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Review Article

Tomatine as an adjuvant in malaria vaccine development

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Introduction

Adjuvants are immunogenic compounds that, when combined with an antigen, potentiate an antigen-specific immune response, *i.e.*, they play the role of immunopotentiator. Adjuvants may not only boost the response of an immunologically weak antigen but also influence the type of immune response elicited (1). They are a critical component of the vast majority of vaccines developed to combat disease caused by a wide range of pathogenic microorganisms, including viruses, bacteria and parasitic protozoa. Not only is the field of adjuvant research extremely active, it has become increasingly important as efforts are made to control emerging and reemerging diseases with biological agents (2).

From an historical perspective, the main focus of adjuvant research has been to assess the ability of compounds to elicit humoral immunity, with cellular immune responses largely neglected. Cellular immunity is mediated by specifically sensitized T lymphocytes that produce their effects by direct action, in contrast to the indirect effect of humoral immunity mediated by antibodies produced by B lymphocytes (3). Aluminium-containing compounds, the only adjuvants currently licensed for human use, are poor at stimulating cell-mediated immunity and so are not suitable for use with all antigens (4).

There is therefore a pressing need for a second generation of licensed adjuvants to improve the effectiveness of many poorly immunogenic vaccines presently undergoing preclinical and clinical trials. This necessitates basic research to identify potential adjuvants, to determine their mechanisms of function and to optimize the immune response that they elicit. We have demonstrated tomatine as a promising novel immunopotentiator, being nontoxic in a mouse model at recommended dose regimens and stimulating both humoral and cell-mediated immune responsiveness when coupled to ovalbumin (ova) (5-7). Our current work is focused on the application of tomatine to immunization with a recognized vaccine candidate antigen of the malaria parasite, *Plasmodium*. An efficacious vaccine for mass immunization of humans against this pathogen is widely considered a holy grail of modern molecular medicine.

Regulation of the immune response

The term cytokine is used today as a generic name for a diverse group of soluble proteins and peptides that act as humoral regulators at nano- and picomolar concentrations and which, under normal or pathological conditions, modulate the functional activities of individual cells and tissues. These proteins also mediate interactions between cells directly and regulate processes taking place in the extracellular environment. In many respects the biological activities of cytokines resemble those of classical hormones, although, unlike for hormones, there

is no single organ source of these mediators (8). In a narrower sense, cytokines comprise interleukins, lymphokines, monokines, interferons, colony-stimulating factors, chemokines and a number of other proteins. These subgroups were initially thought to be produced by specific cells – interleukins exclusively by leukocytes, lymphokines by lymphocytes, and monokines by monocytes – or to perform specific functions, for example, interferons to be exclusively involved in antiviral responses, colony-stimulating factors to support growth of cells in semisolid media and chemokines to induce chemotaxis. Subsequently, it has become apparent that these concepts are restricted and have been expanded (9).

Cellular immune responses generated by T lymphocytes of both $CD4^+$ and $CD8^+$ subsets can generally be classified as either type 1 or 2 as determined by the distinct cytokine profile of the antigen-specific T cells which produce them (10). Type 1 responses are characterized by IL-2 and IFN-γ, mediate macrophage activation and antibody-dependent cellular cytotoxicity and promote production of IgG_{2a} opsonizing and complement-fixing antibodies. Type 2 responses feature IL-4, IL-5, IL-10 and IL-13, and provide help for B cell maturation and production of IgG_1 and IgE. Hence, in both mice and humans, T cells secreting type 1 and 2 cytokines broadly affect cell-mediated and humoral immunity, respectively (11).

Type 1 and type 2 cytokine-secreting cells may influence each other through cross-regulation. IFN-γ, for example, can downregulate type 2 clones while type 2 cytokines, notably IL-10, can suppress type 1 functions (12, 13). IFN-γ has been shown to also inhibit the proliferation of T cells secreting type 2 but not type 1 cytokine profiles (14). It thus appears that these functional T cell subsets are mutually antagonistic, such that the balance of subset predominance during an infection may determine the nature of the immune response and thereby also its outcome. In this context, an understanding of the mechanisms for the induction of type 1 and type 2 responses and their different capacities for adjuvantation becomes an important consideration for vaccine development. In general, type 1 responses are most appropriate for combating viruses and intracellular pathogens, while type 2 responses are best suited to respond to free-living bacteria and large, extracellular parasites (15).

Participation of adjuvants in rational vaccine design

Successful elimination of a pathogen following prophylactic immunization depends to a significant degree on the ability of the host's immune system to recognize when it is necessary to become activated and how to respond most effectively, preferably with minimal injury to healthy tissue. In the design of effective, nonreplicating vaccines, in addition to the antigens themselves, immunological adjuvants serve as critical components that instruct and control the selective induction of the appropriate type of antigen-specific immune response (16). Adjuvants are defined as a group of structurally heterogeneous compounds used to evoke or increase an immune response to an antigen (17).

Traditionally, vaccines come in several forms: live-attenuated, replicating pathogens and nonreplicating, inactivated pathogens or their subunits. The latter, nonviable category is the safer, but, because of poor or no immunogenicity, often requires adjuvants (adjuvare is Latin for "to help") to elicit an adequate immune response. A lack of responsiveness may occur in the absence of adjuvant since naive antigen-specific T cells may recognize the antigen but become tolerized (2). There is an increasing trend away from classical attenuated or killed whole pathogen vaccines towards developing chemically defined preparations. Paradoxically, in order to be effective, these synthetic vaccines require incorporation into adjuvants, of which little is actually understood about their mode of action (18). This is an example of the empiricism and exercise of judgement on which vaccine research has been largely based since its infancy. Examples of classic adjuvants include oil emulsions, saponins, aluminium or calcium salts, nonionic block polymer surfactants, derivatives of lipopolysaccharide (LPS) and mycobacteria.

Empirical vaccine investigations were driven mainly by the testing of various antigen preparations and by variations in the choice of adjuvant, dose, route of administration and vaccination schedule without knowledge of protective immune responses. Nevertheless, this trial-and-error approach has resulted in remarkably effective vaccination programs employed against some pathogens of human and veterinary importance (19). In contrast, the rational design of vaccines involves initial identification of immunological correlates of protection, namely the immune effector mechanism(s) responsible for protection against disease, and the subsequent selection of the antigen(s) that elicit the desired adaptive response. A vaccine must then be developed to induce the protective immune responses against the identified target antigen(s) and be delivered effectively to the host's immune system. To this end, advances in biochemistry and molecular biology have resulted in new and refined approaches for antigen preparation. In addition, there has been significant progress in the identification of novel gene products involved in the regulatory pathways governing the immune response (16). The advent of these modern techniques provides the basis to enable the rational design of new adjuvants to direct immune responses during vaccination.

The need for a malaria vaccine

Malaria is caused by protozoan parasites of the genus *Plasmodium*. Each year, 2-3 million children die as a result of *P. falciparum* malaria, and up to 500 million people throughout the world suffer clinical disease (20). Malaria thus ranks alongside acute respiratory infections, measles and diarrheal diseases as a major cause of mortality worldwide. The situation has gradually worsened in

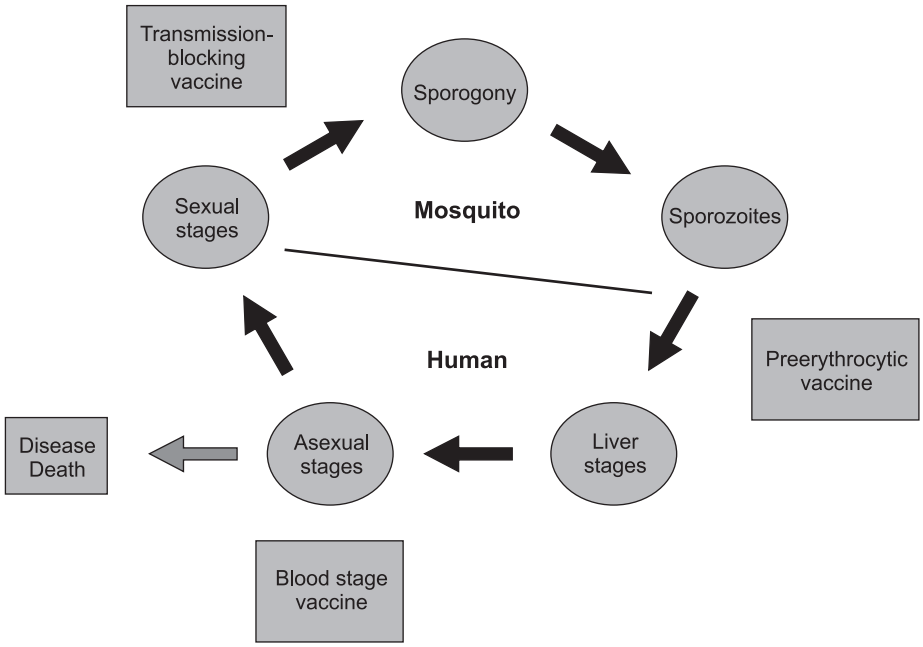

Fig. 1. Schematic representation of the life cycle of the malaria parasite, *Plasmodium*, indicating where vaccination may be expected to intervene.

recent years because of increasing resistance of the anopheline mosquitoes that transmit malaria to insecticides, and of the parasites themselves to antimalarial chemotherapy (21). Thus, the development of an effective malaria vaccine represents a high priority intervention strategy to control both the transmission of infection and the impact of disease (22).

Unlike many acute viral diseases that produce lifelong resistance to reinfection, malaria only elicits immunity after several years' continuous exposure, during which time recurring infections and illness occur. Immunity to malaria acquired in this way is only partially effective and results in milder, sometimes asymptomatic infections in spite of harboring low blood-borne parasitemias. This immunity is short-lived unless reinforced through frequent reinfection and is therefore only acquired by so-called "semi-immune" adults resident in malaria-endemic areas. Consequently, in order to be effective, a malaria vaccine must boost this immune steady state by potentiating the native immune mechanisms through which this has been achieved to rapidly induce extensive and long-lasting protection. In endemic areas, an effective vaccine should protect not only semi-immune persons, but also those who most frequently develop severe forms of malaria (23). More susceptible groups include pregnant women, who are selectively immunosuppressed by a type 2-biased cytokine imbalance (24), and young children, whose maternally derived antibodies have waned but who have not had enough exposure to develop effective antiparasite immunity of their own (25). A prophylactic vaccine would also protect individuals resident in non-malarious areas, who have little or no specific acquired immunity, from contracting severe disease while travelling through regions of high endemicity. These include transmigrants, refugees, tourists and military personnel.

A malaria vaccine could act at one of several distinct stages of the *Plasmodium* life cycle (Fig. 1), but for prophylactic purposes a preerythrocytic vaccine is required as it aims to prevent or reduce the acquisition of clinical infection. By preventing either invasion of hepatocytes by sporozoites (antibodies) or exoerythrocytic stage development within hepatocytes (T cells, cytokines and perhaps antibodies), a vaccine targeting preerythrocytic stages in the liver would preclude both the progression of disease, since clinical symptoms of malaria manifest only during the subsequent erythrocytic stage, and parasite transmission, since no gametocytes would develop (26, 27). This would benefit individuals who either are malaria-naive or who have lost their previously acquired immunity. It would also enhance the naturally acquired protective immune response of individuals resident in malaria-endemic countries that is achieved upon prolonged exposure, in order to either prevent blood stage infection or to reduce the numbers of parasites that emerge from the liver (28).

The advent in recent years of molecular tools to identify, produce and study malarial antigens has encouraged construction of subunit vaccines containing multiple targets at each stage of parasite development. In a bid to overcome the dual difficulty of poor immunogenicity and parasite diversity, much experimental work is now focused on complex antigenic constructs delivered as synthetic peptides, recombinant proteins, live vectors or naked DNA, using different adjuvants and/or prime-boost strategies (29).

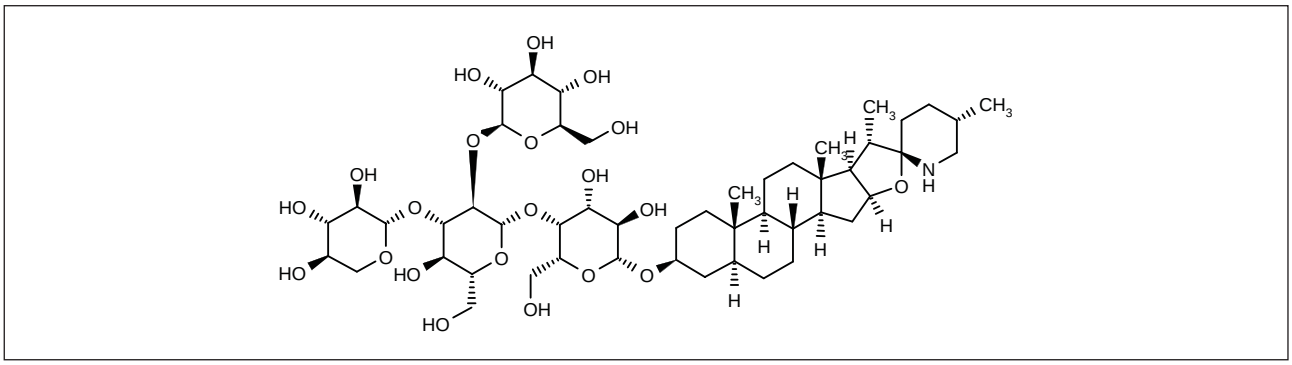

Fig. 2. Structure of tomatine, also known as lycopersicin. Molecular formula: $C_{50}H_{83}NO_{21}$. Tomatine consists of 1 mol tomatidine linked to a tetrasaccharide composed of 2 mol D-glucose, 1 mol D-xylose and 1 mol D-galactose.

Immunity to preerythrocytic malaria

Studies of individuals naturally infected with malaria (30-33), of volunteers immunized with attenuated sporozoites (34, 35) and of model malaria systems (36-39) indicate that antigen-specific $CD8^+$ T cells are effective at eliminating preerythrocytic stage parasites that reside in the liver.

Antigens expressed by malaria-infected hepatocytes are processed via the endogenous pathway and presented to $CD8^+$ T cells in association with major histocompatability complex (MHC) class I molecules. It is generally thought that a liver stage vaccine against the *Plasmodium* parasite will require the induction of antigen-specific $CD8^+$ T cells (27). However, most vaccines deliver exogenous antigen to the host's immune system by the MHC class II pathway, thereby eliciting a humoral and cellular $CD4^+$ T cell response but not a $CD8^+$ T cell response. This is problematic for vaccine design against pathogens that require a protective $CD8^+$ T cell response, including intrahepatic malaria parasites, as soluble antigens are mainly presented by MHC class II molecules (40). Hence, there is a pressing need to develop and validate novel adjuvants that allow synthetic peptides to access the MHC class I antigen processing pathway and thereby potentiate $CD8^+$ T cell-mediated immunity. While a handful of such adjuvants have been described over the past decade (1), very few have been evaluated in malarial vaccine models. Here, we describe a novel formulation based on a plant extract known as tomatine which has powerful adjuvant properties.

The natural history of tomatine

Tomatine, also called lycopersicin (Fig. 2), is an alkaloid glycoside that is found abundantly in the leaves of the wilt-resistant wild tomato *Lycopersicon pimpinellifolium* (41). Extracted concentrations are typically 1000 mg/kg fresh weight leaves (42). It is also present in high levels (≤ 500 mg/kg fresh weight fruit) in immature green-colored tomato fruit, where its toxicity acts as a resistance mechanism against predation from both invertebrate and vertebrate pests (41, 43). As the tomato ripens to red, tomatine is partly degraded to concentrations (< 5 mg/kg fresh weight) that are no longer toxic to consumption by birds and mammals, egestion from which is the main route of seed dispersal. Domesticated tomatoes may also contain tomatine but it has been eliminated from commercially grown varieties through plant selection.

Discovery of tomatine as a potential novel adjuvant

In vitro studies have previously revealed that tomatine forms a strong 1:1 complex with cholesterol in aqueous media (44), following which tomatine has attracted interest as a possible agent to reduce dietary cholesterol absorption (45, 46). Our interest in tomatine started through a collaboration with Dr. George Attard, Department of Chemistry, University of Southampton, U.K. Together, we carried out a systematic study of the relationship between the chemical structure of tomatine and other amphiphilic alkaloid glycosides, the types of aggregation structures they form and their adjuvant potency (5-7). Upon mixing an aqueous solution of tomatine with cholesterol, a fine precipitate was obtained which consisted of a molecular mixture of the two components. Electron microscopy (EM) that was carried out on ova formulated with tomatine revealed several micro- and nanostructures. The adjuvant-antigen mixture was separated by isopycnic ultracentrifugation followed by freeze fracturing and then subjected to transmission and scanning EM. The major fraction was shown to consist of cylindrical needle-like microcrystals, approximately 100-160 nm in width and 2-4 μm in length. The tomatine crystals were shown to be elongated hollow tubular crystals in length, along which *n*-octyl-β-glucopyranoside was speculated to serve as a seeding microtemplate for gel crystallization of protein complexes. Indented marks within the gel phase were observed in the freeze-fractured replicas of the adjuvant, suggesting that protein complexes may have crystallized within the gels. Several other forms of micro- and nanostructures were also observed,

showing multiple dispersion features with gel characteristics. While the biophysical properties of tomatine are still undergoing investigation, we postulate that the presence of gel crystalline and multiple dispersed phases contribute to the sustained immunopotentiation it appears to induce (47). For the purposes of application to a synthetic vaccine, this structure would promote a more efficient transportation around the body from the site of inoculation than would otherwise be achieved.

Tomatine causes no significant side effects

As a prerequisite to investigating tomatine as a potential adjuvant for vaccine delivery, preliminary toxicity studies were performed. We have shown that tomatine is safe and well tolerated in mice as it does not elicit hemolytic activity, granuloma formation or tissue damage at the site of inoculation. However, mononuclear cells infiltrate within 24 h postimmunization, indicating the recruitment of immunological mediators (5). The adjuvant-antigen preparation consists of a colloidal suspension of solid-state aggregates (0.1-4.0 μm) containing the antigen, tomatine and cholesterol (47).

Adjuvanticity of tomatine using a model antigen

We initially used the model antigen ova to assess the adjuvanticity of tomatine in mice by comparison to the benchmark adjuvants Freund's incomplete adjuvant and alum (6, 7). The cytokine profiles of splenocytes derived from immunized mice following restimulation with ova *in vitro* indicated that whereas Freund's incomplete adjuvant and alum both elicited a type 2-specific response, tomatine potentiated predominantly type 1-specific immunity. Assessment of the adjuvant's ability to induce an ova-specific cytolytic response showed that only splenocytes derived from ova-tomatine immunized mice were able to lyse target cells *in vitro* (6). The response was shown to be MHC class I-restricted, ova peptide-specific and $CD8^+$ T cell-dependent (6). This provided a rationale for using tomatine as an adjuvant for a preerythrocytic malaria vaccine that would specifically promote a $CD8^+$ T cell-dependent type 1-polarized response. From our previous findings (48, 49), we believe that $CD8^+$ T cell release of IFN-γ is a critical component of immunity induced by liver stage malaria, and that successful immunization of humans with vaccines designed to elicit protective immunity will require induction of specific $CD8^+$ T cells that home to the liver (50).

Vaccination with *Plasmodium* circumsporozoite protein

There are many species of malaria parasite that infect mammals other than humans. *Plasmodium berghei* is one of four species that have been described in rodents of central Africa. These rodent parasites are not of direct concern to man or his domestic animals, but serve as valuable practical models for the experimental study of human malaria. They have proved to be analogous to the malarias of man and other primates in most essential aspects of structure, physiology and life cycle (51). For this reason, they have been used extensively to investigate parasite-host interactions, vaccine development and drug testing. With particular regard to gaining an insight into natural or vaccine-induced protective immunity *in vivo*, murine malarias offer an advantage over simian models in that they are easy and cheap to maintain, the mouse immune system is well characterized and large-scale dissective or intervention studies of a nature not ethically permissible in humans or practicable in nonhuman primates may be performed (52). Thus, preclinical vaccine trials are routinely conducted in murine models to validate candidate antigens prior to testing in humans. One such antigen that has been studied extensively is the immunodominant circumsporozoite (CS) protein that is the major surface protein of sporozoites, the hepatocyte-invasive stage of the *Plasmodium* life cycle (53). Vaccination with the defined *P. berghei* CS peptide SYIPSAEKI (9-mer aa 252-260) confers protection against homologous challenge in mice which express MHC class I molecules of the H2-k^d haplotype when CS peptide-specific $CD8^+$ T cells are elicited (37, 54, 55). As both the target antigen and the protective immune response to it in this malaria model are characterized this provides a powerful tool for vaccinologists, as the potential of novel adjuvants to elicit an antigen-specific MHC class I-restricted cytotoxic response may be assessed.

The objectives of our present work (56, Heal *et al*., unpublished data) are to evaluate the ability of tomatine to potentiate a cytotoxic T cell response against the *P. berghei* CS peptide and to determine if and to what degree such a response confers protection *in vivo* to homologous parasite challenge. The most successful vaccine against human malaria to date, protecting 6 of 7 volunteers, contains regions of *P. falciparum* CS protein that stimulate peptide-specific $CD8^+$ T cell responsiveness (57).

Preparation and immunization of the antigen-tomatine vaccine

Tomatine was prepared with the *P. berghei* CS 9-mer peptide, as described previously (5, 7). Briefly, the adjuvant comprised two solutions, A and B, which were formulated as follows. A: 6.25% (v/v) tomatine (Fluka), 31.25% octylglucopyranoside (Sigma) and 0.75% phosphatidyl-ethanolamine (PE) (Fluka) in sterile saline, which was vortexed, heated to 60 °C until clear, then cooled to room temperature. B: 2.1% cholesterol (Sigma), 41.7% octylglucopyranoside and 1.0% PE in sterile saline, the solution was then prepared as for A and allowed to cool to 37 °C. *P. berghei* CS peptide SYIPSAEKI was prepared by solid phase chemical synthesis and confirmed as

> 80% full-length product by high performance liquid chromatography and mass spectrophotometry. 2.5 mg peptide was dissolved in 3 ml sterile saline and added to 4 ml of solution A, vortexed and incubated at 37 °C for 10 min, after which 3 ml of solution B was added, vortexed and incubated at 37 °C for a further 30 min. After vortexing, the completed formulation was placed at 37 °C for 24 h after which the resultant cloudy solution was dialyzed against sterile saline using a 10,000 MW cut-off membrane (Slide-a-lyzer, Pierce) to remove any unassociated octylglucopyranoside. A saline-tomatine adjuvant control was prepared by an identical method but without the addition of the CS peptide.

Experimental groups comprised naive mice or mice immunized with a preparation of either *P. berghei* CS peptide-tomatine or saline-tomatine. BALB/c ($H2^d$) inbred strain mice (Harlan Olac) were used when 6-8 weeks old. Female mice were injected subcutaneously in the scruff of the neck with 200 µl of the antigen-adjuvant preparation (50 µg peptide/mouse) on day 0 and then again 28 days later (56).

Cytokine production following stimulation with *P. berghei* CS peptide

Spleens were aseptically removed from naive mice and from immunized mice 14 days after the boost immunization was administered. To generate cytokine-containing supernatants, single cell suspensions were adjusted to a final concentration of 5 x 10^6/ml in RPMI 1640 (Gibco) supplemented with 10% (v/v) heat-inactivated fetal calf serum (complete medium) and 100 µl aliquots placed in 96 well flat-bottom tissue culture plates, to which were added 100 µl volumes of complete medium alone, or containing final concentrations of one of the following: 25 µg/ml *P. berghei* CS 9-mer peptide; 1 µg/ml concanavalin A (Con A; Sigma); 25 µg/ml *Escherichia coli* lipopolysaccharide (LPS; Sigma) (58). Cultures were incubated for 6 days (37 °C, 5% CO_2), supernatants were removed, centrifuged at 300x g for 5 min and stored at –20 °C until assayed. Levels of the cytokines IL-4, IL-10, IL-12, IFN-γ and TNF-α were quantified by two-site sandwich enzyme-linked immunosorbent assay (ELISA) (59).

Following restimulation *in vitro* with *P. berghei* CS 9-mer peptide (25 µg/ml), splenocytes from mice immunized with CS peptide-tomatine showed significantly upregulated production of IFN-γ when compared to splenocytes from either tomatine-saline-immunized mice or naive controls (Fig. 3). Production of the other type 1 cytokines measured, IL-12 and TNF-α, was similar for all three groups (56). The relatively low antigenicity of tomatine on its own was exemplified by the similar production of each of IL-12, IFN-γ and TNF-α by splenocytes from saline-tomatine-immunized mice compared to controls (Fig. 3). This therefore demonstrated the capacity of tomatine to act as an adjuvant for delivery of *P. berghei* CS peptide, notably for the induction of the pronounced production of antigen-specific IFN-γ.

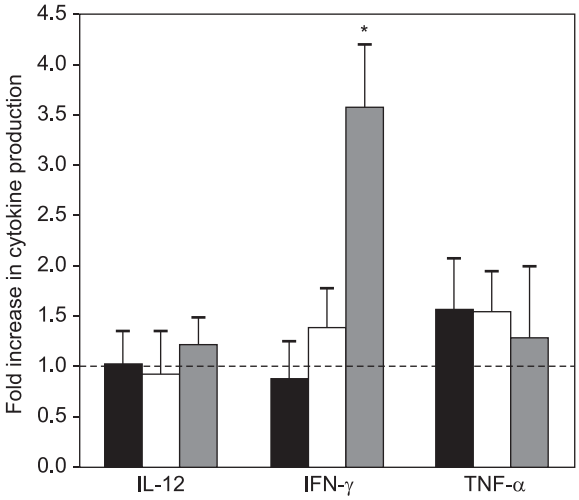

Fig. 3. Type 1 cytokine profiles of splenocytes from mice immunized with antigen-adjuvant preparations of *P. berghei* CS peptide aa 252-260-tomatine (▒) or saline-tomatine (□) following *in vitro* restimulation with *P. berghei* CS peptide. Control cytokine production of similarly stimulated splenocytes from naive mice is also shown (■). Data represent fold increases in secretion of each cytokine over that of splenocytes from identically immunized mice within each group but which were not restimulated *in vitro*, for which by convention, the value of cytokine production = 1.0 (depicted as a horizontal line). Cytokine production by splenocytes not restimulated *in vitro* were similar for all three treatment groups; pooled mean ± SD (pg/ml) were IL-12, 97.8 ± 21.2; IFN-γ, 153.1 ± 32.7; TNF-α, 39.1 ± 5.6. Data shown represent the mean ± 1 SD of 4 mice, assayed individually in triplicate, from 1 of 4 similar experiments. IFN-γ production: *p <0.05 *vs.* □; p <0.04 *vs.* ■. Modified from ref. 56.

In the presence of homologous peptide (25 µg/ml), splenocytes from CS peptide-tomatine immunized mice did not elicit a type 2-specific cytokine profile when compared to the response of similarly stimulated splenocytes from the adjuvant control and naive mice (56). IL-4 and IL-10 were measured in all supernatants but production of each was below the level of detection of the respective ELISA. Stimulation with the mitogens Con A or LPS induced splenocytes from each experimental group to produce predominantly type 1 and type 2 responses, respectively.

Cytolytic activity following stimulation with *P. berghei* CS peptide

The cytotoxic T lymphocyte (CTL; cytolytic) activity of splenocytes was assessed by coincubation with P815 target cells labeled with ^{51}Cr (Amersham Int.) and loaded with peptide. P815 cells express $H2\text{-}k^d$ molecules (37). In the presence of homologous peptide (25 µg/ml), cells derived from *P. berghei* CS 9-mer peptide immunized mice produced significantly raised peptide-specific CTL activity at 100-50:1 E:T ratios compared to splenocytes

from either saline-tomatine-immunized mice or from naive controls (shown for 100:1 E:T ratio in Fig. 4) (also $p < 0.025$ and $p < 0.05$, respectively, at a 50:1 E:T ratio). In contrast, splenocytes derived from mice immunized with the saline-tomatine preparation showed very low CTL activity, significantly less than that exhibited by naive control cells (Fig. 4) (56). Restimulation of splenocytes with tomatine *in vitro* led to uniformly residual CTL activity, thereby providing further indication of tomatine's adjuvant but not antigenic properties. Also of note was the increased target cell lysis upon incubation with splenocytes from CS peptide-immunized mice without restimulation *in vitro*, compared to that for cells from naive or saline-tomatine-immunized mice similarly cocultured with unloaded target cells (Fig. 4). This suggested that *in vivo* priming to the CS 9-mer peptide was sufficient to elicit at least some CTL activity without homologous restimulation *in vitro*. The cytolytic T cell clone CS.C7 specific for CS aa 252-260 (37), which was assayed as a positive control, elicited 81.2% and 31.6% P815 cell lysis at an E:T ratio of 100:1 and 25:1, respectively.

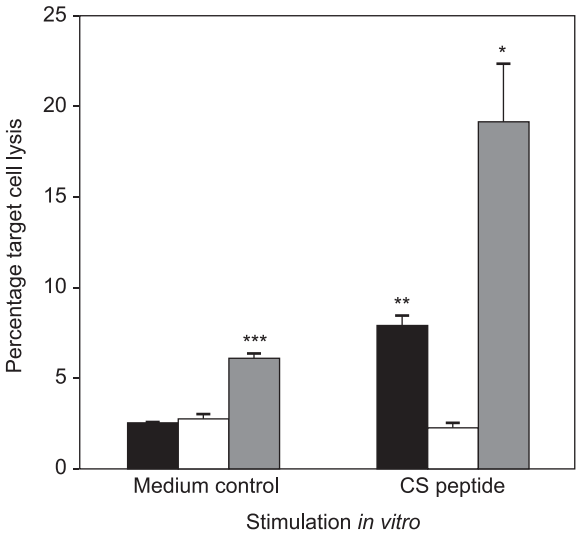

Fig. 4. Cytolytic activity of splenocytes from mice immunized with adjuvant-antigen preparations of *P. berghei* CS peptide aa 252-260-tomatine (▩) or saline-tomatine (□) following *in vitro* restimulation with *P. berghei* CS peptide. Control cytolytic activity of similarly stimulated splenocytes from naive mice is also shown (■). Data represent percentage specific lysis of P815 target cells loaded with *P. berghei* CS peptide or with no peptide (medium control) at an effector:target cell ratio of 100:1. Similar results were obtained at an effector:target ratio of 50:1. Data shown represent the mean ± 1 SD of 4 mice, assayed individually in triplicate, from 1 of 4 similar experiments. IFN-γ production following peptide stimulation: $*p < 0.025$ *vs.* □; $p < 0.05$ *vs.* ■; $**p < 0.05$ *vs.* □. IFN-γ production in the absence of peptide stimulation: $***p < 0.05$ *vs.* □ and *vs.* ■. Modified from ref. 56.

Postvaccination challenge with *P. berghei* sporozoites

Sporozoite challenge experiments were conducted to determine whether the enhanced cytotoxic immunity that tomatine promoted translated directly to a protective effect *in vivo*. 14 or 21 days after the boost immunization, naive mice and mice immunized with *P. berghei* CS peptide-tomatine or saline-tomatine were infected with *P. berghei* by mosquito bite. Infectious, *P. berghei* sporozoite-bearing *Anopheles stephensi* mosquitoes (17 days old) were allowed to feed on the shaved abdomen of mice for 15 min. The development of blood-borne parasitemias was determined daily by examination of Giemsa's stained thin blood smears by light microscopy for parasitized erythrocytes (60). Approximately 80% of mosquitoes fed to engorgement, indicative of successful transmission of sporozoites to the host animal, and mice were monitored daily thereafter for the development of parasitemia.

All mice challenged with *P. berghei* sporozoites developed clinical disease following the release of parasites from the liver into the bloodstream. However, mice immunized with *P. berghei* CS peptide-tomatine showed a markedly delayed onset of erythrocytic infection, indicative of an effective but nonsterilizing immune response to preerythrocytic parasites in the liver (56). Hence, this group of mice consistently had a significantly extended time to reach 2% parasitemia (that parasite density at which disease manifestations start to be observed) compared to either naive mice or those immunized with a saline-tomatine preparation (Table I). Moreover, while mice in these latter two groups showed parasitemias > 2% from 9 days postchallenge, those inoculated with the adjuvant-antigen maintained subclinical parasitemias for between a further 2-6 days, depending on the timing of challenge (Table I). In contrast, there was no significant difference for either of the parameters measured between naive and saline-tomatine-immunized mice, suggesting that despite its demonstrable adjuvanticity, tomatine alone does not protect against preerythrocytic malaria.

Tomatine holds promise for malaria vaccine adjuvantation

The purpose of an adjuvant is to elicit an appropriate and effective immune response against the antigen(s) with which it is administered. Protective antigens generally require a specific type of response to be induced if the immunized host is to effectively combat a challenge infection. Studies in various murine malaria models have shown that animals immunized with radiation-attenuated sporozoites develop parasite-specific $CD8^+$ T cells and that depletion of such cells abrogates protection (36, 61, 62). Therefore, a vaccine targeting the liver-dwelling stage of the *Plasmodium* life cycle should theoretically favor a vaccine-adjuvant formulation that induces a $CD8^+$ T cell-biased response by antigen delivery via the MHC class I pathway.

Immunization regimens were performed in mice with the defined antigen *P. berghei* CS 9-mer peptide

Table I: Determination of protective capacity of CS peptide-tomatine immunization by challenge of vaccinated mice with Plasmodium berghei *sporozoites.*

Time of challenge[a] (days postbooster)	Parameter measured[b]	Experimental groups (preparation immunized) Naive	Saline-tomatine	CS 9-mer-tomatine
14	Time to 2% parasitemia	9 ± 0	9 ± 0	11 ± 0*
21	(days postchallenge)	9 ± 0	9 ± 1	14 ± 1^{+}
14	% parasitemia	2.64 ± 0.53	2.58 ± 0.47	1.41 ± 0.32*
21	9 days postchallenge	2.71 ± 0.49	2.47 ± 0.68	0.37 ± 0.45^{+}

[a]Female BALB/c mice were immunized subcutaneously with 200 µl antigen-adjuvant preparation (50 µg peptide/mouse) on day 0 and again on day 28 (booster). Either 14 or 21 days later, mice (6 per group) were challenge infected by exposure to *P. berghei* sporozoite-infected *Anopheles stephensi* mosquitoes. [b]Erythrocytic parasitemias were determined daily by examination of Giemsa's stained thin blood smears by light microscopy. Values are mean ± SD of results pooled from 6 repeat experiments. *$p < 0.005$ compared to other experimental groups; $^{+}p < 0.0025$ compared to other experimental groups.

aa 252-260 to examine the capacity of tomatine to potentiate antigen-specific cellular immune responses to preerythrocytic malaria. As described above, when restimulated with homologous peptide *in vitro*, splenocytes derived from mice immunized with the CS peptide-tomatine vaccine upregulated production of the type 1 cytokine IFN-γ and elicited a peptide-specific cytolytic response. This supports our hypothesis that immunization with the CS peptide-tomatine vaccine might elicit a $CD8^+$ T cell response if this peptide is presented in association with MHC class I molecules (50, 56).

Passive transfer of a CTL clone recognizing *P. berghei* CS peptide aa 252-260 derived from mice immunized with irradiated *P. berghei* sporozoites conferred a high degree of protection to mice against homologous sporozoite challenge (37). Further work has shown that immunity against preerythrocytic stages of the malaria parasite is dependent upon the activation of $CD8^+$ T cells (31, 34, 35, 38, 39). Candidate antigens for a malaria preerythrocytic vaccine are commonly tested for their ability to induce $CD8^+$ T cells using a CTL assay. CTL activity against target cells presenting the *P. berghei* CS 9-mer peptide indicates that MHC class I presentation was induced through immunization with the peptide-tomatine preparation. The percentage specific cell lysis elicited by the CS peptide-tomatine vaccine is comparable with that engendered by other vaccine delivery systems when this peptide was presented alone (54, 63).

The pivotal importance of IFN-γ to the immune response to preerythrocytic malaria is well established. Recombinant IFN-γ inhibits the *in vitro* development of intrahepatic parasites (64, 65), while anti-IFN-γ monoclonal antibody treatment abrogates protection in mice immunized with radiation-attenuated sporozoites (36, 66). Administration of IFN-γ or IL-12 protects mice (64, 67) and monkeys (68, 69) against preerythrocytic malaria, and in mice the protection is reduced when the synthesis of nitric oxide (NO) is inhibited (67). These findings suggest that $CD8^+$ T cells may additionally perform a noncytolytic role in this protective immunity. Following induction by IL-12, $CD8^+$ T cells produce IFN-γ which stimulates the production of inducible NO that subsequently mediates the elimination of the liver stage parasite (70, 71). Indeed, perforin-deficient, CD95- and CD95L-mutant mice immunized with irradiation-attenuated sporozoites were each shown to be protected against a *P. berghei* challenge infection (72), indicating that this response alone is protective. It is probable, therefore, that sensitization of a $CD8^+$ T cell population to produce high levels of IFN-γ promotes the induction of both cytolytic and noncytolytic mechanisms of protective immunity (50).

Protection by CS peptide-tomatine vaccination

Mice vaccinated with the *P. berghei* CS 9-mer peptide-tomatine preparation not only elicited pronounced IFN-γ and CTL responses, but also showed a significant degree of protection in as much as there was a delay of 2-6 days in time to 2% erythrocytic parasitemia (56). Despite this, immunity to preerythrocytic parasites was not sterilizing and blood stage infection was eventually established. However, we consider that this delayed onset of erythrocytic parasitemia provides an extremely promising premise for further vaccine evaluation for two reasons. First, in these experiments peptide-tomatine was immunized in the absence of a T helper epitope, the inclusion of which may be expected to contribute to the expansion of $CD8^+$ CTL through the priming of $CD4^+$ T cells (27, 48). Therefore, while $CD8^+$ T cells are critical effector cells in the malarious liver, $CD4^+$ T cells may be required for the optimization of the protective response (50). Second, although adjuvants based on polymer microspheres (63), lipopeptides (73, 74), DNA (55), cationic lipids (75), *Salmonella* (76) and the viruses adenovirus (77), influenza (78) and vaccinia (56, 78) have been shown previously to elicit a CTL response against *Plasmodium* CS protein, none of these formulations alone was able to induce complete protection against sporozoite challenge. Indeed, our results stand comparison with any reported homologous immunization regime (50). The only CS-peptide-based vaccine protocol able to confer sterile immunity against malaria reported to date used a heterologous prime-boost strategy of DNA

followed by live, attenuated recombinant vaccinia virus (55). The successful vaccines contained either the entire *P. berghei* CS sequence or the CS peptide aa 252-260, but in each case together with a string of *P. falciparum* preerythrocytic stage T cell epitopes. Such a construct containing the CS 9-mer peptide is currently undergoing clinical trials in Oxford, U.K. and The Gambia (79).

Putative mechanism of action of tomatine

The mechanism by which tomatine is able to deliver antigen in order that it is presented by MHC class I molecules to $CD8^+$ T cells has yet to be established. The glycoalkaloid group of compounds to which tomatine belongs has been shown to disrupt mammalian cell membranes by interacting with sterols (80, 81) and tomatine has specifically been reported to increase the permeability potential of the cell membrane (82). Properties of an adjuvant that change the membrane of a cell may have a role in the delivery of an antigen to it and affect the pathway by which that antigen is processed. We have recently shown that depletion of splenic or lymph node macrophages does not affect the CTL activity detected after immunization with the ova-tomatine vaccine (Sheikh *et al*., unpublished data). This provides indirect evidence that by using tomatine as an adjuvant, dendritic cells rather than macrophages may principally present exogenous antigen to $CD8^+$ T cells.

Future research

Current experiments are exploiting the *P. berghei* CS 9-mer peptide to optimize the use of tomatine as a vaccine delivery system and to identify cytokine production of defined lymphocyte populations. Preliminary findings indicate that when splenocytes are separated into specific T cell fractions the major source of IFN-γ is the $CD8^+$ T cell population (Heal *et al*., unpublished data). Additional work is required to determine the conditions under which this vaccine, either as a homologous or a heterologous immunization regimen, may prevent or further delay the onset of blood stage malaria infection.

Conclusions

We have previously demonstrated that the novel glycoalkaloid tomatine, derived from leaves of the wild tomato *Lycopersicon pimpinellifolium*, can act as a powerful adjuvant for the elicitation of antigen-specific $CD8^+$ T cell responses to the model antigen ova (5-7). In order to validate tomatine as an adjuvant for cellular immune responsiveness against infectious diseases of medical or veterinary importance, we have exploited an established malaria infection system in mice to evaluate immunity to a major preerythrocytic stage malaria vaccine candidate antigen when administered with tomatine (56). The defined MHC H-$2k^d$ class I-binding 9-mer peptide aa 252-260 from *P. berghei* CS protein was prepared with tomatine to form a molecular aggregate formulation and this was used to immunize BALB/c (H-$2k^d$) mice. Antigen-specific IFN-γ secretion and CTL activity *in vitro* were both significantly enhanced compared to responses detected from similarly stimulated splenocytes from naive and saline-tomatine-immunized control mice. Hence, the processing of the *P. berghei* CS 9-mer peptide as an exogenous antigen and its presentation via MHC class I molecules to $CD8^+$ T cells led to an immune response that is an *in vitro* correlate of protection against preerythrocytic malaria. Moreover, when challenged with *P. berghei* sporozoites, mice immunized with the CS 9-mer peptide-tomatine preparation had a significantly delayed onset of erythrocytic infection compared to controls. The protective capacity of this CS peptide-tomatine combination upon *in vivo* immunization is thus indicated. These findings merit further work to optimize the use of tomatine and derivative preparations as adjuvants in malaria vaccine development. In addition, as tomatine is nontoxic and inexpensive to manufacture, we have the potential to develop and commercialize a family of compounds that may enhance the efficacy of synthetic vaccines against other important intracellular pathogens, including HIV and *Mycobacterium tuberculosis*.

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