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Research Article

Tomatine Adjuvantation of Protective Immunity to a Major Pre-erythrocytic Vaccine Candidate of Malaria is Mediated via $CD8^+$ T Cell Release of IFN-γ

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The glycoalkaloid tomatine, derived from the wild tomato, can act as a powerful adjuvant to elicit an antigen-specific cell-mediated immune response to the circumsporozoite (CS) protein, a major pre-erythrocytic stage malaria vaccine candidate antigen. Using a defined MHC-class-I-restricted CS epitope in a *Plasmodium berghei* rodent model, antigen-specific cytotoxic T lymphocyte activity and IFN-γ secretion ex vivo were both significantly enhanced compared to responses detected from similarly stimulated splenocytes from naive and tomatine-saline-immunized mice. Further, through lymphocyte depletion it is demonstrated that antigen-specific IFN-γ is produced exclusively by the $CD8^+$ T cell subset. We conclude that the processing of the *P. berghei* CS peptide as an exogenous antigen and its presentation via MHC class I molecules to $CD8^+$ T cells leads to an immune response that is an in vitro correlate of protection against pre-erythrocytic malaria. Further characterization of tomatine as an adjuvant in malaria vaccine development is indicated.

1. Introduction

Adjuvants are immunogenic compounds that, when combined with an antigen, potentiate an antigen-specific immune response. Adjuvants may not only boost the response of an immunologically weak antigen but also influence the type of immune response elicited [1]. While it has been know for many years that formulating antigen(s) with adjuvants may potentiate the magnitude of vaccine-elicited immune response, traditionally accepted adjuvants such as alum and various oil-in-water emulsions have failed to induce cytotoxic T lymphocyte (CTL) responses. However, the ability of new generation adjuvants to deliver soluble protein to the major histocompatibility complex (MHC) class I processing pathway, thereby inducing antigen-specific CTL responses, has been recognized recently [1, 2]. The development of tomatine as an immunostimulating agent was initiated as a direct response to the hypothesis that reagents that were capable of delivering soluble protein into the class I pathway would induce antigen-specific CTL responses, and thus were likely to be powerful adjuvants [3].

The adjuvant tomatine is based upon the glycoalkaloid lycopersicon ($C_{50}H_{83}NO_{21}$), which is derived from the leaves and unripe fruit of the wilt-resistant wild tomato species *Lycopersicon pimpinellifolium.* This compound has been shown to have membrane-disrupting qualities [4, 5], similar in character to that of saponins which have long been established as potent immunostimulators [6]. In its naturally occurring form tomatine is known to be a primary toxicity-based plant defense mechanism against viral and bacterial pathogens; furthermore, it prevents infestation by arthropods and discourages ingestion (of unripe tomatoes) by vertebrates [7]. In spite of this, tomatine is safe and well tolerated in mice as it does not elicit haemolytic activity, granuloma formation, or tissue damage at the site of inoculation. However, mononuclear cells infiltrate within

24 hours post immunization, indicating the recruitment of immunological mediators [8]. The adjuvant-antigen preparation consists of a colloidal suspension of solid-state aggregates (0.1–2 μm) containing the antigen, tomatine and cholesterol [8].

The malignant tertian malaria of humans, *Plasmodium falciparum*, kills 2-3 million people and causes a further 500 million clinical infections annually. Due to increased resistance of the parasite to available drugs and of the mosquito vector to insecticide treatment there is a pressing need for novel control measures, one of which is an antimalarial vaccine [9]. For prophylactic purposes, a pre-erythrocytic vaccine is required as it aims to prevent or reduce the acquisition of clinical infection. By preventing either invasion of hepatocytes by sporozoites or pre-erythrocytic stage development within hepatocytes, a vaccine targeting 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 sexual stages would develop [10]. This would benefit individuals who either are malaria naive or 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 [11].

Preclinical vaccine trials may be conducted in murine malaria 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 [12]. Passive transfer of a CTL clone recognizing *P. berghei* CS peptide SYIPSAEKI (aa 252–260) derived from mice immunized with irradiated *P. berghei* sporozoites conferred a high degree of protection to mice against homologous sporozoite challenge [13]. Furthermore, vaccination with this defined *P. berghei* CS peptide conferred 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 were elicited [14]. 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 CTL response may be assessed.

The objective of this study was to exploit the ability of the novel adjuvant tomatine to potentiate a CTL response against the *P. berghei* CS peptide in order to identify cytokine production of defined lymphocyte populations. This would have the dual effect of both helping to evaluate further the mechanism of action of tomatine as a vaccine adjuvant and to elucidate the mechanism of protective immunity against pre-erythrocytic malaria. The most successful vaccine against human malaria to date, protecting six of seven volunteers, contains regions of CS protein that stimulate peptide-specific CD8^{+} T cell responsiveness [15, 16].

2. Materials and Methods

2.1. Preparation of Tomatine-Antigen Vaccine. Tomatine was prepared with the *P. berghei* CS peptide by minutesor modification of a protocol described in detail previously [8, 17]. Briefly, the adjuvant comprised two mixtures, A and B, which were formulated as follows. *Mixture A*. 25 mg tomatine (Fluka, Gillingham, UK), 125 mg octylglucopyranoside (Sigma, Poole, UK) and 3.1 mg phosphatidylethanolamine (PE) (Fluka) were added to 4 mL sterile saline, vortexed and heated to 60°C until a clear solution was obtained, which was then allowed to cool to room temperature. *Mixture B*. 6.25 mg cholesterol (Sigma), 125 mg octylglucopyranoside and 3.1 mg PE were added to 3 mL sterile saline, the solution then prepared as for mixture A and allowed to cool to 37°C. *P. berghei* CS peptide SYIPSAEKI (aa 252–260) was prepared in-house by solid phase chemical synthesis and confirmed as 75% 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 mixture A, vortexed and incubated at 37°C for 10 minutes, after which mixture B was added, vortexed and incubated at 37°C for a further 30 minutes. After vortexing, the completed formulation was placed at 37°C for 24 hours after which the resultant cloudy solution was dialyzed against sterile saline using a 10000 MW cutoff membrane (Slide-a-lyzer, Pierce, Chester, UK) to remove any unassociated octylglucopyranoside. A tomatine-saline adjuvant control was prepared by an identical method but without the addition of the CS peptide.

2.2. Immunization. Three experimental groups comprised naive mice or mice immunized with a preparation of either tomatine-*P. berghei* CS peptide or tomatine-saline. BALB/c (H2^{d}) inbred strain mice (Harlan Olac, Bicester, UK) were used when 6–8 weeks old. Female mice (four per group) were injected subcutaneously in the scruff of the neck with 200 μL of the adjuvant-antigen preparation (50 μg peptide/mouse) on day 0 and then again 28 days later.

2.3. Preparation of Splenic Lymphocytes. Mice were sacrificed on day 42 postprimary immunization (14 days after boosting). Spleens were aseptically removed and single cell suspensions in RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (complete medium) prepared using a 20 μm sieve from each mouse individually as described previously [18, 19]. Erythrocytes were lyzed with 0.17 M Tris-buffered ammonium chloride and membrane debris was removed by filtration through sterile gauze. Viability determined by trypan blue exclusion was routinely >95%.

2.4. Lymphocyte Subset Depletion. Lymphocyte subset depletion was performed by immunomagnetic cell sorting to >98% purity on splenocytes from mice immunized with tomatine-*P. berghei* CS peptide following ex vivo restimulation with homologous peptide (25 μg/mL), as previously

Table 1: Cytolytic activity of splenocytes from mice immunized with adjuvant-antigen preparations of tomatine-*P. berghei* CS peptide or tomatine-saline following ex vivo restimulation with *P. berghei* CS peptide (25 μg/mL). 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) over a range of effector: target cell ratios. Data shown represent the mean of four mice, assayed individually in triplicate, from one of four similar experiments. Cytolytic activity following CS peptide stimulation (shown in bold): $^{*}P < .025$ versus tomatine-saline group, $P < .05$ versus naive group, $^{**}P < .05$ versus tomatine-saline group, determined by the Mann-Whitney U-test. The positive control cytolytic T cell clone CS.C7 specific for *P. berghei* CS peptide elicited 81.2 and 53.0% P815 cell lysis at an effector: target ratio of 100 : 1 and 50 : 1, respectively.

	Percentage target cell lysis			
Effector: target cell ratio	Naive	Tomatine saline	Tomatine-CS peptide	Medium
100 : 1	6.8 (0.9)	3.7 (0.2)	**19.2** (3.6)*	2.5 (0.1)
50 : 1	6.1 (0.3)	4.8 (0.9)	**16.4** (1.4)*	4.3 (0.1)
25 : 1	5.1 (0.5)	3.0 (1.0)	**9.7** (0.2)**	4.3 (0.5)
1 : 1	7.7 (0.8)	4.0 (0.2)	2.5 (0.8)	4.8 (0.5)

Table 2: Type 1 cytokine production by splenocytes from mice immunized with adjuvant-antigen preparations of tomatine-*P. berghei* CS peptide or tomatine-saline following ex vivo restimulation with *P. berghei* CS peptide (25 μg/mL). 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 ex vivo (absolute levels shown in italics). Data shown represent the mean of four mice, assayed individually in triplicate, from one of four similar experiments. IFN-γ production (shown in bold): $^{*}P < .05$ versus tomatine-saline group; $P < .04$ versus naive group, determined by the Mann-Whitney U-test.

Cytokine	Naive	Tomatine saline	Tomatine-CS peptide	Unstimulated cells (pg/mL)
IL-12	1.02 (0.32)	0.92 (0.44)	1.22 (0.27)	*97.84 (21.22)*
IFN-γ	0.88 (0.38)	1.48 (0.40)	**3.48** (0.64)*	*153.17 (32.74)*
TNF-α	1.66 (0.61)	1.54 (0.41)	1.28 (0.71)	*39.08 (5.61)*

described [20, 21]. Lymphocytes were prepared from single-cell suspensions of spleen cells by Ficoll gradient centrifugation (Lympholyte-Mammal; Tebu-Bio, Peterborough, UK). Immunomagnetic cell sorting was performed for B, T, $CD4^+$ and $CD8^+$ T cells using Dynal mouse cell negative isolation kits (Invitrogen, Oxford, UK), following manufacturer's instructions for binding, washing and elution. The specificity of each depletion treatment was assessed by flow cytometry. Cells were incubated with rat IgG_{2b} monoclonal antibody (mAb) specific for murine CD20 (pan-B), CD3 (pan-T), CD4, and CD8 (AbD Serotec, Oxford, UK). Negative controls of cells incubated with normal rat serum in place of a primary mAb and of untreated cells were included. FITC-conjugated goat anti-rat IgG (Sigma) was used as the secondary mAb. Labelled cells were analyzed on a FACSCalibur flow cytometer (Becton-Dickinson, Oxford, UK) after correction for nonspecific fluorescence of controls and exclusion of dead cells and granulocytes on the basis of forward and right-angle light scatter.

2.5. Measurement of Cytolytic Activity. To assay for a CTL response, the cultured spleen cells were used as effector cells against P815 target cells labelled with ^{51}Cr (Amersham Int., Little Chalfont, UK). P815 cells, which express H2-k^d molecules [13], were loaded with *P. berghei* CS peptide. A cytolytic T cell clone (CS.C7) specific for this peptide [13] was similarly assayed as a positive control. Three aliquots of 1×10^6 cells were each labelled with 1 mCi ^{51}Cr in 1 mL. Five micrograms CS peptide or 5 mL saline was added to the aliquots. The cells were then incubated for 1 hour at 37°C, after which they were washed twice with 10 mL complete medium. Effector and target cells were incubated at ratios of 100 : 1–1 : 1 in V-bottomed microtitre plates (Gibco) for 4 hours at 37°C. The cells were pelleted by centrifugation at 200 × g for 2 minutes and 100 μL of the supernatant was added to 100 μL of scintillant (Optiphase HiSafe 3, Wallac, Milton Keynes, UK) in a 96-well plate (Isoplate, Wallac), mixed well and ^{51}Cr activity was measured on a beta counter (1450 Microbeta, Wallac). Cytolytic activity was calculated using the following formula to determine target cell lysis:

$$\%\ \text{lysis} = \frac{(\text{test release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})}.$$

2.6. In Vitro Restimulation for Cytokine Production. To generate cytokine-containing supernatants, spleen cell suspensions were adjusted to a final concentration of 5×10^6/mL in complete medium and 100 μL aliquots placed in 96-well flat-bottom tissue culture plates (Nunc, Roskilde, Denmark), 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 peptide, 1 μg/mL concanavalin A (Con A; Sigma), 25 μg/mL *Escherichia coli* lipopolysaccharide (LPS; Sigma) [20]. Cultures were incubated for 6 days (37°C, 5% CO_2); supernatants were removed, centrifuged at 300 × g for 5 minutes, and stored at −20°C until assayed.

2.7. Cytokine Measurement. Levels of the type 1 cytokines IL-12, IFN-γ and TNF-α were quantified by two-site sandwich enzyme-linked immunosorbent assay (ELISA) [22], using

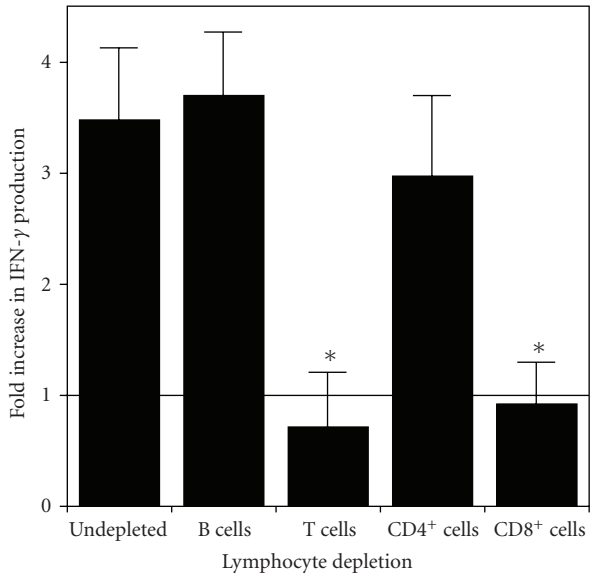

FIGURE 1: IFN-γ production by splenocytes from mice immunized with tomatine-*P. berghei* CS peptide following ex vivo restimulation with *P. berghei* CS peptide (25 μg/mL). Lymphocyte subset depletion was performed by immunomagnetic cell sorting to >98% purity. Data are fold increases in cytokine secretion over that of splenocytes from identically immunized mice not restimulated ex vivo (by convention, value = 1.0, depicted as a horizontal line). Data shown represent the mean of four mice, assayed individually in triplicate, from one of four similar experiments. $^*P < .05$ versus other groups, determined by the one-way ANOVA test.

DuoSeT^{TM} matched antibodies (Genzyme, West Malling, UK) and following manufacturer's instructions. Reactivity was visualised using 3,3′,5,5′-tetramethylbenzidine (TMB) in 0.05 M phosphate-citrate buffer and 0.014% (v/v) hydrogen peroxide (Sigma) as substrate. Optical densities were determined at 450 nm using an Emax plate reader (Molecular Devices, Crawley, UK). Recombinant murine cytokines (Genzyme) were used for calibration. Control samples of spleen cell culture supernatants from naive, nonimmunized mice, derived under identical conditions to experimental samples, showed low background cytokine levels to specific antigen (<5 ng/mL; SI $\ll$ 2). The minimum level of detection for each cytokine was between 30–60 pg/mL.

3. Results

3.1. Cytolytic Activity of Splenocytes after Stimulation with P. berghei *CS Peptide.* The cytolytic activity of splenocytes was assessed by coincubation with P815 target cells loaded with peptide. In the presence of homologous peptide (25 μg/mL), cells derived from *P. berghei* CS-immunized mice produced significantly raised peptide-specific CTL activity at 100–50 : 1 effector: target ratios compared to splenocytes from either tomatine-saline immunized mice ($P < .025$) or from naive controls ($P < .05$) (Table 1). At a 25 : 1 effector: target ratio peptide-specific CTL activity was significantly greater for splenocytes from tomatine-CS peptide-immunized mice than for splenocytes from mice immunized with tomatine-saline ($P < .05$) but not from naive controls ($P > .05$) (Table 1). There was no significant difference between groups at a 1 : 1 effector: target ratio ($P > .05$).

3.2. Cytokine Production from Splenocytes After Stimulation with P. berghei *CS Peptide.* Following restimulation ex vivo with *P. berghei* CS peptide (25 μg/mL), splenocytes from mice immunized with tomatine-CS peptide showed significantly upregulated production of IFN-γ when compared to splenocytes from either tomatine-saline-immunized mice ($P < .05$) or from naive controls ($P < .04$) (Table 2). Production of the other type 1 cytokines measured, IL-12 and TNF-α, was similar for all three experimental groups ($P > .05$). 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 tomatine-saline-immunized mice compared to controls ($P < .05$) (Table 2). 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.

In the presence of homologous peptide (25 μg/mL), splenocytes from tomatine-CS peptide-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. 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, (data not shown).

3.3. IFN-γ Production by Lymphocyte Subsets After Stimulation with P. berghei *CS Peptide.* Following restimulation ex vivo with *P. berghei* CS peptide (25 μg/mL), splenocytes were depleted of B, T, CD4$^+$ and CD8$^+$ T cell fractions by immunomagnetic cell sorting. In each case contamination with residual cells was <2% of the undepleted population, as determined by flow cytometry. Splenocytes depleted of either B cells or CD4$^+$ T cells produced similar levels of IFN-γ to undepleted splenocytes ($P > .05$). In contrast, depletion of either T cells or the CD8$^+$ T cell subset ablated the IFN-γ response to the CS peptide ($P < .05$) (Figure 1).

4. Discussion

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 combat effectively 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 [23–25]. Moreover, this has been

corroborated by the recent development of genetically-attenuated sporozoites [26].

In the present study, immunization of mice with the defined *P. berghei* CS peptide SYIPSAEKI (aa 252–260) was used to examine the capacity of the novel adjuvant tomatine to potentiate antigen-specific cellular immune responses to pre-erythrocytic malaria. When restimulated with homologous peptide ex vivo, splenocytes derived from mice immunized with the tomatine-CS peptide vaccine elicited a peptide-specific CTL response and upregulated production of the type 1 cytokine IFN-γ. This supported our hypothesis that immunization with the tomatine-CS peptide vaccine might elicit a $CD8^+$ T cell response if this peptide were presented in association with MHC class I molecules.

The pronounced production of IFN-γ by splenocytes from tomatine-*P. berghei* CS peptide-immunized mice upon CS peptide stimulation has particular significance, since this is recognized as a major host defence mechanism against liver stage malaria [10, 23]. The $CD8^+$ T cell epitope for BALB/c mice within the *P. berghei* CS peptide appeared to elicit IFN-γ production by tomatine-CS peptide-immunized splenocytes ex vivo since when the cells were depleted of specific lymphocyte fractions, removal of $CD8^+$ T cells ablated the IFN-γ response.

The pivotal importance of IFN-γ to the immune response to pre-erythrocytic malaria is well established. Recombinant IFN-γ inhibits the in vitro development of intrahepatic parasites [27, 28], while *anti*-IFN-γ mAb treatment abrogates protection in mice immunized with radiation-attenuated sporozoites [23, 29]. Administration of IFN-γ or IL-12 protects mice [27, 30] and monkeys [31, 32] against pre-erythrocytic malaria, and in mice the protection is reduced when the synthesis of nitric oxide (NO) is inhibited [30]. 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 [33, 34]. 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 [35], 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 [12, 36].

5. Conclusions

The release of IFN-γ by $CD8^+$ T cells is considered a critical component of immunity induced by liver stage malaria [36], and successful vaccination of humans with vaccines designed to elicit protective immunity will require induction of specific $CD8^+$ T cells that home to the liver [37]. In this context, our findings validate the use of tomatine to potentiate a cellular immune response to antigenic stimulus by testing in an important biologically relevant system [38, 39]. Specifically, the processing of the *P. berghei* CS peptide as an exogenous antigen and its presentation via MHC class I molecules to $CD8^+$ T cells led to IFN-γ secretion that is an in vitro correlate of protection against pre-erythrocytic malaria [10, 40]. This was confirmed by the protective capacity of the tomatine-CS peptide combination upon in vivo immunization [19]. These findings merit further work to optimize the use of tomatine as an adjuvant in malaria vaccine development. Future studies are required to understand the mechanism by which tomatine generates antigen-specific CTL when formulated with soluble protein. In particular, the processing pathways used to load the MHC class I molecules and which antigen-presenting cells are involved are being investigated.

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