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EDITORIAL

Parasite Vaccines with Special Reference to Toxoplasmosis

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Parasites cause some of the most devastating and prevalent diseases in humans and animals. Moreover, parasitic infections increase mortality rates of other serious non-parasitic infections caused by pathogens such as HIV-1. The impact of parasitic diseases in both industrialised and developing countries is further exacerbated by the resistance of some parasites to anti-parasitic drugs and the absence of efficacious parasite vaccines. Despite years of research, much remains to be done to develop effective vaccines against parasites. This editorial focuses on the more recent vaccine strategies such as DNA and viral vector-based vaccines that are currently being used to develop vaccines against parasites as well as the current status of development of a vaccine for toxoplasmosis.

For many years parasitologists have been searching for an effective way of protecting man and his domestic animals from the numerous parasitic diseases affecting them. Infections caused by parasites are responsible for some of the most devastating and prevalent diseases of humans, livestock and companion animals. The malaria parasite, *Plasmodium*, is responsible for the third most common cause of human mortality globally[1] Other similarly devastating parasitic diseases of humans include cysticercosis (including neurocysticercosis), toxoplasmosis, schistosomiasis and leishmaniasis, which cause significant morbidity and mortality.[2,3,4,5]

In many tropical and subtropical countries parasitic diseases exact a toll of human life and health grave enough to constitute a serious threat to economic and social development. Infections caused by *Toxoplasma* spp. *Schistosoma* spp. and Neospora spp. are widely associated with significant economic loss in livestock and associated industries.[6,7,8] Whilst several cheap and effective vaccines do exist for parasites of livestock, resistance and public concerns about chemical residues in animal products mean safer and more effective vaccines are still needed even for those parasites currently under control.[9]

Using a conventional approach live attenuated vaccines have been produced for the protozoan Theileria and for the nematode Dictyocaulus. Live attenuated larvae are not very popular. Some of them are due to their stages which have to be isolated form natural sources, limited shelf life and attenuation is a critical step in the production of this type of vaccine. A vaccine against the dog hookworm, Ancylostoma caninum was developed in the USA in the 1970's, based on X-ray attenuated infective larvae. It was eventually withdrawn because of the small number of infections arising from the attenuated larvae. Subunit vaccines (based on a single recombinant protein have been developed against ticks (tickGARD). Initially it was found that you could vaccinate cattle using whole tick homogenates, the protective antigen was narrowed down to the gut, and eventually a single protein Bm86. It was released in Australia in 1994 after 12 years of development and trials involving 18,000 cattle. Production has now stopped because it was no longer a commercial proposition. Another sub-unit vaccine was developed against Taenia ovis in sheep. The adult tapeworm occurs in dogs, sheep are the intermediate host. In sheep the parasite develops into a cystic stage in the muscles, carcasses are condemned. Once infected sheep show a strong resistance to re-infection. A recombinant vaccine gave 90 % protection in lambs, but it was never developed commercially because of the difficulty of stabilizing the recombinant protein.

GENERAL TYPES OF VACCINES

In most cases, parasitic infections do confer immunity to subsequent infections by the same parasite in the host, thus demonstrating the potential for a vaccine strategy[10]. Generally vaccines can be classified into different genre.

1. Killed whole organisms. In general killed organisms do not seem to work with parasites, although tickGARD started as whole homogenates.
2. Attenuated organisms. The key seems to be that the attenuated organism follows the same migration route in the host, but does not mature. Attenuated vaccines exist for *Dictyocaulus, Theileria, Ancylostoma, T. ovis, Eimeria*.[11,12] Interestingly infection with irradiated malarial sporozoites or with irradiated schistosome cercariae induces strong resistance, whereas natural infections do not (although there may be a slow acquired immunity).
3. Defined vaccines. These can be based on purified parasite proteins, or more usefully on recombinant proteins.

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Naturally isolated parasite antigens require infected animals to produce the parasites, natural antigens often show batch variation and cannot be easily produced in large amounts. In contrast, recombinant antigens are well characterised and can be produced consistently, in large quantities and relatively cheaply.[13, 14] A possible problem with recombinant antigens is if the target protein turns out to be polymorphic so the vaccine is ineffective against some strains of parasite. In addition, a simple defined antigen may not stimulate all of the host immune components needed to give an effective response. One possible way round this is to use peptide vaccines. These are short synthetic sequences which cover the immuno-dominant epitopes. Being synthetic there is no danger of contamination with other proteins. An approach being tried with malaria is to incorporate a series of such epitopes, from different proteins, into a single construct. By choosing the epitopes it is possible to bias the immune response towards antibody production or a cell mediated response.

4. Recombinant vector vaccines. Cell mediated responses occur in particular in response to intercellular antigens which are processed and presented on the cell surface in association with Class 1 MHC proteins.[15, 16] This MHC 1 pathway is usually activated by intracellular viruses or bacteria. Extracellularly applied vaccine antigens do not normally enter the cell and so do not enter the MHC 1 pathway, and tend to produce an antibody rather than a cellular response. In recombinant vector vaccines, DNA encoding the major parasite antigenic determinants is introduced into the genome of an attenuated virus or bacterium which will replicate inside host cells and express the required gene product. The most promising vector is attenuated vaccinia virus (cow pox). The vaccinia virus can be engineered to express several recombinant genes. Another possible vector is attenuated *Salmonella*, which is useful because it can induce mucosal immunity. The advantage of recombinant vector vaccines is that they induce both a cellular and an antibody response. The disadvantage is that they may not be safe to use with immunocompromised individuals and you could develop immunity to the vector, and could not have a second vaccinations with the same vector.
5. DNA Vaccines. The principal behind DNA vaccines is in some way the same as recombinant vestor vaccines, by introducing a plasmid containing the immunodominant sequence into a cell, the antigen is produced intracellularly and enters the MHC 1 pathway thus provoking a strong cellular response. Prolonged expression of the plasmid encoded peptide means there should be no need for boosters. DNA vaccines are usually circular plasmids encoding the target antigen (or antigens) under the control of a promoter active in human cells.[17, 18]

There are several methods in delivery of DNA vaccines taking into account that direct injection of DNA into the muscle often get poor results. Methods currently in use include Gene gun, whereby the plasmid is coated onto gold particles which are 'fired' into the skin by compressed air (uses much less DNA); complexed DNA, when the plasmid is complexed with a carrier such as a liposome or a specific ligand which can target the DNA to a specific tissue or cell type and electroporation, using electric current to make cells permeable, used widely in bacteria, experimental stage in animals.

There are advantages of DNA vaccines. They are safe, can be used in immunocom-promised individuals and pose no danger of contamination with foreign protein; protection conferred should be long lived as the infected cells produce antigens continuously thus boosting to immune system; inexpensive to make and able to generate a cell mediated as well as an antibody response [19]

However there are also potential problems with DNA vaccines. There is danger that immune cells will become sensitized to DNA and could result in auto-immune disease; foreign DNA could be incorporated into the host genome and could cause cancer and antibiotic resistance genes used in the generation of plasmids might 'escape'. Whatever methods that shall be selected for production of subunit vaccines, recombinant vector vaccines, DNA vaccines all depend on the identification of the key protective antigens and this remains a major challenge.

VACCINATION PROSPECTS AGAINST TOXOPLASMOSIS

Treatment of this disease is difficult due to toxic effects of available drugs, and reinfection occurs rapidly. Under the present scenario, development of either new antitoxoplasma drugs or a vaccine is an attractive alternative. In the last few years, there has been considerable progress towards the development of a vaccine for toxoplasmosis, and a vaccine based on the live attenuated S48 strain was developed for veterinary uses.[20] However, this vaccine is expensive, causes side effects and has a short shelf life. Furthermore, this vaccine may revert to a pathogenic strain and therefore is not suitable for human use. Various experimental studies have shown that it may be possible to develop a vaccine against human toxoplasmosis. In recent years, significant progress has also been made in the identification of vaccine candidates which can induce a protective immune response. Most of the work has focused on surface antigens of tachyzoites. SAG1 (30 kDa), SAG2 (22 kDa), SAG3 (43 kDa), which are major surface antigens of tachyzoites.[21] Amongst them, SAG1 is the predominant vaccine candidate. Excretory secretory antigens of *T. gondii* also play an important role in the stimulation of the protective immune system.[22, 23] These antigens are expressed by both tachyzoites and encysted bradyzoites. The major components of excretory secretory antigens are GRA molecules.[24]

GRA1 (23 kDa), GRA4 (40 kDa) and GRA7 (29 kDa) have also been identified as vaccine candidates. Recently, ROP2 (56 kDa) antigen (expressed by tachyzoites, bradyzoites and sporozoites) has also been proposed as a vaccine candidate against toxoplasmosis.[25]

Humans are generally infected by ingesting oocysts released in cat faeces or consuming meat from infected animals containing the long-lived tissue cysts. After ingesting infective tissue cysts or oocysts, tachyzoites are released which first invade and multiply in intestinal epithelial cells. In the lumen of the gut, intra-epithelial lymphocytes are located among the epithelial cells.[26,27] They participate in modulating host immunity through the release of various cytokines, most apparently interferon-c (IFN-c) and induce immunity. Similarly to intra-epithelial lymphocytes, mesenteric lymph node lymphocytes may migrate to the gut and prevent parasite invasion.[28] From the gastro-intestinal tract, tachyzoites are disseminated to other organs of the human body.

T. gondii induces a potent cell-mediated immune response. Tachyzoites stimulate macrophages to produce interleukin-12 (IL-12) and tumour necrosis factor-alpha (TNF-a).[29,30] IL-12, in turn, activates natural killer cells and T cells to produce IFN-c, which is crucial for killing the parasite, hence confering resistance against subsequent infection. IFN-c and TNF-a act synergistically to mediate killing of tachyzoites by macrophages. The combination of these two cytokines results in the enhanced production of free radicals and nitric oxide (NO), which also can affect parasite killing.[31, 32] Nitric oxide (NO) is produced as a result of activation of inducible nitric oxide synthase (iNOS). iNOS is induced in *T. gondii* replication in murine cells but not in human macrophages. In an *in vivo* study with iNOS-deficient mice, the protective role of NO against *T. gondii* infection was tissue specific rather than systemic.[33] Other in vitro studies demonstrated that IFN-c-induced antitoxoplasma activity in human cell depends on the induction of indoleamine 2,3 dioxygenase (IDO), which is the rate-limiting enzyme for the L-tryptophan-L kynurenine pathway. In the absence of tryptophan, parasitic growth becomes restricted. Thus, IDO and iNOS are involved in the immuno-modulatory role of IFN-c. Recent studies suggest that there is an anti-toxoplasma mechanism of cross-regulation between iNOS and IDO and that the expression of the main antiparasitic effector mechanism for iNOS or IDO may vary among mouse tissues. Among the T cell population, CD8+ T cells are considered to be the major effector cells responsible for protection against *T. gondii*, with CD4+ T cells playing a synergistic role. Furthermore, the depletion of the total T cell population abrogates protective immunity against toxoplasma challenge in the immunised animals.[34] CD8+ T cells from TS-4 vaccinated animals are able to proliferate and secrete IFN-c in response to recall antigen for a longer duration than the CD4+ T cells from the same mice. Immune CD8+ T cells from both infected mice and humans secrete IFN-c and exhibit *in vitro* cytotoxicity towards infected cells. The CD4+ Th1 cells exert their protective effect through the production of IFN-c and IL-2. Conversely, the cytokines produced by CD4+ Th2 cells, such as IL-4, IL-5 and IL-10, are associated with down-regulation of protective cell-mediated immune responses. These T cell subsets are able to cross-regulate their activity. The host immune response blocks the multiplication of tachyzoites, resulting in the formation of cysts containing bradyzoites in all the tissues. A variety of stress conditions including pH shock, heat shock, mitochondrial inhibitors, chemical stress and NO induced bradyzoite formation.[35]

Control of multiplication of tachyzoites is largely dependent on endogenous INF-c, with partial involvement of TNFRp55 and iNOS. In contrast, induction of bradyzoite-specific antigen expression and cyst formation during toxoplamosis seem to be dependent on INFc, but independent of TNFRp55 and iNOS functions. The development of large numbers of cysts are controlled by major histo-compatibility complex class I gene, Ld, in mice However, toxoplasma strain-specific differences have been observed. Recurrence of *T. gondii* infection and consequent disease is common in congenitally infected individuals. Immuno-suppressive therapy favours disease reactivation. Murine studies have found that neutralisation of IFN-c or TNF-a or inhibition of NO production leads to relapse of chronic infection, resulting in active disease with numerous tachyzoites.[36] Detailed studies are therefore required for the determination of the mechanism of conversion from bradyzoite to tachyzoite. *T. gondii* mutant strains, viz., S48, TS-4 and T-203, have been identified as vaccine candidates.[37] The live tachyzoites of strain S48 have been used as the commercial vaccine for ovine toxoplasmosis. Following subcutaneous inoculation of naive sheep (sero-negative for *T. gondii*) with the commercial vaccine, the parasite multiplies in the local draining lymph node, causing a mild fibrile response. Peak titres of antibody are reached by 6 weeks. The immunity induced by this vaccine is likely to involve both CD4+ and CD8+ T cells and IFN-c. However, such a vaccine is not suitable for human use due to reactivation to the pathogenic form.

SAG1 plus SBAs1 (Th1 response inducer adjuvant) induced significant protection against materno-foetal transmission in guinea pig.[38] DNA vaccine has been shown to be a powerful method for the induction of specific humoral and cellular immune responses. It is a novel method involving the injection of the naked DNA plasmid into the host, whose cells express the encoded protein. Mice immunised with plasmid encoding the SAG1 gene (Pit SAG1) showed 80–100% protection. It influenced the immune response towards a Th1 type. Furthermore, DNA vaccine encoding SAG1 increased the survival time of animals and reduced the number of brain cysts in rodents. However, there was a slight increase in survival days of infected mice immunised with pGRA4 or plasmid encoding GRA1, GRA7 and ROP2 or BCG strain secreted GRA1.[39] Recently, genetically modified skins have been developed,

and these grafts produced some therapeutic proteins. The SAG1 gene-vaccinated mouse skin graft was effective for protection against challenge infection.[40, 41] However, the molecular basis of the gene vaccine effect against *T. gondii* infection remains to be determined.

ADJUVANTS

A current area of intense interest is trying to manipulate the response to the vaccine. In particular trying to increase the response and also trying to steer it towards a cell mediated Th1 response rather than an antibody response. Adjuvants are used when the antigen is only weakly immunogenic or there are only small amounts of antigen available. It is not entirely clear how they work. In part they may act as depositories and slowly release the antigen over a period of time. Some antigens are known to bind to Toll like receptors on dendritic cells and macrophages. Activated dendritic cells and macrophages are more phagocytic than their unstimulated counterparts and express higher levels of the molecules that trigger co-stimulation and enhancement of the T-cell response. So in the presence of adjuvant antigen presentation and co-stimulation signals are increased. Only one adjuvant is widely used for human use- alum (aluminium potassium sulphate) which probably primarily acts as a depot. Much more efficient adjuvants are used in animals, these are usually water in oil mixtures (Freund's). Many of these exerimental or veterinary adjuvants have side effects which make them unsuitable for human use.

We have performed experiments on co-immunisation of rSAG1 plus IL-12 adjuvant against lethal challenge with *T. gondii*. Immunisation with rSAG1 alone induces specific humoral type 2 immunity. The results obtained after immunisation of mice by rSAG1 alone were comparable to those obtained with the purified natural protein.[42] The presence of specific anti-rSAG1 IgG1 (titer, 3.8 ± 1.1) and the absence of IgG2a suggest that rSAG1 preferentially induces type 2 humoral immunity. The cytokine pattern produced ex vivo by splenocytes in response to TLA stimulation indicates a decreased type 1 response with reduced IL-2 and IFN-γ production relative to that of the vehicle-treated mice. However, IL-4 production by splenocytes was not significantly modified by rSAG1 immunisation. Addition of IL-12 as an adjuvant abolished the IL-4 production. No IL-10 was detected. These results are reminiscent of those reported by Godard *et al.*, who fragmented SAG1 into five peptides and found that four peptides induced type 2 humoral immunity (exclusive IgG1 production) when they were administered subcutaneously or intravenously to mice.[43] The rSAG1-IL-12 combination reorients the immune response toward the type 1 pattern. In response to TLA stimulation, splenocytes of mice treated with rSAG1 plus IL-12 produced significantly more IL-2 and IFN-γ than the vehicle control group. In contrast, the production of the type 2 cytokine IL-4 was totally abolished in both control mice treated with IL-12 alone and mice treated with the combination rSAG1 plus IL-12. In the IL-12 control group, the production of IL-2 was unchanged relative to that in the vehicle control group, whereas IFN-γ production was curiously reduced. In response to *in vitro* TLA stimulation, we did not observe any exacerbated inflammatory response in the rSAG1-IL-12-treated group, because IL-6 and TNF-α production remained unchanged relative to that in the vehicle control group. No IL-12 production was detected under our experimental conditions, even when a short incubation time (6 h) and different TLA concentrations (0.1 to 10 μg/ml) were used. Nevertheless, this does not rule out very early production after initial contact with the antigen. Some observations indeed suggest that this cytokine is produced within the first few hours of infection, and then production ceases.[44] In addition, rSAG1 plus IL-12 immunisation reduces brain parasite load after peroral infection. Immunisation with rSAG1 alone did not modify the number of cysts compared with that in control mice. In contrast, in the group treated with rSAG1 plus IL-12, parasite load was significantly reduced by 40%.

The natural site of infection for *T. gondii* is the mucosal surface of the intestine. Protective immunity obtained after natural infection with *T. gondii* points to the importance of developing a vaccine that stimulates mucosal defence. When lysate of tachyzoites with cholera toxin (mucosal adjuvant) was administered orally, significant protection was noted in mice. The intranasal route of administration of vaccine is more effective than intragastric immunisation, as it generates an earlier, strong mucosal response. When SAG1 plus cholera toxin was administered intranasally, it provided significant protection and reduced the cyst burden. However, cholera toxin cannot be included in vaccine formulation for use in humans due to its toxicity. LTR72 and LTK63 are the two non-toxic mutants of heat liable enterotoxin which have been used with bacterial antigens and showed protection against different infections after intragastric or nasal vaccination. Mice immunised with SAG1 plus these mucosal adjuvants had significantly fewer cysts. These combinations induced strong systemic and mucosal humoral response. Salbutamol, currently used as an anti-asthmatic drug, was also tested for its potential adjuvant activity for nasal vaccination. Mice vaccinated with SAG1 with salbutamol showed significant decrease in cerebral cysts.[45] Nasal delivery of the antigen SAG1 plus LTR72 as the adjuvant produced a cellular response in local (mesenteric lymph node) and systemic sites. This cellular response was important for protection against infection with *T. gondii*. Now these non-toxic mutants are the most attractive candidates for the development of mucosally delivered vaccine. One of the goals of a vaccination protocol is to be able to appropriately direct the T helper response. In naturally occurring *T. gondii* infection, the Th1 immune response is predominant. Therefore, a vaccination protocol that directs immune response to the Th1 type is desirable. Significant

protection was found with SAG1 in liposome. Liposomes are known to be a particularly effective stimulator of CD8+ T lymphocytes.[46]

FUTURE DIRECTION AND CHALLENGES

There is a strong argument in support of developing vaccines for parasitic diseases. Vaccines have a number of advantages over chemotherapy, in theory a single treatment gives life long protection. Chemotherapy requires repeated doses and for something like malaria you may be taking the drug continuously for years: so there are worries about effects on the host of long term treatment and the accumulation of drug residues in tissues and milk. Long term drug treatment can also be expensive and drug resistance is widespread.

Initially the success of anti-viral and anti-bacterial vaccines suggested that vaccine development for parasitic diseases would be straight forward. However, to date there has only been very limited success in producing effective anti-parasite vaccines.

For successful development of any anti-parasite vaccine, we first need to understand the life cycle of the parasite in order to identify which is the best target stage. For example in malaria an anti-sporozoite vaccine would block infection and be very attractive. An anti-gametocyte vaccine would block transmission, but not do much for the host. Secondly we need to understand the immune mechanisms stimulated by the parasite. Pertinent research question arising shall be whether the effective response is an antibody mediated response or is it primarily a cell- mediated response or does it involve the innate immune response?

Despite the huge investment in time and money there is no commercial vaccine for a human parasitic infection. There are a number of reasons for this failure. Parasites have strategies to avoid and confuse the host immune response, including things like non specific activation of B-cells, so the host produces large amounts of irrelevant antibodies. Moreover, many parasites produce factors (possibly cytokine mimics) which down regulate the cellular response. Protein polymorphism, particularly of surface proteins is common in parasitic infections, so a vaccine against one serotype is ineffective against others. However, an exception is in tickGARD whereby the Bm86 showed very little polymorphism.

Much vaccine development is done using laboratory strains of parasites which may have become atypical and have lost their natural degree of polymorphism. Laboratory passage itself selects for certain phenotypes, for example trypanosomes which have been kept in culture for many years loose the ability to develop in the tsetse fly.

Parasite antigens are complex and difficult to characterise. A nematode may contain 20,000 different proteins, added to which may be post-translational modifications. Carbohydrate side chains are often important in determining antigenicity but such proteins are extremely difficult to analyse. The traditional approach to identifying potential antigens for vaccine development is Western Blotting. The parasite proteins are separated on a gel, blotted onto a membrane and the membrane probed with hyper-immune serum. Bands which give a strong response are subsequently isolated and cloned. The problem is that whilst many proteins are immunogenic few of the antibodies are protective. Quite a lot of the proteins identified by Western Blotting turn out to be internal proteins which are probably released when the parasite die and have nothing to do with immunity. Henceforth, the identification of protective antigens is extremely difficult.

The other classical approach is to start by immunising with whole parasite extracts, if protection is achieved then fractionate the extract until the key protective antigen is found. This approach worked for tickGARD, but often it is the synergy between proteins that leads to protection and fractionation leads to a loss of efficiency.

Much of the research on parasite immunology is done with rodent models and the immune response of rodents are very different from those of man. For example in the rodent, immune killing of schistosomulae takes place primarily in the lungs, in man it takes place in the skin.

The immune response to parasites is often multi-faceted and involves a range of mechanisms, so it may be necessary to activate several different pathways. Different breeds of animals may respond differently. In sheep, for example, the Indonesian thin tailed show a high resistance to *Fasciola gigantica* compared to European breeds, but is equally susceptible to *Fasciola hepatica*. It has been found that in thin- tailed sheep the oxidative burst of macrophages and neutrophils is much more intense than in other breeds. But *F. hepatica* has much higher levels of protective enzymes, particularly glutathione transferase than *F. gigantica*. So *F. hepatica* can cope with the oxidative burst but *F. gigantica* succumbs.

Vaccine development is expensive, registration, particularly for use in humans can be time consuming and difficult, so there has to be a sufficient market to cover this. With human vaccines there is always concern if anything goes wrong or is perceived to go wrong you may be sued. Nevertheless, more research especially into immunologically relevant vaccine antigens and vaccine modalities that work against particular parasites, including the use of adjuvants to improve vaccine efficacy, is needed.

In conclusion, these vaccine candidates (alone or in cocktail) demonstrated the development of significant protection in animal models. The route of administration of vaccine candidates and use of suitable adjuvants have been shown

to be important in eliciting an immune response. Recent progress in characterising potentially protective defence mechanisms during naturally acquired and experimental toxoplasmosis will help to define the immune response which should be elicited by vaccination. Future vaccine design has to account for the stage-specific regulation of immunity against *T. gondii* to prevent abortion and damage as well as to reduce cyst formation in the intermediate host.

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