# 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*

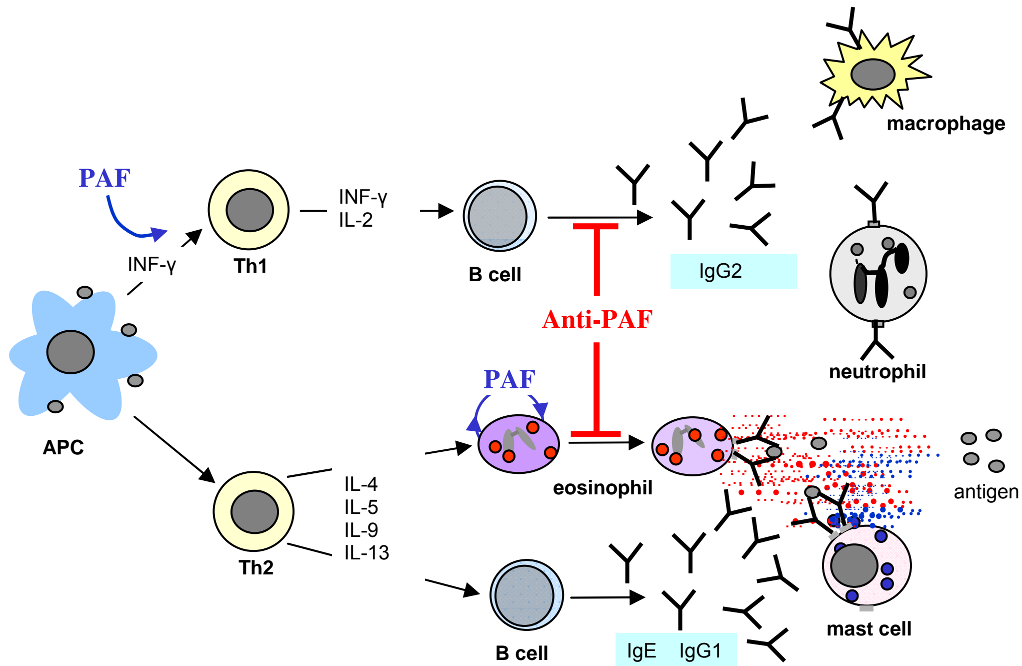
Because the glycans were the immunogenic part of the glycoprotein in primary infected and vaccinated animals, we characterized the N-glycan moiety of GP300 in more detail (Chapters 3 and 4). The successful one-step purification of GP300 by WGA lectin affinity chromatography indicated the presence of 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.

## References


1. **Black, S., A. Wilson and D. Samols.** 2005. An intact phosphorylcholine binding site is necessary for transgenic rabbit C-reactive protein to protect mice against challenge with platelet-activating factor. 2005. J. Immunol. **175:** 1192-1196.

2. **Blaxter, M., P. De Ley, J. R. Gareys, L. X. Liu, P. Scheldeman, A. Vierstraete, J. R. Vanfleteren, L. Y. Mackey, M. Dorris, L, M, Frisse, J. T. Vida and W. Kelley Thomas.** 1998. A molecular evolutionary framework for the phylum Nematoda. Nature **392:** 71-75.

3. **Britton, C., G. J. Canto, G. M. Urquhart and M. W. Kennedy**. 1993. Characterization of excretory-secretory products of adult Dictyocaulus viviparus and the antibody response to them in infection and vaccination. Par. Immunol. **15:** 163-174.

4. **van Die, I., V. Gomord, F. N. Kooyman, T. K. van den Berg, R. D. Cummings and L.Vervelde.** 1999. Core alpha 1→3 fucose is a common modification of N-glycans in Parasitic helminths and constitutes an important epitope for IgE from *Haemonchus contortus* infected sheep. FEBS Lett. **463:** 189-193.

5. **Gånheim, C., J. Höglund and K. Persson Waller.** 2004. Acute phase proteins in response to *Dictyocaulus viviparus* infection in calves. Acta Vet. Scand. **45:** 79-86.

6. **Haslam, S. M., G. C. Coles, E. A. Munn, T. S. Smith, H. F. Smith, H. R. Morris and A. Dell.** 1996. *Haemonchus contortus* glycoproteins contain N-linked oligosaccharides with novel highly fucosylated core structures. J. Biol. Chem. **271:** 30561-30570.

7. **Höglund, J. C. Ganheim and S. Alenius**. 2003. The effect of treatment with eprinomectin on lungworms at early patency on the development of immunity in young cattle. Vet. Par. **114:** 205-214.

8. **Houston, M. K., R. Sutharsan, C. N. Steiger, H. Schagter and W. Harnett.** 2008. Gene inactivation confirms the identity of enzymes involved in nematode phosphorylcholine N-glycan synthesis. Mol. Biochem. Parasitol. **157:** 88-91.

9. **Jarrett, W. F. H., F. W. Jennings, W. I. M. McIntyre, W. Mulligan and G. M. Urquhart.** 1955. Immunological studies on *Dictyocaulus viviparus* infection. Passive immunisation. Vet. Rec. **67:** 291-296.

10. **Johnson, M., R. E. Labes, M. J. Taylor and C. G. Mackintosh**. 2003. Efficacy trial of an irradiated cattle lungworm vaccine in red deer (*Cervus elaphus*). Vet. Parasitol. **117:** 131-137.

11. **Lochnit, G., R. D. Dennis and R. Geyer.** 2000. Phosphorylcholine substituents in nematodes: structure, occurrence and biological implications. Biol. Chem. **381:** 839- 847.

12. **McKeand, J. B., D. P. Knox, J. L. Duncan and M. W. Kennedy.** 1994. The immunogenicity of the acetylcholinesterases of the cattle lungworm *Dictyocaulus viviparus*. Int. J. Parasitol. **4:** 501-510.

13. **McKeand, J. B., D. P. Knox, J. L. Duncan and M. W. Kennedy.** 1995. Protective immunisation of Guinea pigs against *Dictyocaulus viviparus* using excretory/secretory products of adult parasites. Int. J. Parasitol. **25:** 95-104.

14. **McKeand, J. B., D. P. Knox, J. L. Duncan and M. W. Kennedy.** 1995. Immunisation of guinea pigs against *Dictyocaulus viviparus* using adult ES products enriched for acetylcholinesterases. Int. J. Parasitol. **25:** 829-837.

15. **Nor, Z. M., K. M. Houston, E. Devaney and W. Harnett**. 1997. Variation in the nature of attachment of phosphorylcholine to excretory-secretory products of adult *Brugia pahangi*. Parasitology **114:** 257-262.

16. **Paschinger, K., D. Rendic, G. Lochnits and V. Jantsch**. 2004. Molecular basis of anti-horseradish peroxidise staining in *Caenorhabditis elegans.* J. Biol. Chem. **279:** 49588-49598.

17. **Petersen, H. H., J. P. Nielsen, P. M. H. Heegaard.** 2004. Application of acute phase protein measurements in veterinary clinical chemistry. Vet Res. **35:** 163-187.

18. **Pöltl, G., D. Kerner, K. Paschinger and I. B. H. Wilson**. 2006. N- glycans of the porcine nematode parasite *Ascaris suum* are modified with phosphorylcholine and core fucose residues. FEBS J. **274:** 714-726.

19. **Scott, C. A., J. B. McKeans and E. Devaney.** 1996. A longitudinal study of local and peripheral isotype/subclass antibodies in *Dictyocaulus viviparus*-infected calves. Vet. Immol Immunopathol. **53:** 235-247.

20. **Skuce, P. J., G. F. J. Newlands, E. M. Stewart, D. Petit, S. K. Smith, W. D. Smith and D. P. Knox**. 2001. Cloning and characterisation of thrombospondin, a novel multidomain glycoprotein found in association with a host protective gut extract from *Haemonchus contortus.* Mol. Biochem. Parasitol. **117:** 241-244.

21. **Smith, S. K. and W. D. Smith**. 1996. Immunisation of sheep with an integral membrane glycoprotein complex of *Haemonchus contortus* and with its major polypepetide components. Res. Vet. Sci. **60:** 1-6.