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Invited review

Exploiting natural immunity to helminth parasites for the development of veterinary vaccines

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Abstract

The development of subunit vaccines against most parasitic helminth infections will require a better understanding of the different components of a natural rejection process including (1) recognition of parasite antigens; (2) induction of protective immune response phenotypes; and (3) activation of appropriate immune effector mechanisms. While novel technologies have allowed significant progress to be made in the identification of candidate vaccine antigens, the large scale production of these antigens and their presentation to the host with appropriate adjuvant systems remains a major problem in vaccine research. Identification of the molecular interactions involved in the innate immune response to helminth infections and the application of new genomic and proteomic technologies are likely to lead to major advances in these research fields. Gastrointestinal nematode parasites and liver fluke are the most important helminth parasites of production animals. In recent years, a lot of new knowledge has been gathered on the immunobiology of the host–parasite interactions in these two infection systems, which has allowed new vaccination strategies to be considered. Functional genomic technologies such as gene expression analysis by microarrays, promise to further advance our understanding of the molecular pathways leading to protection against parasite infections. This will not only have implications for vaccine research, but also provide novel targets for drug development and genetic selection.

Keywords: Gastrointestinal nematodes; Liver fluke; Antigens; Immune response

1. Introduction

Vaccination has made an enormous impact on the control of viral and bacterial diseases in both humans and animal species. The underlying mechanism of protection induced by vaccines is generally considered to be based on the imprinting of the immune system by pathogen-specific molecules presented in a non or poorly infectious form present in the vaccine preparation, followed by the activation of an immune memory response against these molecules upon natural exposure to the live and virulent pathogen. The nature of immunological memory determines the duration of this vaccine-induced immunity and can last for the lifetime of the host. Factors that control the duration of immunological memory are still not fully understood but are influenced by the nature and persistence of the immunising antigens (Zinkernagel, 2000). Despite the long-standing success of bacterial and viral vaccines, parasite vaccines have still not been developed to a point where they have a major impact on disease management. This can be in a large part attributed to the further complexity of parasitic organisms compared to bacteria and viruses and the difficulty in culturing parasites in large quantities.

Most successful veterinary, bacterial and viral vaccines still consist of killed or attenuated whole organisms grown in in vitro culture systems. Initial experiments using cultured parasites attenuated by irradiation or passage through unnatural hosts have shown promising results for the development of vaccines and in some cases, this type of vaccination is still being practiced (e.g. vaccination against *Babesia* and *Anaplasma* with organisms grown in splenectomised calves and against cattle lungworm with irradiated *Dictyocaulus* larvae). It has recently been suggested that irradiated larval vaccines may have further applications in the control of gastrointestinal nematodes of ruminants (Le Jambre et al., 1999). Such an approach may indeed be relevant given the increasing level of anthelmintic resistance and the lack of any useful subunit vaccines against

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these parasites. However, the difficulties in growing parasitic organisms, maintaining reproducible levels of attenuation and short shelf-life may limit the widespread use and commercial exploitation of attenuated parasite vaccines. In the longer term, it would be preferable to have parasite vaccines based on non-infectious parasite molecules that can be produced in commercial quantities by recombinant or synthetic means. A major disadvantage of this subunit vaccine approach is that it cannot rely on the living parasite to orchestrate the necessary immunological events that have to accompany presentation of the appropriate 'protective' parasite molecules at the site of infection. Subunit vaccine preparation will therefore need to contain all the necessary ingredients for inducing the particular immune memory response that can be reactivated upon natural infection to result in parasite rejection. This will require a more rational, rather than hit and miss, approach to vaccine development and a thorough understanding of the molecular and cellular processes associated with the development of parasite immunity in the host animal. In particular, scientists involved in parasite vaccine development will need to identify and incorporate into a vaccine the different components of a natural rejection process which include: (1) recognition of parasite antigens; (2) induction of a protective immune response phenotype; resulting in (3) activation of appropriate immune effector mechanisms and expulsion or killing of the parasite. The present paper will give some thoughts on how these three objectives may be achieved, with examples from work in our laboratories on the helminth parasites of most importance to animal agricultural development worldwide, i.e. gastrointestinal nematodes and liver fluke.

2. Identification of candidate vaccine antigens

A primary aim of vaccination is to prevent or reduce infection of the host. We have, therefore, concentrated our search for protective antigens to those that are expressed by the infective larval stages of the parasite. Infective parasite larvae are also the most vulnerable to immunological attack, as they are much smaller in size than the adult stages and may not have developed the evasion mechanisms that allow adult parasites to persist in the host for prolonged periods of time. A disadvantage of this approach is the limited availability of parasite material for extensive immuno- and biochemical characterisation. This is a particular problem for parasites with complex lifecycles such as liver fluke where infective metacercariae have to be harvested from their snail intermediate host. However, our studies to date on antigenic variation in a range of nematode (Bowles et al., 1995; Jungersen et al., 2001), trematode (Meeusen and Brandon, 1994a) and cestode (Meeusen and Brandon, 1994b) infections, have confirmed the necessity of using larval material, as in each case antigens detected in the infective larval stage were no longer present in the adult parasite. This was most clearly shown with the liver fluke, *Fasciola hepatica*, where all three antigens identified in the newly excysted juvenile stage were no longer detected within 2 days of infection (Meeusen and Brandon, 1994a). In our hands, the identification of stage specific antigens expressed by helminth parasites has been greatly facilitated by the use of a novel technique for antigen identification developed in our laboratory. This technique, based on the use of antigen secreting cell probes (ASC-probes) harvested from activated lymph nodes, has been used and described extensively (Meeusen and Brandon, 1994a,b; Bowles et al., 1995; Meeusen, 1996; Jungersen et al., 2001). Briefly, the technique consists of harvesting draining lymph nodes from the site of a current parasite infection and culturing the lymph node cells with antigens for a short period in vitro to allow spontaneous antibody secretion by in vivo induced ASCs. The supernatant of these cultures, containing the secreted antibodies is then used directly as a probe for identifying antigens using existing technologies such as Western blots. The capacity of ASC-probes to differentiate between antigen recognition profiles in separate tissue compartments and at different stages of infection allowed some novel observations to be made of the molecular interactions between host and parasite. For example, it was shown in several host–parasite systems that the antibody response generated at the early stage of infection was exclusively directed against larval antigens with no reaction at all against adult parasite extract and that the different antibody isotypes were induced in different tissue compartments such as lymph nodes draining lung, liver and gastrointestinal tract. More importantly, because ASC-probes can easily identify antigens of a particular parasite stage at the time that protective immunity is taking place, these antigens are potential candidates for parasite vaccines.

While significant progress has been made in the identification of candidate vaccine antigens, their subsequent isolation, characterisation and recombinant expression still poses major challenges and has become a stumbling block in many laboratories involved in parasite vaccine development. As described in a recent review (Newton and Meeusen, 2003), the rapid development of new proteomic and genomic technologies may offer some novel solutions to these problems in the future.

3. Induction of appropriate immune response

The typical allergic- or Th2-type immune responses that are induced by helminth infections have been well characterised and extensively studied in murine model systems. What has recently become clear, is that (1) the initial recognition of pathogen molecules by host receptors is a crucial event in the initiation of an effective immune response; and (2) the induction of specific immune phenotypes (e.g. Th1/Th2 type responses) is dependent on molecular stimuli of the innate immune system. Host

molecules involved in innate immunity, although of limited genetic diversity, can differentiate between groups of pathogens through recognition of unique pathogen-associated molecular structures. Activation of a particular set of host pattern recognition receptors (PRRs) can then drive the strength and nature of a subsequent adaptive immune response into a specific direction (Janeway and Medzhitov, 2002). While many of these pathogen-associated molecular patterns (PAMPs) and PRRs have now been identified for bacterial and protozoan pathogens (Teixeira et al., 2002), few have been described for the major helminth parasites and the unique immune responses they induce.

All bacterial and protozoan PAMPs share some important characteristics (Medzhitov and Janeway, 2000; Teixeira et al., 2002). First, they are only produced by the pathogen and not by host cells, thus ensuring self/non-self discrimination by the host. Second, they represent conserved molecular patterns whose expression is essential for the survival of the pathogen. This feature prevents the development of mutants, which could escape recognition by the host's immune system. Finally, they are often shared by large groups of micro-organisms, and show little variation among micro-organisms of a given group. Thus a limited number of host PRRs can recognise a variety of PAMPs and can provide information to the adaptive immune system on the type of invading pathogen.

Identification of parasite-specific PAMPs may lead to improved vaccination strategies. A number of immunostimulatory adjuvants are derived from bacteria and protozoa, and often represent PAMPs (e.g. LPS, MPL, CpG DNA). These adjuvants activate the cells of the innate immune system, which drive and focus the acquired immune response (O'Hagan et al., 2001). As yet no PAMPs have been identified for any of the veterinary parasites. The use of parasite specific PAMPs as immunostimulatory adjuvants in conjunction with parasite antigens, may generate a highly targeted immune response to vaccination and elicit a more efficacious protective response when compared to conventional adjuvants, which are mainly designed for bacterial vaccines. New proteomic and genomic technologies could again play a major role in identifying PAMPs and PRRs that determine parasite–host recognition events (Newton and Meeusen, 2003).

4. Activation of effective parasite rejection mechanisms

Although in many cases, the exact mechanisms of pathogen killing achieved by existing vaccines are not known, the complexity of host–parasite interactions and the current paucity of effective parasite vaccines would indicate that a better understanding of the rejection process could lead to novel approaches for improving parasite vaccines. For example, the selection of a particular rejection response as a target for vaccine development would influence the selection of the parasite stage to be targeted and the immune response to be promoted.

While the immune response induced by natural helminth infections has often been described as a stereotypic response, with the activation of 'typical' cellular (e.g. mast cells, eosinophils) and humoral (e.g. IgE) effector pathways, the susceptibility of different parasite species and parasite stages to these effector mechanisms is very individual and depends both on the make-up of the organism and the microenvironment it occupies within the host tissues (Balic et al., 2000). It is therefore important to study these rejection processes in the definite host–parasite system to be targeted. Recent progress in this field for gastrointestinal nematode parasites and liver fluke infections in sheep will be described in the following section.

4.1. Gastrointestinal nematode parasites of sheep

As detailed in a recent review (Balic et al., 2000), a range of manifestations of resistance to gastrointestinal nematode parasites exist, even against the one developmental stage of the same parasite species. It is therefore likely that a range of immunological mechanisms are involved in the rejection process, some of which may be more suitable for inducing long-lasting immunity through vaccination or more amenable to immune modulation and genetic selection. In particular, rejection of infective larvae involving two different cellular kinetic mechanisms can occur.

(a) Rapid expulsion or immune exclusion may occur where infective larvae are rejected within hours after infection of hyperimmunised animals, before they reach their tissue niche. This phenomenon is associated with mucosal mast cell hyperplasia and the appearance of mast cell-derived intraepithelial globule leucocytes in the gastrointestinal tissues (Miller, 1996). The speed of the response follows the release of potent preformed mediators stored in cytoplasmic granules (histamine, heparin, proteases), within minutes of mast cell stimulation. The effect of these primary and subsequent secondary mediators, on the intestinal tissues results in a hypersensitivity-type response and expulsion of the incoming larvae. While most of these studies used high challenge doses shortly after hyperimmunisation of sheep by repeated infections, recent studies in our laboratory suggest that rapid expulsion may also occur after lower challenge infections and several months after the last sensitising infection (Balic et al., 2002). A prerequisite for this phenomenon to occur seems to be persistence of sufficient numbers of intraepithelial globule leucocytes in the gastrointestinal tissues that prevent the entry of larvae into the tissue. The exclusion of larvae from their tissue niche also precludes the development of a local immune response and eosinophil recruitment (Balic et al., 2002). A negative correlation between globule leukocyte numbers and tissue eosinophilia has recently been observed (Balic and Meeusen, unpublished observation).

(b) Delayed rejection has been observed in some experimental systems where rejection of a proportion of challenge larvae occurs over a period of days, after larvae have reached their tissue niche. The immune mechanisms responsible for this delayed type expulsion of nematode larvae are unknown. In recent experiments, we have demonstrated a pronounced infiltration of lymphocytes and eosinophils, only when challenge larvae reach their tissue niche (Balic et al., 2002). In addition, our studies have demonstrated that eosinophils can damage and kill *Haemonchus* larvae both in vitro (Rainbird et al., 1998) and in vivo (Meeusen and Balic, 2000; Balic et al., in preparation). On the basis of these results, we have suggested that the delayed rejection of infective larvae in immunised sheep could be mediated by 'activated' eosinophils (Meeusen and Balic, 2000).

For vaccination purposes, it is important to consider which rejection mechanisms are most useful as targets in the vaccine. A delayed-type rejection response may be more relevant, as it persists longer and allows for restimulation of the immune system during natural infections. However, a major consideration for this scenario is to promote adequate activation and targeting of eosinophils as the presence of eosinophils has also been associated with pathological conditions such as scouring in sheep (Larsen et al., 1999). An eosinophil-mediated response leading to rejection is likely to involve the mobilisation of other known mediators including antibody and complement, both essential components for the in vitro killing of *H. contortus* larvae by eosinophils (Rainbird et al., 1998). These in vitro killing assays also revealed that activation of eosinophils was critical to their effector function and could be achieved either by repeated in vivo challenges or in vitro incubation with known stimulators such as IL5. In addition, other molecules may also be involved in creating the right environment for eosinophil-mediated killing, such as the recently identified galectins (Dunphy et al., 2000, 2002).

4.2. Liver fluke infection in sheep

Until recently, sheep were considered to be unable to generate protective immunity to liver fluke infections (reviewed in Spithill et al., 1997). This would seem to preclude the development of anti-fluke vaccines based on natural immunity. Recent studies of liver fluke infections in breeds of sheep with different genetic backgrounds have, however, revealed some interesting findings, which may impact on future vaccine development as well as genetic selection strategies.

Previous studies have shown differences in recoveries of flukes in different sheep breeds infected with *Fasciola hepatica* or *Fasciola gigantica* (reviewed in Spithill et al., 1999). In particular, Wiedosari and Copeman (1990) were the first to report that Indonesian Thin Tail (ITT) sheep had very low rates of mature worm burdens following infection with *F. gigantica* metacercariae when compared to other susceptible sheep breeds, such as the Merino. Subsequent work in our laboratories established that the mechanism of resistance to *F. gigantica* infection by ITT sheep was immunologically mediated and identified the peritoneum as an important potential site of killing of the recently excysted juvenile or early immature parasite (reviewed in Spithill et al., 1999). The immunity of ITT sheep against invading *F. gigantica* parasites is likely to involve complex processes with numerous pathways and effector mechanisms mediating parasite death (Fig. 1). One strategy we used to unravel the resistance mechanism active in ITT sheep was to focus on which cellular responses occur at the time when killing of the parasite takes place. Initial experiments identified macrophages and eosinophils as the two major immune cell types within the peritoneum during the early migration of *F. gigantica* parasites in ITT sheep. Subsequent in vitro studies demonstrated that each of these immune cell populations from the ITT host could indeed effectively kill ($>70\%$) immature parasites of *F. gigantica* but this ability was critically dependent on the presence of sera from *F. gigantica*-infected ITT sheep (Piedrafita et al., in preparation). By using a series of inhibitors we were further able to determine that the major cytotoxic molecules mediating parasite killing, and produced by these cells, were superoxide radicals.

Two critical observations from in vivo studies supported the proposed resistance mechanism of ITT sheep to *F. gigantica* infections identified by the in vitro experiments. First, eosinophil numbers were significantly elevated at the time of *F. gigantica* parasite killing in the ITT host relative to the *F. gigantica*-susceptible Merino host and second, IgG_2 responses were positively correlated with susceptibility to infection in the Merino host (Hansen et al., 1999). The production of IgG_2 has been shown to be strongly up-regulated by IFN-γ in mice, humans and cattle (Reviewed in Hansen et al. 1999) and it is therefore likely that the elevated IgG_2 levels in the susceptible Merino sheep are related to elevated IFN-γ levels. This observation is intriguing given our findings (D. Piedrafita, personal communication) and others (Bielefeldt et al., 1984) that IFN-γ, a typical Th1-type cytokine, decreases superoxide production by sheep immune cells. Thus, the immunological pathways that could inhibit our proposed effector mechanism in the resistant ITT sheep, appear to be up-regulated in the susceptible Merino host. On the other hand, the low production of IgG_2 antibodies in resistant ITT sheep suggests that Th_1-like responses may be downregulated in this breed during infection with *F. gigantica*. In addition, there was a trend for higher levels of IgM, IgG_1, IgE antibodies, and, significantly, the percentage of eosinophils in blood (a key hall mark of Th2 responses) was elevated in ITT sheep compared to those in Merino sheep. Taken together, these observations indicate that the enhanced resistance of ITT sheep to *F. gigantica* infection is concomitant with a Th2-like pattern of immune responses.

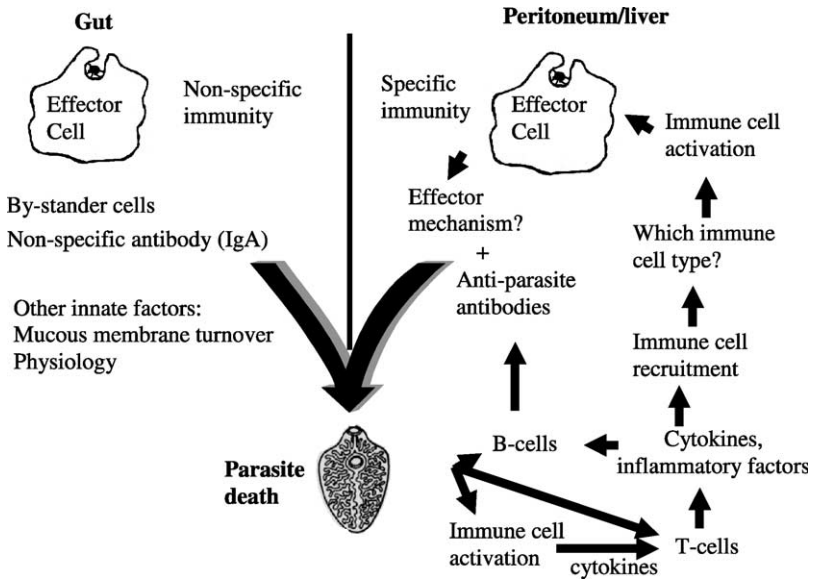

Fig. 1. Current evidence suggests that the protective responses employed by some hosts to kill incoming *Fasciola* parasites involves two basic mechanisms. The first involves the gut wall where innate factors such as non-specific and hypersensitivity reactions of resident effector cells, natural antibodies and mucus may limit the number of parasites penetrating the gut wall after initial infection. The second mechanism involves the induction of T-cell dependant adaptive immune responses, following parasite migration in the peritoneum and liver. Parasite-specific antibody and as yet unidentified effector molecules from activated immune cells ensue in parasite killing in these body compartments. In addition, once elicited, components of adaptive immunity may cooperate with the innate response mechanisms to limit infection and kill parasites at the level of the gut (reviewed in Mulcahy et al., 1999; Spithill et al., 1999).

Infections of sheep with *F. hepatica* parasites are also known to induce a strong Th2-like immune response, however, these parasites are not killed by the immune response of ITT sheep that remain completely susceptible to primary and subsequent challenge infections (Roberts et al., 1997). Direct comparative studies between juvenile *F. gigantica* and *F. hepatica* parasites demonstrated that *F. hepatica* immature parasites were not susceptible to the superoxide-dependent killing mechanisms of macrophages or eosinophils from ITT sheep that were able to kill *F. gigantica* juveniles. In addition, our previous studies had demonstrated that *F. hepatica* juvenile parasites are highly resistant to free radical killing, including superoxide radicals (Piedrafita et al., 2000, 2001). The finding that *F. hepatica*, in contrast to *F. gigantica*, is not susceptible to free radical damage by ITT immune cells suggests that *F. hepatica* has effective defences against these toxic molecules. Oxidant scavenger enzymes are critical defence molecules against a large range of immune-mediated toxic metabolites (reviewed in Piedrafita and Liew, 1998) and their importance in *Fasciola* is demonstrated by the fact that these enzymes are excreted/secreted by the juvenile parasites (summarised in Piedrafita et al., 2000). We have shown that inhibition of antioxidant enzyme activity renders *F. hepatica* juveniles susceptible to killing by reactive oxygen intermediates (Piedrafita et al., 2000) and have preliminary evidence to suggest that levels of some of these antioxidant enzymes, including glutathione *S*-transferase/peroxidase and superoxide dismutase, are lower in *F. gigantica* parasites. Interestingly, glutathione *S*-transferase (GST) levels are elevated in *F. hepatica* adult parasites isolated from susceptible hosts (sheep, mice) and lowered in parasites from resistant hosts (cattle, rats) (Miller et al., 1993). These results show that expression of defence enzymes is variable between *Fasciola* species and may imply that the level of defence enzymes is one of the factors determining the ability of the parasite to survive in different hosts.

Fasciola hepatica and *F. gigantica* are recognised as two of the most economically important helminth parasites of production animals. Recent studies, briefly summarised here, indicate that resistance to *Fasciola* infection is determined in part by biochemical differences between species of *Fasciola* and not just differences in the host immune response. By focusing on these inherent differences between the parasites and their ability to modulate the immune response we hope to identify mechanisms that play a role in either host protective immune responses (*F. gigantica*) or immune evasion (*F. hepatica*).

5. Conclusion

The search for immunological control strategies for helminth parasites has often followed the much better explored fields of bacterial and viral research. Advances in immunology, and in particular the advent of cytokine biology, have however highlighted the distinct immune regulatory pathways that lead to protection of helminth infections and the need for basic studies in the natural host–parasite systems. Many new advances have been made in our understanding of the final effector mechanisms associated with the rejection or killing of helminth parasites

by the immune host, with a few specific examples presented in this paper. It is likely that novel parasite control strategies will emerge from a better understanding of the basic biology of host–parasite interactions, as clearly exemplified by the liver fluke studies where parasite defence molecules are identified as new targets for vaccine or drug-based control.

Many of the underlying molecular pathways that contribute to protective immunity or chronic disease have not been fully identified. This is in part due to the complexity of host–parasite biology and the limitations of the available research tools. High through-put analysis of gene expression profiles at different stages of infection is now possible with the development of new functional genomic technologies such as gene microarrays. While these new tools promise to dramatically expand our ability to identify molecular processes associated with parasite immunity, their application will need to be carefully targeted to reduce the complexity of the host response to well defined biological outcomes. The identification of key molecules associated with the protective response by the host and with the defence mechanisms evolved in the parasite may not only lead to more effective vaccines, but also deliver new targets for drug development and genetic selection.

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