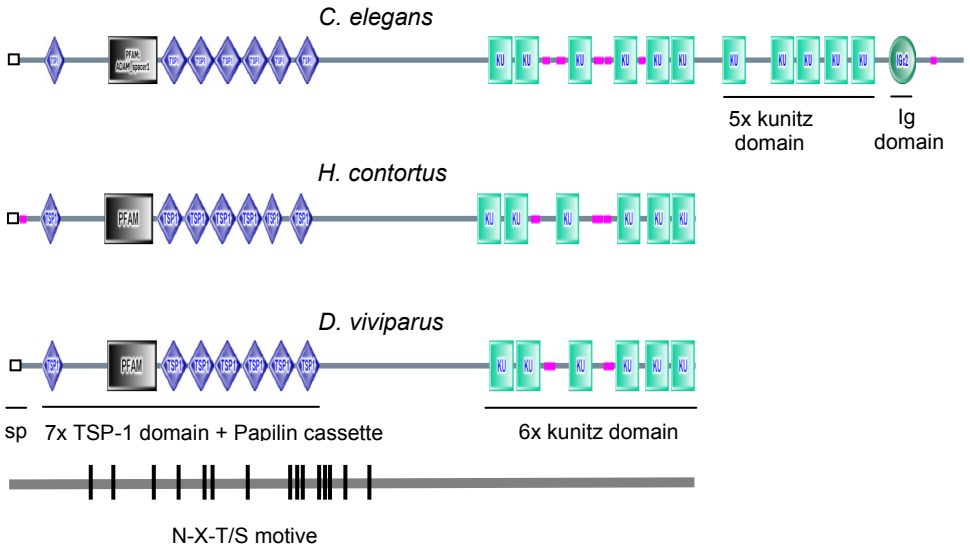

Fig. 3. Structural domains of GP300 homologues of *C. elegans* (NM_072616), *H. contortus* (AF043121) and *D. viviparus*. *In all nematodes, the protein carries a signal peptide (sp, open square) followed by seven TSP domains (diamonds). A papilin cassette (black square) is located between the first and second TSP domain. The C-termini contain a variable number of kunitz domains (green rectangles), 6 in D. viviparus and H. contortus and 11 in C. elegans, interspaced by regions of low complexity (small pink rectangles). C. elegans posseses at its C-terminal an immunoglobulin (Ig) domain. The 15 putative N-glycosylation sites with the N-X-S/T motif, are indicated for D. viviparus with vertical bars.*

Detailed protein sequence analysis of GP300 (Fig. 3) indicated the presence of a signal peptide of 19 amino acids at the N-terminus, suggesting that the protein enters the secretory pathway. Further analysis showed that the aminoterminal segment of the GP300 contains 7 thrombospondin domains (TSP1) which in other organisms are known to play a role in cell-cell interaction. Between the first and second TSP1 domain there is a cys-rich region, which together with the adjacent TSP domains forms the “papilin cassette” (9). All 15 potential N-glycosylation

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Fig. 2. Alignment of amino acid sequences of GP300 homologues from nematodes (left page). *D. viviparus (Dv) GP300, H. contortus (Hc), accession number AF043121 and C. elegans (Ce), accession number NM_072616, truncated at amino acid 1558.*

sites based on the presence of the N-X-S/T motif are located on the aminoterminal half of the protein. The carboxyterminal part of the GP300 consists of six closely packed kunitz-type serine protease inhibitor domains.

Localization and heterogeneity of GP300 in D. viviparus

As thrombospondin-like proteins in other nematodes are considered ECM proteins and the use of GP300 as a putative candidate vaccine antigen requires surface exposure of the antigen, immunohistochemistry was applied to localize the protein in adult *D. viviparus*. As no other adult *D. viviparus* proteins contain PC or react

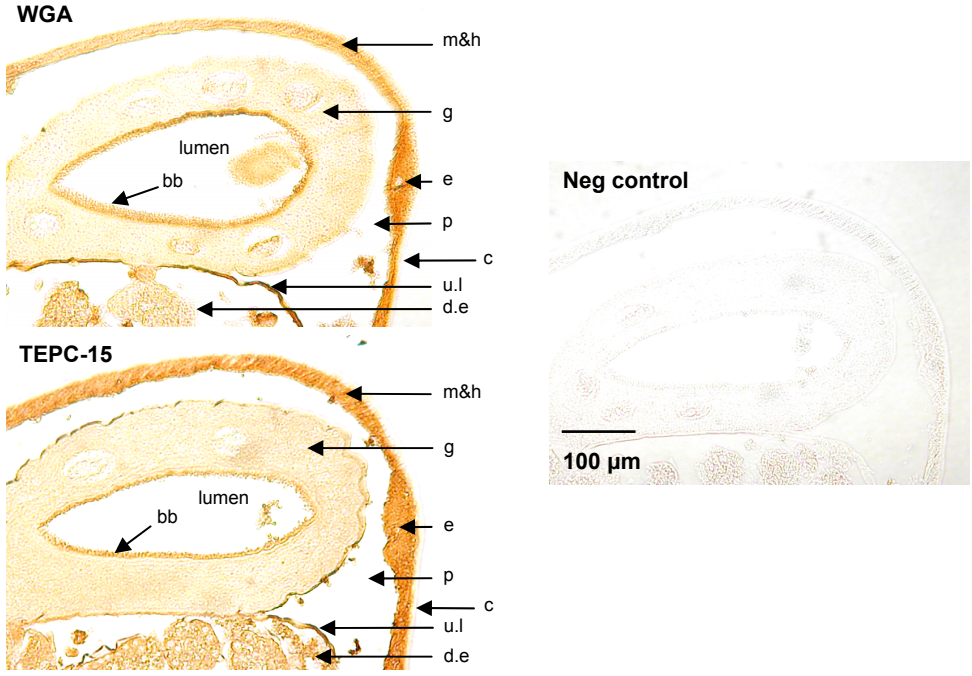


Fig 4. Localization of GP300 in adult female *D. viviparus*. *De-paraffinated mid-sections of an adult female worm were incubated with buffer (negative control), the lectin WGA, or the PC-specific antibody TEPC-15. (bb) brushborder of the gut, (c) cuticle, (d.e) developing eggs, (e) excretory canal, (g) gut, (m&h) muscle & hypodermis, (p) pseudocoel, (u.l) uterus lining.*

with WGA (23), the PC-specific antibody TEPC-15 and the lectin were used for this purpose. Immunochemistry on cross-sections demonstrated reactivity for both probes with the hypodermis, muscle, lining of the uterus, and the brushborder of the gut, but not in the gut itself (Fig. 4). The staining by the lectin and TEPC-15 was blocked in the presence of 1 M of GlcNAc and 10 mM of PC, respectively (not shown).

Western blots with water-insoluble extracts of individual male worms and the antibody TEPC-15 as a probe yielded no differences in GP300 reactivity between worms (Fig. 5A). Similar results were for female worms (not shown).

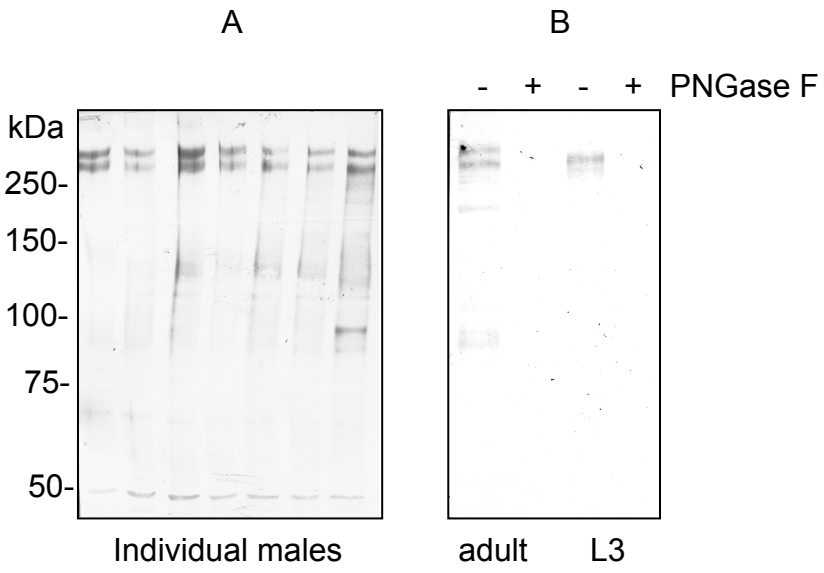


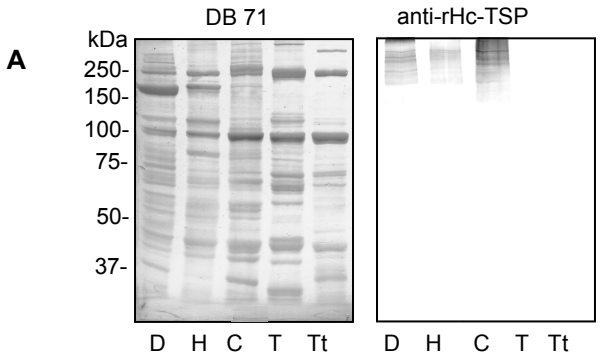
Fig 5. Expression of GP300 in male adult worms and in L3 larvae of *D. viviparus*. *(A) Western blot of SDS-PAGE-separated water-insoluble extracts of seven individual male worms probed with the PC-specific antibody TEPC-15. GP300 stained as doublet in all males. (B) Western blot of water-insoluble extracts of pooled adult worms and L3 larvae were treated with PNGase F (+) or buffer (-) and probed with TEPC-15. Note that the L3 larvae expressed only a single reactive GP300 band. Molecular weight markers are indicated in kilodaltons (kDa).*

These results indicate that GP300 is present in all adult worms and consistently migrates as a double band on SDS-PAGE. In contrast, L3 extracts probed with anti-GP300 showed only one reactive band that was sensitive to deglycosylation (Fig. 5B). These results suggest that the appearance of GP300 in SDS-PAGE as a single or double band may vary with the stage of worm development.

Isolation of the GP300 homologue of H. contortus

The sequence similarity of GP300 with thrombospondin of *H. contortus* led us to investigate whether the unique PC-substitution of the N-linked glycans of GP300 was perhaps also present on the closely related homologues in other nematodes. Hereto, we determined the reactivity of water-insoluble extracts of five different helminths (3 trichostrongyloids, 1 ascarid and 1 cestode) with WGA, anti-rHc-TSP and TPEC-15. As shown in Fig. 6, all tested trichostrongyloids (*D. viviparus, H. contortus* and *C. oncophora*) carried multiple reactive high molecular thrombospondin-like proteins with attached N-linked glycans that react with WGA and that are substituted with PC. Deglycosylation of the blots with PNGase F resulted in loss of reactivity with WGA and TEPC-15 (Fig. 6B and C), indicating the absence of core α(1,3) fucosylation. The extract derived from the ascarid *T. canis* did not react with anti-rHc-TSP (Fig. 6A), but did contain a high molecular weight glycoprotein that was recognized by WGA and TEPC-15 (Fig. 6B, C).

Fig. 6. Presence of GP300 homologues in other nematodes (right page). *Western blots of SDS-PAGE separated water-insoluble extracts of D. viviparus (D), H. contortus (H), C. oncophora (C), T. canis (T) and T. taeniaeformis (Tt) were:* ***(A)*** *stained for total protein with Direct Blue 71 (DB71) or probed with antibody directed against recombinant H. contortus thrombospondin (anti-rHc-TSP),* ***(B)*** *treated with PNGaseF of buffer (mock) and probed with TEPC-15, and (**C**) treated with PNGase F or buffer (mock) and probed with the lectin WGA. A blot of H. contortus extract incubated with the conjugate streptavidin-HRP served as negative control (neg). Molecular weight markers are indicated in kilodaltonds (kDa).*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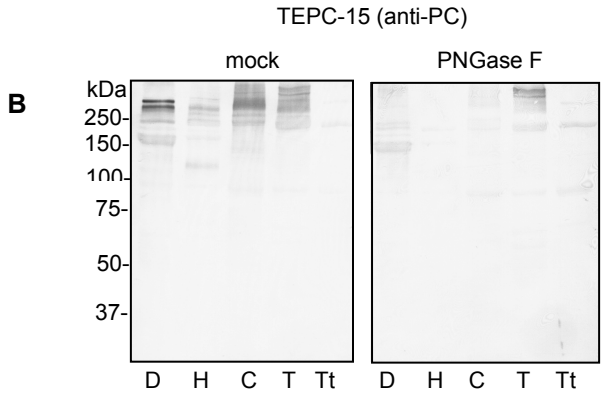
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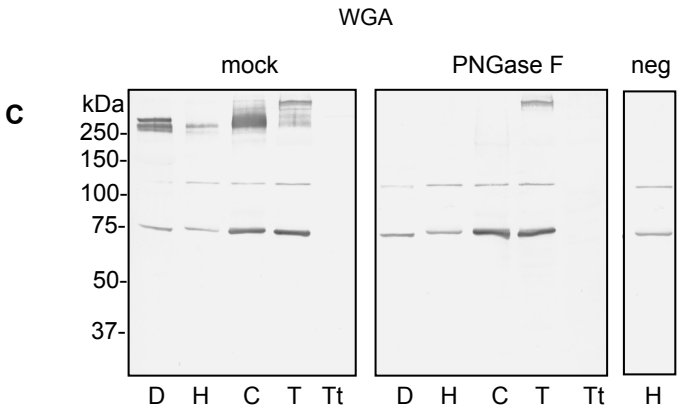
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50-
37-
D H C T Tt
D H C T Tt
H

Deglycosylation of this material resulted in loss of a single, but not all WGA and anti-PC positive bands. The extract of the cestode *T. taeniaeformis* did react with neither anti-rHc-TSP, WGA nor TEPC-15 (Fig. 6).

To ensure that the identified PC-containing N-glycan structures were attached to thrombospondin-like proteins, we purified the protein from *H. contortus* using WGA affinity chromatography. After deglycosylation with PNGaseF, the protein migrated on SDS-PAGE as three separate high molecular bands (Fig. 1C, right). Mass spectroscopy revealed 23, 18, and 19 peptide sequences obtained for band 1, 2 and 3 that fully matched *H. contortus* thrombospondin. These data confirm that the purified glycoprotein was thrombospondin, and thus that nematodes other than *D. viviparus* also decorate their GP300 homologue with PC-containing glycans.

Discussion

The glycoprotein GP300 of *D. viviparus* carries *N*-linked glycans substituted with PC, has immune-modulating activity, and elicits a powerful immune response in infected calves. Antibodies directed against the PC moiety of GP300 cross-react with and possibly neutralize platelet-activating factor (PAF) (23), while GP300-specific IgE antibody titers correlate with protection against infection (24). In the present study, we identified the protein backbone of GP300 as a thrombospondin-like molecule and provide evidence that other nematode species carry related PC-containing glycoproteins. The observed cross-reactivity of antibodies and lectins directed against the protein backbone, glycan moiety as well as the PC group is particularly important considering the potential of GP300 as an immune modulatory and protection-inducing antigen in *D. viviparus*.

The evidence that GP300 represents a thrombospondin-like molecule is based on the results of mass spectrometry on purified, deglycosylated protein and confirmed by the discovery and nucleotide sequencing of the corresponding gene.

The thrombospondin-like nature of GP300 was further underpinned by the immunological reactivity of the protein with antibodies directed against recombinant thrombospondin of the nematode *H. contortus*. This protein also served as a valuable tool to amplify and sequence the cDNA encoding *D. viviparus* GP300. Sequence analysis predicts that GP300 consists of 1540 amino acids. The presence of a putative signal peptide, an array of TSP-1 domains, and the apparent absence of a transmembrane domain fit the hypothesis that GP300 is an ECM protein. This is in agreement with the finding that the protein is found in the water-insoluble extract of *D. viviparus* and localizes in muscles, hypodermis, lining of uterus and brushborder of the gut, but not in the gut itself (in nematodes the cells from the gut forms a syncytium, without ECM) (Fig. 4). The possible function of GP300 as an extracellular matrix protein may seem at variance with its presence in ES products of *D. viviparus* (23). However, it has been demonstrated that antibodies bound to the surface of adult *D. viviparus* can be shed into the environment via an active metabolism-dependent mechanism (32). Alternatively, it can be imagined that turnover of ECM proteins or active secretion of the protein results in the release of protein, reminiscent of the release of ECM proteins by mammalian cells.

Analysis of the N-terminal region of the GP300 protein sequence revealed seven TSP-1 domains (40) These domains have been identified in many different proteins of protozoa and animals (1) and play a role in cell-cell interactions and the binding of matrix glycoproteins and glycosaminoglycans. Between the first and second TSP-1 domain there is a C-rich region. This region forms together with the adjacent TSP domains the “papilin cassette”, a sequence homologous to the non-catalytic C-terminal part of ADAMTS metalloproteinase. Papilin from *Drosophila* as well as the papilin cassette itself inhibit procollagen N-proteinase, an ADAMST metalloproteinase (25). This protease inhibition has been suggested to play a role in the regulation of embryonic development. In embryo’s of the tobacco hornworm

(*Manduca sexta*) papilin appears to play an important role in the remodeling of the basal laminae (33). The function of GP300 in nematode development has not been investigated.

At its carboxy-terminus GP300 carries six closely packed kunitz domains. These domains are assumed to confer serine protease inhibitor activity (29). Serine proteases are widely present in the host and have many functions. For example, the release by granulocytes and the role of the serine proteases thrombin in blood clotting may be relevant in the defence against infection and may limit blood and tissue feeding by parasites. For *D. viviparus* serine protease inhibitors have thus far not been identified. Recently, a multidomain kunitz-type inhibitor has been found in the hookworm *Ancylostoma caninum* (15).

As expected, the amino acid sequence of GP300 was predicted to carry a large number (15) of potential N-glycosylation sites. Most sites located within the thrombospondin domains. The large number of putative glycosylation sites is in agreement with the size difference of GP300 before and after deglycosylation with PNGase F (~ 70 kDa). As more glycans can be attached to one single thrombospondin protein and each *N*-glycan may contain more than one PC molecules (14), the thrombospondin molecule can be loaded with a considerable amount of PC. A function of PC can be, apart from its immunomodulatory role (23), the inhibition of attachment of the parasite to host cells, not unlike the inhibition of cell attachment and thrombus formation on PC coated surgical implants (41).

The protein sequence of GP300 provided no basis for its migration in SDS-PAGE as multiple bands, even after deglycosylation. Mass spectrometry on each of the bands yielded similar peptides, indicating that both bands represent thrombospondin-like proteins. Furthermore, no difference in protein mobility was found between female and male worms. In contrast, L3 larvae showed only one size of GP300 protein, suggesting that the composition of the protein may vary

during the life cycle. For papilin of *Drosophila*, splice variants have been demonstrated and linked to the number of kunitz domains at the C-terminus. (26). Thus, it can be imagined that *D. viviparus* produces different splice variants of GP300 dependent on the developmental stage.

Several lines of evidence indicate that GP300 is a conserved member of a glycoprotein family of nematodes typified by a PC-containing glycan moiety that lacks core α(1,3) fucosylation, attached to a thrombospondin-like protein backbone. Thus far, PC has not been identified as a constituent of N-glycans on thrombospondin-like proteins in nematodes. Yet, our Western blotting results unequivocally demonstrate that besides *D. viviparus*, also *H. contortus* and *C. oncophora* contain a WGA reactive and PC substituted glycans attached to a thrombospondin-like core protein (Fig. 6). Recently, characterization of an acetylglucosaminyltransferase *C. elegans* knockout also revealed a PC group linked to the terminal GlcNAc residue (17). Furthermore, in *C. elegans*, a potential homologue of GP300 has been described that terminates behind the 6th kunitz domain and yields a protein similar in size to GP300 *D. viviparus* and thrombospondin of *H. contortus.* Whether this protein serves as a backbone for the identified PC containing-glycan is unknown.

Our finding that all tested trichostrongyloids GP300 homologues display reactivity with WGA as well as PC is consistent with the notion that PC substitution to GlcNAC residues and α(1,3) fucosylation are mutually exclusive due to the constraints of the involved enzymes. Interestingly, the ascarid worm *T. canis* contained proteins that were tested positive with TEPC-15 and WGA, but not with anti-rHc-TSP and a high molecular weight protein was resistant to PNGase F treatment, indicating decoration with O-glycans or N-glycans with core α(1,3) fucosylation. O-linked glycans carrying PC are expected to exist in filarial worms (34), which belong to the same clade III as the ascarids (5).

The identification of GP300 as a thrombospondin-like molecule opens new perspectives to test this protein as a candidate vaccine antigen. In *H. contortus* thrombospondin is part of a galactose-containing glycoprotein complex (H-gal-GP) (36) that governs partial protection against infection (37). It has been argued that thrombospondin is most likely not the most protective component because of its limited immunoreactivity (36). However, this immunoreactivity was determined with anti-rHC-TSP, which does not recognize the immunodominant PC epitope. Our results in *D. viviparus* infected calves indicate that PC on thrombospondin-like protein is an immunodominant epitope and that (IgE) antibodies directed against GP300 correlate with protection against infection (24). The present purification of GP300 and the cloning of its protein backbone provide the opportunity to test the vaccine potential of GP300 to protect against infection by *D. viviparus* and, possibly, related nematodes.

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CHAPTER 6

Summarizing discussion

1. Introduction

D. viviparus is the etiological agent of parasitic bronchitis in cattle. The most efficient way to prevent disease is by vaccination. To date, only one vaccine is commercially available in a number of countries. The vaccine consists of irradiated L3 larvae isolated from infected cattle. This production method has serious disadvantages. Apart from concerns about animal welfare, the use of the L3 larvae from infected animals is troublesome as the vaccine has an undefined composition, may be contaminated with non-larval molecules, and can be stored for only limited time. Another major disadvantage is that the available vaccine provides only short-lived protection. A natural boost with *D. viviparus* is required to obtain long-lasting immunity.

Modern vaccine design requires knowledge of the factual protection-inducing antigens and suitable adjuvants. In the present study we aimed to identify candidate vaccine antigens for *D. viviparus*. As the genome sequence of *D. viviparus* is still unknown, we followed the classical vaccine design approach guided by the knowledge that passive immunisation can harness protection against infection (9). As described in this thesis, systematic analysis of the natural immune response and the antigens recognized, led to the discovery that a glycoprotein present in ES and L3 extracts of the nematode play a major role in the pathobiology of *D. viviparus* infection and in the development of protection. We have been able to separate the immune response directed against the carbohydrate and peptide backbone of the glycoprotein recognized by immunoglobulin isotypes involved in protection and to characterize the candidate vaccine antigen. Furthermore, based on the nature of the decoration of the carbohydrate moiety with phosphorylcholine, it is proposed that the glycoprotein has a strong immunomodulatory activity that may add to the successful parasitism of nematodes.

2. Relationship between levels of ES-specific immunoglobulin isotypes and protection against *D. viviparus* infection

The first step in the identification of novel candidate vaccine antigens of *D. viviparus* was the dissection of the protective antibody response during experimental infection (Chapter 2). As the protective response may well be related to specific immunoglobulin isotypes and analysis of the overall immunoglobulin (Ig) response may mask correlations between distinct isotypes and protection, we investigated the reactivity of Ig isotypes with isolated ES and L3 extracts. ES was chosen as antigen as immunisation of Guinea pigs with ES protected against challenge infected with *D. viviparus* (13).

2.1 Correlation between bovine IgE levels and protection against infection

Antibodies of the IgE isotype are important in the host response against helminth infections in several species. As no tools were available to measure bovine IgE levels, we first developed monoclonal and polyclonal antibodies against bovine IgE. These antibodies recognized bovine IgE and displayed no cross-reactivity with other Ig isotypes (Chapter 2). With these novel tools we determined the IgE response during *D. viviparus* infection. In two independent infection experiments, total IgE levels clearly positively correlated with protection against infection (Chapter 2). Although parasite specific IgE levels could not be measured in the sera of individual animals most likely because of competition with other more abundant isotypes, assays with affinity purified IgE derived from pooled sera indicated that the best protected calves had much higher levels of parasite specific IgE than the least protected animals from the same experimental group.

2.2 Bovine IgA, IgG1 and IgG2 responses against ES of D. viviparus

During infection with *D. viviparus,* cattle also developed other Ig (non-IgE) isotype specific responses against ES of adult worms. In a previous study, *D. viviparus* infection increased parasite specific IgA, IgG1, IgG2 and IgM levels (IgE was not measured) both in both serum and bronchoalveolar lavage fluid (BALF), but no positive correlation of this humoral response with protection (larval excretion) was found (19). In our hands, parasite specific IgG1 levels on Day 42 and parasite specific IgG2 on Day 70 correlated with protection, suggesting that besides IgE also IgG may contribute to protection.

3. Antigen specificity of the immune response against ES and L3 extracts of *D. viviparus*

The results described above were obtained for the total complex mixture of ES antigens rather than for specific antigens. In the experiments described in Chapter 3, we used both ES and L3 extracts as well as antigen profiling methods to further dissect the immune response. Antibody responses against (deglycosylated) ES and L3 extracts were measured in sera from calves that were infected and challenged with a 73 day interval in stead of the previously used 35 days to allow solid differentiation of primary and secondary Ig responses. Furthermore, as infection but not vaccination of calves results in long-lasting protection, we compared antibody responses in infected and vaccinated calves.

The Ig response after primary infection and vaccination displayed grossly similar patterns with respect to the antibody titers to ES and L3 extracts. Antigen profiling indicated a series of immune reactive proteins. As antibodies may be directed against protein as well as carbohydrate epitopes, we particularly focused on the glycoproteins bearing in mind that the carbohydrate moiety of glycoproteins is often immunogenic and /or has immunomodulatory activity. PNGase F treatment of *D. viviparus* revealed the presence of N-linked glycoproteins in both ES and L3

larval extracts (Chapter 3). Deglycosylation of antigens resulted in substantially reduced IgA, IgG1, IgG2 and IgE immunoreactivity against the ES and L3 larval extracts in the sera of both primary infected and vaccinated animals. This for the first time indicated that part of the immune response against *D. viviparus* is directed largely against N-linked glycans.

After challenge infection, the antibody responses of the infected calves differed from those of the vaccinated ones. The infected calves showed a booster response for the IgG1 and IgE isotypes. Interestingly, this booster response was mainly directed against the deglycosylated ES and L3 proteins (Chapter 3). The booster effect was virtually absent in the previously vaccinated animals. This may be explained by the fact that the vaccinated animals were at day of slaughter free of worms (10), whereas in the infected group a mean of 13 worms per calf were found (7). The lack of protection after challenge of vaccinated animals may thus have resulted from the lack of exposure to adult worm antigens. This difference in antigen exposure may be quantitative rather than qualitative as an adult worm is about 100 times longer than a L3 larvae, making that its biomass is about a million times that of a L3. The fact that there was also a booster response in infected calves against L3 antigens also points in that direction.

4. Selection of vaccine candidates

Western blots loaded with ES or L3 extract and probed with serum IgG1 derived from boostered infected animals indicated several immunodominant glycoproteins. The proteins in ES had molecular masses of 42, 67 and >300 kDa in ES, while in larval extracts and 30, 67 and > 93 kDa glycoproteins were recognized (Chapter 3, Figs. 1 and 2). The 42 kDa ES protein likely resembles the protein described by Britton *et al.* (3) as the only glycoprotein in ES of *Dictyocaulus*. The 67 kDa ES protein is likely the acetylcholinesterase (AChE) previously described to elicit a strong immune response in infected, but not in vaccinated animals (12).

Immunizations with an AChE-enriched fraction has been demonstrated to gave partial protection against *D. viviparus* infection in guinea pigs and thus may have vaccine potential (14). The most predominant immunoreactive glycoprotein recognized by IgG1 from sera from vaccinated and infected calves was a high molecular weight protein later designated as GP300. Interestingly, PNGase F treatment of the antigen indicated that the IgG1 booster response was also directed against the protein backbone of GP300 (Chapter 4).

Analysis of the specificity of the IgE antibodies after boostering of the infected animals indicated that also IgE antibodies recognized the high molecular weight ES protein (GP300) as the primary antigen. The protein was present in different developmental stages of *D. viviparus*. In L1 and crude worm extract of adults, it appeared virtually the only protein that was recognized by IgE, despite its low abundance. Because of its apparent conservation during the different growth stages, its immunodominance, and its recognition by IgE antibodies that positively correlated with protection against *D. viviparus* infection, we considered GP300 as a prime candidate vaccine antigen.

5. Characterization of the candidate vaccine antigen GP300

5.1 Analysis of the GP300 protein backbone

The GP300 protein was successfully purified by WGA lectin affinity chromatography (Chapter 4). PNGase F treatment and Western blotting confirmed that the purified protein was the immunodomaint antigen recognized by IgE and IgG1 of infected animals. Mass spectrometry revealed high similarity of GP300 with a protein of the sheep parasite *Haemonchus contortus*, designated as thrombospondin (20, Chapter 5). Sequencing of the *D. viviparus* cDNA encoding GP300 indicated 78% similarity at the amino acid level with thrombospondin of *H. contortus*. The protein contained a number of thrombospondin and kunitz domains,

the function of which still remain to be defined. Histoimmunochemistry using GP300 specific probes indicated that in *D. viviparus* thrombospondin is localized in the brushborder of the gut, but also in the hypodermis and the lining of the uterus (Figure 4, Chapter 5). *D. viviparus* thrombospondin was also found in ES, most likely derived from turnover of the hypdermis or brushborder. In all cases, this localization suggests that there may be direct contact between the thrombospondin of the parasite and the host.

The similarity between thrombospondin of the different trichostrongyloids species, was further confirmed by the reactivity of antibodies directed against recombinant thrombospondin of *H. contortus*. The cross-reactivity of these antibodies with similarly sized proteins of *D. viviparus* and *Cooperia oncophora* indicates that the thrombospondin-like GP300 protein is conserved among trichostrongyloids and thus may fulfil an important biological function. In *H. contortus* thrombospondin is present in a lectin (peanut agglutinin) purified experimental vaccine (H-gal-GP) derived from the membrane of the gut of the adult worm and this vaccine is partial protective (21).

5.2 Characterization of the N-glycan moiety

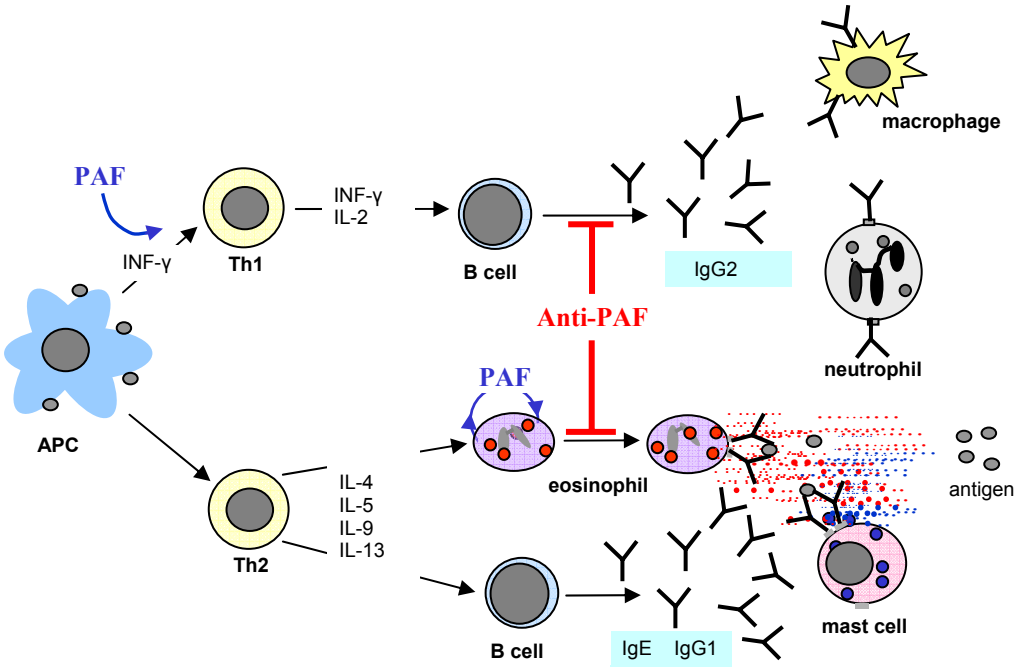
Because the glycans were the immunogenic part of the glycoprotein in primary infected and vaccinated animals, we characterized the N-glycan moiety of GP300 in more detail (Chapters 3 and 4). The successful one-step purification of GP300 by WGA lectin affinity chromatography indicated the presence of N-acetylglucosamine (GlcNAc) at the antenna. Furthermore, phosphorylcholine (PC) was identified as a component. The complete removal of N-glycans of the protein by PNGase F indicated that absence of a core α(1,3) fucose (chapter 3). This seems remarkable as α(1,3) fucose is a common and very immunodominant and allergenic epitope in other nematodes (4, 6, 18). However, as nematode core α(1,3) fucosyltransferase cannot use N-glycans with a GlcNAc at the α1,3-antenna as

substrate (16) and a terminal GlcNAc at the α1,3-antenna is a prerequisite for substitution with PC (8), it seems that the substitution with PC and the process of α(1,3) fucosylation are mutually exclusive. At this point it should be noted that in *Toxocara canis* part of the glycans that carry PC cannot be cleaved by PNGase F. The most likely explanation for this phenomenon is that PC is substituted onto O-glycans as have been demonstrated for filarial worms (15) which belong to the same clade III as *Toxocara canis* (2).

5.3 Role of phosphorylcholine in D. viviparus infection

The presence of PC on GP300 was of particular interest because of is immunogenic and immunomodulating properties (11). The reactivity of a PC-specific monoclonal antibody with GP300 of *D. viviparus* led us to hypothesize that N-glycan-directed antibody of *D. viviparus* infected animals may cross-react with PC-containing molecules in the host. One such a molecule is the pro-inflammatory mediator platelet-activating factor (PAF). Indeed, we were able to demonstrate that GP300-specific antibodies purified from infected animals bind PAF. Furthermore, we discovered a negative correlation between the level of PC-specific antibodies and known PAF-mediated effects such as IgG2 and IgA responses in serum and eosinophilia in the BALF. Based on these results it can be hypothesized that antibodies directed against the PC-moiety of GP300 inhibit PAF function and thus may limit PAF-mediated eosinophilia and inflammation in infected animals. On the other hand, the reduction of the IgG2 (and IgA) responses due to the presence of anti-PC antibodies may limit protection against infection as we found that te IgG2/IgG1 ratio was correlated with protection against *D. viviparus*. Thus, anti-PC is likely not protective for the host in terms of worm counts, but may be protects the host in terms of inflammation and immunity-induced pathology. Interestingly, this scenario of limiting inflamation and infection pathology by neutralizing PAF activity resembles the function of the acute phase protein, C-reactive protein

(CRP). This protein also binds to PAF and prevents PAF-induced death in mice via a PC-dependent mechanism (1). In cattle, CRP is not an acute phase protein (5, 17), which makes putative neutralisation of PAF activity by anti-PC antibodies even more relevant.



Model of the effect of D. viviparus-induced PAF-neutralizing antibodies on the immune response and immunopathology. PAF induces attraction and activation of eosinophils and stimulates the production of IgG2. Neutralization of PAF by anti-PAF antibodies will therefore result in decreased eosinophilia and decreased IgG2 levels.

6. Concluding remarks and perspectives

The results presented in this thesis may contribute to the development of a novel vaccine against *D. viviparus* that might replace the current vaccine which consists of irradiated larvae, provides only short-lived protection and, barely acceptable nowadays, requires infection of cattle for its production. Our data indicate that, during natural infection, the development of antibodies of the IgE and IgG1

immunoglobulin classes directed against the protein backbone of glycoproteins, correlate with a long-lasting protective immune response. One antigen that is recognized is the 67 kDa putative AChE protein, which induces protection in *D. viviparus* infected Guinea pigs (14). Another identified prime candidate vaccine antigen is GP300, the thrombospondin-like protein of *D. viviparus*. Clearly, the protective effect of this glycoprotein needs to be evaluated in a vaccination trial. For an initial trial the use of purified GP300 from *D. viviparus* is an option as 10 g of adult worms will yield ~100 μg of GP300 and the effect of the PC moiety can be demonstrated by vaccination with glycosylated and deglycosylated antigen. Challenge infection will demonstrate whether there is (IgE mediated) protection or not. After challenge infection the inflammation parameters (eosinophils, IgG2, respiration frequency) and acute phase proteins (5) are expected to be downregulated in the animals vaccinated with the intact GP300. In this study, special attention is required for monitoring mast cell activity as GP300 may act as an allergen causing asthma-like symptoms. Measuring IgE bound to mast cells may require a novel assay as the novel IgE assay used in the work described in this thesis relies on a monoclonal antibody that is produced against the third and fourth constant domain of the heavy chain of IgE (C3 and C4 of ε-chain), the same part that also binds to the FcεRI. Alternatively, it can be considered to test the vaccine potential of GP300 in other trichostrongyloid animal model systems such as *Nippostrongylus brasiliensis* infection in rats. Our results suggest that, in addition to GP300 and the 67 kDa AChe protein, *D. viviparus* may express other candidate vaccine antigens. Additional experiments are needed to identify the nature of these deglycosylated proteins that were recognized by IgG1 (or IgE) after re-infection (Chapter 3). This can likely simply be achieved with the strategy successfully applied in this thesis for GP300.

The striking finding that *D. viviparus* elicits antibodies that cross-react and likely neutralize the inflammatory and immunomodulatory mediator PAF adds a

new dimension to the basic understanding of how this parasite and, perhaps nematodes in general, establish true parasitism. The strategy to take advantage of the host immune response to elicit antibodies that bind PAF and thus limit the inflammatory response as well as the production of harmful antibodies may contribute to prolonged survival of the parasite, without causing too much immunopathology in the host. We found PC attached to thrombospondin-like molecules in all tested trichostrongyloids. It can be imagined that in these species PC has a similar function as discovered for *D. viviparus* if not *via* the anti-PAF mechanism than *via* one of the other mechanisms as described for the PC conjugated ES-62. This definitively deserves further investigation. One relatively simple strategy to assess the contribution of PC to infection by other trichostronglyloids is the transfer of purified anti-PC antibodies prior to challenge infection. In this way, it is possible to make a distinction between the role of PC-containing glycoproteins and the antibodies induced by it. One potential problem here, when performed in calves, may be the availability of bovine anti-PC antibody, although animals vaccinated with the current vaccine may be a valuable antibody source.

Perhaps the most important conclusion of the work described in this thesis is real scientific progress can be made via thorough study of how nature has equipped the parasite and host to deal with each other. In this respect, there is still much to learn. An ever intriguing phenomenon is the susceptibility of horses but tolerance of donkeys for *Dictyocaulus arnfieldi* infections. Decreased inflammation in the donkey is associated with increased worm burden without clinical signs. Can this be associated with the presence of PC? If so, we may better learn from evolution and consider to solve the *D. viviparus* problem not only by preventing infection but also by modulating the immunity of cattle in such a way as to make a donkey out of a horse.

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Samenvatting in het Nederlands

Inleiding

Dictyocaulus viviparus (longworm) is de veroorzaker van parasitaire bronchitis bij het rund. De worm behoort tot de superfamilie van de Trichostrongyloidea binnen het phylum Nematoda (rondwormen). De volwassen longwormen zijn van gescheiden geslacht en leven als enige parasitaire nematode in de longen van runderen. De vrouwtjes produceren eieren die zich binnen 24 uur tot eerste stadium larven (L1) ontwikkelen. De eieren en L1's worden door de gastheer opgehoest en ingeslikt, waarna ze worden uitgescheiden met de faeces. Daar vindt de ontwikkeling plaats tot het infectieuze 3^{e} stadium (L3). Deze L3 larven kruipen in de sporangiofoor van de schimmel *Pilobolus* en deze verspreidt zijn sporen samen met de larven over het grasland. Na het eten van besmet gras penetreren de opgenomen larven de darmwand van het rund en migreren via de mesenteriele lymfeklieren en de bloedcirculatie naar de longen, waar ze zich ontwikkelen tot volwassen wormen. Ongeveer 24 dagen na het opnemen van de larven beginnen de dan volwassen wormen met het produceren van eieren en kan de levenscyclus zich weer herhalen.

De pathologie van longworminfecties wordt veroorzaakt door de stadia die zich in de longen bevinden: eieren, L1 en ontwikkelende en volwassen wormen. De aanwezigheid van wormen induceert een influx in de longen van cellen van het immunsysteem, met name eosinofielen. Deze cellen blokkeren de luchtpassage naar de alveolen (longblaasjes) en hierdoor klappen de alveolen in. Voorts komen door activeren van eosinofielen maar ook andere aanwezige cellen van het immuunsysteem zoals mest cellen (mast cells), toxische stoffen vrij die zijn bedoeld om pathogenen uit te schakelen. Bij een overmatige reactie kunnen deze stoffen echter ook de lichaamseigen cellen beschadigen. Het tekort aan zuurstof

veroorzaakt door de beschadiging van de alveolen kan leiden tot hart falen, waardoor de gasuitwisseling in de longen nog verder verslechterd. Bij ernstige worminfecties kan de dood van de gastheer intreden nog voordat de wormen volwassen zijn.

Momenteel worden longworm- en andere nematoden infecties veelal bestreden met anti-worm middelen (anthelmintica). Het grote nadeel hiervan is dat resistentie tegen alle groepen van anthelmintica tegenwoordig wijd verspreid is en nog steeds toeneemt. Hoewel anthelminticum resistentie bij longwormen nog niet ondubbelzinnig is vastgesteld, zou vaccinatie een betere oplossing zijn. Er is al meer dan 40 jaar een vaccin tegen *D. viviparus* op de markt (Huskvac, Intervet). Dit is het enige commercieel verkrijgbare vaccin tegen een parasitaire nematode. Het vaccin bestaat uit L3 larven die zijn verzwakt door bestraling. Deze larven leven lang genoeg om immuniteit te genereren, maar te kort om ziekte te veroorzaken. Er zijn echter belangrijke nadelen verbonden aan dit levende vaccin zoals de noodzaak om donorkalveren te gebruiken voor de productie, de korte houdbaarheid van het vaccin, en het onvermogen van dit vaccin om immunologisch geheugen te induceren. Hierdoor is een booster infectie in de vorm van een natuurlijke infectie op het weiland nodig. Ook zijn de bestanddelen van het vaccin die bescherming geven onbekend. Vanwege deze nadelen is de ontwikkeling van een nieuw vaccin met een gedefinieerde samenstelling en bereid zonder tussenkomst van dieren, wenselijk.

Antilichamen (immunoglobulinen, Ig) spelen een belangrijke rol bij de immuniteit tegen *D. viviparus*. Dit is aangetoond met passieve immunisatie: door het inspuiten van serum van immune kalveren naar naieve kalveren kon bescherming tegen infectie worden overgebracht. De specificiteit en het Ig isotype van de beschermende antilichamen is echter niet bekend. Veel van de antilichamen die tegen de worm zijn gericht zullen geen effect hebben op de parasiet, bijvoorbeeld omdat ze gericht zijn tegen antigenen die bij levende wormen niet op

het oppervlak liggen en dus niet toegankelijk zijn voor het immuunsysteem. Excretie/secretie (ES) producten van parasitaire nematoden bevatten veelal eiwitten met essentiële enzymatische functies of eiwitten afkomstig van de oppervlakte van de worm. Deze eiwitten zijn vaak immunogeen en vaccinatie met ES van diverse parasitaire nematoden geeft gedeeltelijke bescherming tegen infectie.

Het belangrijkste doel van het onderzoek beschreven in dit proefschrift is het ontdekken van componenten van de longparasiet die bescherming kunnen geven tegen infectie en dus onderdeel zouden kunnen zijn van een nieuw veilig, effectief, en diervriendelijk geproduceerd vaccin. Aangezien de herkenning door antilichamen is gecorreleerd met bescherming tegen infectie, is eerst het ontstaan van antilichamen gericht tegen ES producten tijdens natuurlijke *D. viviparus* infectie onderzocht (Hoofdstuk 2). Vervolgens zijn het isotype en de specificiteit van de antistoffen die worden geïnduceerd tijdens de natuurlijke infectie en na vaccinatie onderzocht (Hoofdstuk 3). Potentieel interessante componenten van de parasiet die werden herkend door de antilichamen zijn geïdentificeerd, gezuiverd en onderzocht op mogelijk immunomodulerende eigenschappen (Hoofdstuk 4). Een voor vaccinontwikkeling veelbelovend antigeen (GP300) is vervolgens gekloneerd, gelokaliseerd, en vergeleken met soortgelijke eiwitten in andere nematoden (Hoofdstuk 5).

De humorale immune response tegen ES in geïnfecteerde kalveren

Van alle Ig isotypes bij het rund (IgA, IgE, IgG1, IgG2 en IgM) is IgE het sterkst geassocieerd met parasitaire infecties. In serum komt IgE in zeer lage concentraties voor, maar is toch van groot belang omdat het bindt aan IgE receptoren met hoge affiniteit (FcεRI) die voorkomen op mest cellen en geactiveerde eosinofielen. Omdat er geen methode beschikbaar was om runder (bovine) IgE eenvoudig te bepalen, zijn eerst bovine IgE-specifieke poly- en monoklonale antilichamen

geproduceerd (Hoofdstuk 2). De nieuw ontwikkelde bovine IgE assay werd vervolgens gevalideerd en gebruikt om IgE hoeveelheden te meten in de sera van geïnfecteerde runderen in twee onafhankelijk experimenten. Zoals beschreven in Hoofdstuk 2 werd in beide experimenten een positieve correlatie gevonden tussen de hoeveelheid totaal IgE in serum en bescherming tegen *D. viviparus* infectie. Parasiet specifiek IgE kon alleen worden gedetecteerd in dieren met een hoge immuniteit. In dieren uit dezelfde groep met een lage immuniteit kon geen parasiet specifiek IgE worden aangetoond. Voor wat betreft de andere Ig klassen werd slechts voor IgG1 en IgG2 op 1 dag in het verloop van de infectie een positieve correlatie gevonden tussen de Ig titers en bescherming. Het beschermende IgE bleek vooral te reageren met een eiwit met een hoog moleculair gewicht, GP300.

Parasitaire glycoproteïnen als antigenen in geïnfecteerde en gevaccineerde kalveren

Nader onderzoek naar de aard van de antigenen die werden herkend door de Ig tijdens een eerste en tweede infectie richtte zich op zowel parasitaire eiwitten als de aan sommige eiwitten gekoppelde suikers of andere moleculen (Hoofdstuk 3). Hiertoe werd de immuun respons gemeten tegen het volledige antigeen, maar ook na deglycosylering van de ES of L3 fracties. De gebonden glycanen werden verwijderd door behandeling met PNGaseF. Dit enzym verwijdert alle N-gebonden glycanen die geen core α(1,3) fucosylering hebben. Om een beter onderscheid te maken tussen de immuun response na een eerste infectie en de booster response door een tweede infectie werd de Ig gemeten in sera van dieren die met een grotere tussenpoos waren geïnfecteerd dan die beschreven in Hoofdstuk 2.

Na primaire infectie en ook na vaccinatie bleken de N-glycanen de dominante epitopen voor alle Ig isotypen behalve IgM. Vooral GP300 werd zeer sterk herkend ondanks de relatief lage expressie van het eiwit. Na herinfectie van

de dieren bleek dat er geen booster response optrad tegen het glycaan deel, maar wel tegen het eiwit deel. Deze booster respons werd alleen gezien voor IgG1 en IgE. Dit suggereert dat alleen voor deze isotypen immunologisch geheugen was ontstaan. Opvallend was dat de kalveren die werden gevaccineerd met bestraalde L3 larven (waarvan bekend is dat dit geen immunologisch geheugen induceert) ook geen IgG1 en/of IgE boosterreactie ontwikkelden. Het gedeglycosyleerde eiwit in ES dat het best herkend werd na herinfectie, maar niet na vaccinatie was een 67 kDa protein. Dit is waarschijnlijk het acetylcholinesterase (AChE), een potentiële vaccincomponent waarvan bekend is dat het wel na infectie, maar niet na vaccinatie wordt herkend.

Karakterisering van GP300

Gezien de mogelijke geschiktheid van GP300 als bestanddeel van een nieuw vaccin tegen *D. viviparus*, werd het glycoproteïne gezuiverd en nader gekarakteriseerd (Hoofdstuk 4). GP300 reageerde met het lectine WGA. Dit wees op de aanwezigheid van N-acetylglucosamine (GlcNAc). GP300 was het enige (glyco)proteïne in volwassen wormen dat reageerde met WGA. Hierdoor was het mogelijk vrijwel 100% zuiver GP300 te isoleren uit het wateronoplosbaar extract van volwassen longwormen door middel van lectine affiniteitschromatografie.

Verrassend was dat GP300 niet alleen één of meerdere suikerketens had, maar ook een aan deze keten gekoppelde phosphorylcholine (PC) groep. De aanwezigheid hiervan kon worden aangetoond met PC-specifieke antilichamen. Wellicht nog interessanter was dat in de *D. viviparus*-geinfecteerde dieren PC de immunodominante epitoop van GP300 was, maar dat de IgG respons hiertegen niet correleerde met bescherming in tegenstelling tot de veel lagere IgE response. Wetende dat PC ook veel voorkomt bij de gastheer, bracht deze bevinding ons op de hypothese dat de door de parasiet opgewekte PC-specifieke antilichamen

wellicht zouden kunnen kruisreageren met soortgelijke moleculen van het rund. Dit kon worden aangetoond voor de ontstekingmediator platelet-activating factor (PAF). PAF heeft een belangrijke functie o.a. in stimuleren van eosinofilie en de productie van IgG2. Op grond van deze bevindingen kan worden verondersteld dat de anti-PC antilichamen die ontstaan tijdens een *D. viviparus* infectie en die kruisreageren met PAF, leiden tot een neutralisatie van PAF functie en dus een verminderde eosinofilie en IgG2 productie. Dit is precies wat waargenomen wordt bij ernstig geïnfecteerde runderen die hoge anti-PC titers hebben op het moment van herinfectie. De PC-specifieke IgG antilichamen zouden aldus enerzijds tot een verminderde effectiviteit van de immuun respons van de gastheer kunnen leiden (door verminderde IgG2 productie), maar tegelijkertijd ook de gastheer kunnen beschermen tegen ernstige pathologie door het neutraliseren van PAF-geïnduceerde ontstekingsmediatoren.

Identificatie en karakterisering van peptide-keten van GP300

PC is alleen aanwezig is op GP300 (hoofdstuk 4) en de IgE respons tegen GP300 correleert met bescherming tegen infectie (Hoofdstuk 2). Hierom is het eiwit gedeelte van GP300 verder onderzocht (Hoofdstuk 5). Massa spectrometrie toonde aan dat GP300 veel overeenkomsten vertoonde met een eiwit van *Haemonchus contortus*, de grote lebmaag worm van schaap en geit. Dit eiwit bevat een aantal thrombospondine en Kunitz domeinen en heeft de benaming *H. concortus* thrombospondine (TSP) meegekregen. Op basis van deze homologie kon het gen dat codeert voor GP300 worden gekloneerd en gesequenced. Dit toonde aan dat GP300 eveneens een aantal thrombospondine- en Kunitz-domeinen bevat, alsmede een signaalpeptide, maar vrijwel zeker geen transmembraan domein. Dit past bij de suggestie dat GP300 een extracellular matrix (ECM) proteïne is. Immuno-histologie toonde aan dat in de volwassen worm GP300 is gelokaliseerd in de

hypodermis, de bekleding van de uterus en de brushborder van de darm, net als bij *H. contortus*. De verwantschap tussen GP300 en TSP van *H. contortus* kon verder worden bevestigd door de reactiviteit van antilichamen opgewekt tegen recombinant *H. contortus* TSP (anti-rHc-TSP) met GP300. Voorts bleken de PC-specifieke antilichamen en het lectine WGA die beide reageren met PC300, eveneens te reageren met *H. contortus* TSP en ook met mogelijke verwante proteïne in andere nematoden. Dit wijst erop dat niet alleen GP300, maar ook soortgelijke eiwitten in andere nematoden PC-bevattende glycanen kunnen hebben.

Conclusies en toekomstig onderzoek

De in dit proefschrift beschreven resultaten hebben enkele belangrijke nieuwe inzichten opgeleverd. De bevinding dat IgE titers sterk correleren met de bescherming tegen *D. viviparus* infectie geeft de mogelijkheid de mate van bescherming van runderen tegen longworm te bepalen. Het bestaan van een immuun respons, maar achterwege blijven van een booster respons na vaccinatie, kan de beperkte werking van het huidige vaccin verklaren. De bevinding dat na herinfectie wel een booster respons (IgG1 en IgE) optreedt die gecorreleerd is met bescherming tegen infectie en die vooral gericht is tegen de peptide-keten van o.a GP300, kan erop wijzen dat de eiwitgedeelte van GP300 van groot belang kan zijn voor de ontwikkeling van een vaccin. Het hier beschreven succes met het kloneren van het gen dat codeert voor GP300 en het bepalen van de gensequentie zijn belangrijke stappen in het ontwikkelen van een recombinant eiwit als onderdeel van een toekomstig vaccin. Vaccinatie experimenten met GP300 zullen uit moeten wijzen of dit glycoproteïne inderdaad tot (door IgE gemedieerde) bescherming leidt.

De bevinding dat PC aanwezig is op GP300, dat dit molecuul sterk immundominant is, en dat opgewekte PC-specifieke antilichamen kruisreageren

met de belangrijke onstekingsmediator PAF, geeft een nieuwe dimensie aan het denken over de pathogenese van *D. viviparus* infectie. Sterker nog, het feit dat de PC-specifieke antilichamen kruisreageren met PC op andere nematoden zou kunnen wijzen op een nieuw universeel mechanisme via welke wormen er tijdens de evolutie in geslaagd zijn hun gastheer te koloniseren zonder ernstige schade toe te brengen. Dit zou nader kunnen worden bevestigd door experimenten uit te voeren met intact en met gedeglycosyleerd GP300. Er zal dan niet alleen gekeken moeten worden naar het effect op de wormlast, maar ook naar ontstekings parameters (eosinofilie, IgG2, acute phase proteins, adenhalings frequentie). De verwachting is dat deze verlaagd zullen zijn na vaccinatie met het intacte GP300, maar niet na vaccinatie met het gedeglycosyleerde GP300. Mogelijk zal vaccinatie met GP300 niet resulteren in lagere worm aantallen, maar wel in afname van de symptomen. De vergelijking dringt zich hier op met *Dictyocaulus arnfieldi,* een verwante soort die ezels en paarden infecteert. Ezels hebben doorgaans grote aantallen van deze wormen in hun longen zonder klinische verschijnselen, terwijl deze soort voor paarden zeer pathogeen is. Er wordt aangenomen dat de ezel de oorspronkelijke en dus oudste gastheer van deze parasiet is en blijkbaar heeft de evolutie er voor “gekozen” om de nadelige gevolgen van de infectie tegen te gaan in plaats van afweer tegen de parasiet op te bouwen. Als dit gerelateerd is aan PC en/of anti-PC, dan is het verschil tussen naieve en GP300 gevaccineerde runderen vergelijkbaar met het verschil tussen paarden en ezels.

Dankwoord

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Curriculum vitae

Frans Kooijman werd geboren op 24 januari 1960 in Utrecht. In 1976 behaalde hij het middelbare school diploma aan de RK-MAVO Rotonde te Utrecht. In datzelfde jaar begon hij de HBO opleiding tot zoologisch analist, eerst aan het Dr. Ir. W.L. Ghijsen instituut (Utrecht) en later aan het Ir. W. van den Broek instituut (Amsterdam), waar in 1979 het diploma werd behaald. Na de militaire dienst begon hij in 1981 te werken bij de toenmalige vakgroep Helminthologie en Entomologie (Parasitologie) van de Faculteit Diergeneeskunde van de Universiteit Utrecht. In deeltijd werd het HBO-B diploma (van Leeuwenhoek instituut, Delft, 1987) en het HLO diploma (Hogeschool Utrecht, 1994) behaald. In 2002 begon hij zijn promotieonderzoek waarvan de resultaten zijn beschreven in dit proefschrift, eerst nog naast zijn werk als analist, later voltijds. Het onderzoek werd gestart onder leiding van Prof. Dr. Albert Cornelissen van de afdeling Parasitologie van het departement Infectieziekten en Immunologie van de Faculteit Diergeneeskunde van de Universiteit Utrecht. Deze afdeling is later deels overgegaan naar de afdeling Infectiebiologie van hetzelfde departement onder leiding van Prof. Dr. Jos van Putten en hier is het latere deel van het onderzoek uitgevoerd.

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