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Article in Expert Review of Vaccines - December 2017
DOI: 10.1080/14760584.2018.1411198

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To cite this article: Kwadwo Asamoah Kusi, Bart W. Faber, Gerrit Koopman & Edmond Joseph Remarque (2017): EDiP: the Epitope Dilution Phenomenon. Lessons learnt from a malaria vaccine antigen and its applicability to polymorphic antigens, Expert Review of Vaccines, DOI: 10.1080/14760584.2018.1411198

To link to this article: https://doi.org/10.1080/14760584.2018.1411198
SPECIAL REPORT

EDiP: the Epitope Dilution Phenomenon. Lessons learnt from a malaria vaccine antigen and its applicability to polymorphic antigens

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ABSTRACT

Introduction: Polymorphism in vaccine antigens poses major challenges to vaccinologists. The Plasmodium falciparum Apical Membrane Antigen 1 (AMA1) poses such a challenge. We found that immunization with a mixture of three variants yielded functional antibody levels to all variants comparable to levels induced by monovalent immunization. The mechanism behind the observed broadening was shown to be an increase in the fraction of cross-reactive antibodies, most likely because strain-specific epitopes are present at lower frequency relative to conserved epitopes.

Areas covered: We hereby introduce the Epitope Dilution Phenomenon (EDiP) as a practical strategy for the induction of broad, cross-variant antibody responses against polymorphic antigens and discuss the utility and applicability of this phenomenon for the development of vaccines against polymorphic antigens of pathogens like Influenza, HIV, Dengue and Plasmodium.

Expert commentary: EDiP can be used to broaden antibody responses by immunizing with a mixture of at least 3 antigenic variants, where the variants included can differ, yet yield broadened responses.

1. Introduction

Apical membrane antigen 1 (AMA1) is a protein of apicomplexan parasites with an essential role in host cell invasion [1]. In Plasmodium falciparum, it is a 622-amino-acid-long, type 1 transmembrane protein, initially expressed as an 83-kDa precursor protein in merozoite micronemes and later processed to a 66-kDa protein by the removal of the N-terminal prosequence [2–5]. The AMA1 ectodomain is an important vaccine target since antibodies against the ectodomain have been shown to prevent red cell invasion in vitro [6–9], and this effect requires immunization with correctly folded AMA1 [10,11]. The challenge that AMA1 vaccine developers are confronted with is its extreme variability. In a single vaccine site in Mali, 214 AMA1 variants were identified among 506 subjects over a 3-year period [12]. With a database currently at 2372 entries, we have identified 117 polymorphic positions in the ectodomain (aa 97–545) and 838 unique AMA1 ectodomain variants.

AMA1 has a unique feature which becomes apparent when projecting the variable positions on the crystal structure; it has a polymorphic and a conserved face (Figure 1) [13–15]. The conserved face has a conformational epitope that is recognized by the 4G2 monoclonal antibody [5,13–15]. This monoclonal antibody neutralizes all strains hitherto tested and this conserved (functional) site may potentially also be exploited for an AMA1-based vaccine. The diversity and amino acid differences among AMA1 variants are presented graphically as a genetic map (Figure 2) using similar methodology as described by Smith et al. [18] (see Supplementary Materials).

Figure 2 shows that the maximal ‘distance’ between two AMA1 variants is about 40 amino acids. A number of variable positions located close to residue 197 appear to be dominant in variant specificity as determined by functional (growth inhibition) assays [19].

Polymorphism results from non-synonymous single-nucleotide substitutions within the single ama1 gene locus [20–23], a phenomenon that has been linked with selective pressure from the host immune system. As such, antibodies raised against one AMA1 variant are only partially effective against parasite strains expressing other AMA1 variants. Thus, vaccines based on a single AMA1 variant are likely to have limited efficacy, as was the case for a Phase II trial of a vaccine with a single AMA1 variant which showed only 17% overall vaccine efficacy, but 64% vaccine efficacy against infections with the homologous variant [24]. With such an extremely variable antigen, a successful AMA1-based vaccine should ideally be able to cover diversity by eliciting a broadly neutralizing antibody response against all variants.

2. Covering polymorphism

Human and animal immunization experiments with single AMA1 variants have shown that about half of the antibodies cross-react (functionally) between variants differing by about 25 amino acids [25–27]. In an attempt to cover AMA1 diversity,
Kennedy et al. immunized rabbits with two AMA1 variants, either single or mixed, keeping an equal total antigen dose (viz. FVO and 3D7 with a 24 amino acid difference; see Figure 2) [26]. The single-antigen immunizations were clearly variant specific with about 50% of antibodies cross-reacting with the other variant in ELISA and functional assays [26]. By contrast, anti-AMA1 titers from animals immunized with the AMA1 mixture were similar to titers from animals with single-antigen immunizations in both ELISA and functional assays with the two vaccine components [26]. However, the broadened response did not extend beyond the two vaccine components as functional titers to variants that were poorly...
inhibited by either component did not improve by mixing antigens [26].

The next attempt to cover AMA1 diversity employed three artificial AMA1 sequences, designed to accommodate a maximum of amino acid variation, based on 355 AMA1 sequences available at the time of design [16]. The antigenicity of the three diversity covering (DiCo) proteins was confirmed by recognition of the conserved face by the conformation-sensitive antibody 4G2 [16]. Rabbits immunized with a mixture of the three AMA1 DiCo proteins (D1, D2, and D3 in Figure 2) produced antibodies that recognized a panel of three natural AMA1 variants in ELISA and functional assays similar to the FVO antibodies titers obtained using a homologous (FVO) immunization regime [16]. Rabbit antibodies to the DiCo mixture significantly inhibited red blood cell (RBC) invasion by five different parasite strains (FCR3, 3D7, HB3, CAMP, and 7G8) [28], and a mixture of three AMA1 DiCo proteins formulated in potent adjuvants was subsequently shown to induce high (functional) responses to a panel of three laboratory strains in nonhuman primates [29]. Of note here is that none of the DiCo proteins had a glutamic acid at the previously defined immunodominant position 197 [19], yet growth inhibition against the 3D7 strain harboring glutamic acid at position 197 was comparable to inhibition of assay strains with a homologous amino acid at position 197 (e.g. FVO; Figure 2) [30,31]. The DiCo AMA1 antigens, formulated as a mixture, are currently in clinical development as a vaccine candidate for P. falciparum malaria [32,33].

The mechanism underlying the broadening of responses when using a mixture of three antigens was investigated in a study in rabbits immunized with a mixture of three natural AMA1 variants (FVO, HB3, and 3D7; Figure 2). Rabbits immunized with a mixture of three AMA1 variants were shown to produce functional antibody levels similar to the homologous vaccination regimes, despite having only one-third of the antigen dose [31]. Moreover, sera obtained after mixed antigen vaccination inhibited parasite growth more potently than a mixture of sera obtained after monovalent vaccination. Sera obtained after mixed antigen vaccination also more efficiently neutralized an outgroup strain (CAMP; Figure 2) not contained in the vaccine formulation [31]. Using competition ELISA and affinity purification experiments, it was shown that immunization with a mixture of three antigens yielded an increased fraction of cross-reactive antibodies, rather than a focus of the antibody response on strain-specific epitopes [28,31]. The increase in cross-reactive antibody responses following vaccination with a mixture of AMA1 variants was later elegantly confirmed using P. berghei AMA1 orthologue scaffolds expressing conserved or variable residues in the correct conformation [31]. It was hypothesized that the broadening observed following immunization with a mixture of three or more variants is due to a dilution of strain-specific epitopes and a focus of the immune response on conserved epitopes [28,31,34].

Based on the observations described above, we have formulated the ‘Epitope Dilution Phenomenon’ (EDiP), providing a potential approach to cover polymorphism in polymorphic vaccine antigens to ensure the efficient generation of broadly neutralizing immune responses (discussed later). In summary, EDiP comes down to the observation that immunization with a combination of highly similar antigens focuses the immune response towards epitopes that are shared (conserved) between the antigens used.

The number of antigenic variants required to cover variation in AMA1 is still under debate. A minimum of three components appear to be required for epitope dilution as two component vaccines did not show broadening beyond the vaccine components [26,34,35]. It was shown that antibody breadth was not significantly improved when three AMA1 DiCo proteins were complemented with four natural alleles (viz. FVO, 3D7, HB3, and CAMP; Figure 2) [28]. Dutta et al. show that addition of a fourth naturally occurring component (W2Mef added to FVO, 3D7, and HB3; Figure 2) yielded a small increment in the breadth of functional responses compared to three components [34], whereas Miura et al. suggested the use of five AMA1 variants to optimally cover antigenic variation [36]. It is however important to note, based on the available evidence, that the number of components required to cover AMA1 appears to be limited.

A three-dose immunization with three natural AMA1 variants, either simultaneously as a mixture or sequentially as single variants in different combinations, did not exert major effects on the patterns of immune response breadth [30]. Of note is that antibodies to cross-reactive epitopes have better functional characteristics than strain-specific epitopes [31], although strain-specific epitopes do show some degree of immunodominance following exposure to a single AMA1 variant [19]. It is therefore tempting to speculate that this immunodominance of strain-specific epitopes may be a pathogen strategy to distract the immune system. In subjects naturally exposed to malaria, the differences in titers between any two AMA1 variants decrease with age, suggesting that ‘a broad composite of antibody is gradually acquired with age-associated repeated antigen exposures [37,38]. This may also be the case for other malarial antigens [39] and indeed antigens of other pathogens. A vaccine capable of eliciting such a broad composite of antibody for influenza was first suggested by Davenport and Hennessy in 1957 [40]. It appears that the EDiP offers opportunities to achieve this for variable vaccine targets like influenza hemagglutinin (HA) and neuraminidase, HIV envelope, and dengue E protein.

3. EDiP for influenza

When comparing maximal amino acid distances between AMA1 and influenza H1 HA (Figures 2 and 3), it is evident that influenza HA is more variable than AMA1 [41,43–45]. Huber et al. have shown that a prime with DNA followed by a live-attenuated virus boost with a mixture of three H3N2 viruses (Hong Kong 1968, Victoria 1975, and Leningrad 1986) covers 20 years of antigenic drift in mice [43]. Carter et al. show that sequential infection in ferrets with three seasonal H1N1 strains (either historical or modern) confers protection against H1N1pdm challenge (not contained in infection series) [41]. Moreover, a mixture of sera obtained after infection with single viruses neutralized H1N1pdm, while none of the separate sera had neutralizing activity [41]. A genetic distance map
for H1N1 viruses is shown in Figure 3, and the variants used by Carter et al. (A/PR/8/34, A/FM/1/47, and A/Denver/1/57 [Historical] or A/Texas/36/1991, A/New Caledonia/20/1999, and A/Brisbane/59/2007 [Modern]) are all very distant (~90 amino acids) from the A/California/7/2009 challenge strain (Figure 3). Prabakaran et al. show that three Modified Vaccinia Ankara-expressed H5 antigens induce broad cross-clade neutralizing responses in mice and guinea pigs [44]. A recent publication by Schwartzman et al. suggest that HA group 1/2 transcending immunity can be achieved in mice by intranasal immunization with a mixture of H1, H3, H5, and H7 virus-like particles (VLPs) [45]. In these experiments, mice were protected from lethal challenge with a number of influenza A viruses not contained in the vaccine [45]. These collective data beg the question whether the underlying mechanism, i.e. EDiP, applies here and whether it may be possible to apply this mechanism to devise a vaccine that provides broad protection against all influenza A viruses [45]. It is not yet clear, however, how many separate components would be required to adequately cover all 16 influenza A HAs.

4. EDiP for soluble versus particulate antigens

AMA1 is a soluble antigen, whereas about 400 HA (and 100 neuraminidase) molecules are presented in a highly organized repetitive form on an influenza virus particle [46]. It is assumed that the antigen in this highly organized repetitive form can more efficiently trigger B cells [47–49]. Indeed, this type of antigen may need to be presented in a multimeric form, on a single particle, to be able to trigger B cells capable of mounting broadly neutralizing responses. Such VLPs are expected to be able to activate B-cells capable of producing broadly neutralizing influenza antibodies [47,48]. VLPs expressing multiple HA molecules (H2, H5, H7 or H1, H3 and B) on the same particle have been produced and shown to be immunogenic [50], and the presentation of multiple molecules on a single particle will be highly cost-effective. These VLPs are, however, not expected to fully exploit epitope dilution as the antigens are from different (sub)types and therefore extremely different and the limited number of components included (3) may not be sufficient for adequate dilution of strain-specific epitopes. In order to obtain broad responses within a subtype, one would have to display several HAs of the desired single subtype on a single polyvalent VLP (we coin ‘polyvalent’ VLP here to describe the fact that multiple antigens are expressed on a single particle, as opposed to ‘multivalent’ which describes a mixture of particles expressing a single antigen). This is the strategy that is being employed in the FP7-funded universal influenza project ‘Combinatorial immunization strategy to educate the immune system towards cross recognition and coverage against antigenic drift in seasonal influenza virus exposure: EDUFLUVAC’ [51]. In this project, HAs from multiple (sub)types of H1, H3, and influenza B will be expressed on single polyvalent VLPs per (sub)type. In addition, the EDUFLUVAC consortium will investigate whether broad group 2 HA coverage can be achieved using polyvalent VLPs expressing all six group 2 HAs [51].

5. EDiP for other variable vaccine antigens

The envelope (E) protein is an important constituent of most flaviviral vaccines. Figure 4 shows an amino acid difference plot for the four dengue serotypes. With a total of 495 amino acid positions in the E protein (DENV3 E protein only has 493 aa), maximum amino acid differences within a serotype are 30, 67, 39, and 21 for dengue serotypes 1–4, respectively. Average amino acid differences between serotypes are as follows: 157.6 between 1 and 2, 112.8 between 1 and 3, 181.5 between 1 and 4, 159.4 between 3 and 2, 178.8 between 2 and 4, and 183.1 between 3 and 4. Despite these extensive amino acid differences (early), neutralizing responses following primary and secondary infections are cross-reactive and cross-neutralizing between serotypes [53–55].

Sanofi’s recently licensed Dengvaxia (CYD-TDV), a tetravalent live-attenuated virus vaccine expressing the pre-membrane (prM) and E proteins of the four dengue serotypes on
a yellow fever virus backbone, confers solid protection against dengue serotypes 3 and 4. However, the protection against serotypes 1 and 2 is only moderate or low [56]. This is a complicating factor, especially in the case of dengue, as suboptimal levels of neutralizing or high levels of non-neutralizing antibodies against one subtype lead to a phenomenon known as antibody-dependent enhancement (ADE), where preexisting antibodies increase the infection rate of Fc-γ-receptor-positive cells by other dengue virus serotypes [57]. The follow-up of clinical phase III trials of Dengvaxia showed an increase in hospitalizations of vaccinated children below 9 years and especially those under two-and-a-half years of age, which could be the result of such ADE-like phenomena [58].

The inter-serotype variation of the E-proteins (Figure 4) provides a potential explanation for the lower protection of the Dengvaxia vaccine with respect to specific serotypes. In this case, serotype 2, just by assuming that the serotype 2 virus, that infected the vaccines, had an E-protein not very close to the E-protein sequence that was used in the vaccine (PUO218 for DENV2, only five amino acids of the consensus sequence for DENV2 E-protein [59]). Of note is that DENV2 is more variable as compared to the other serotypes with the most distant strains differing by 67 amino acids compared to 30, 39, and 21 for DENV1, 3, and 4, respectively. The efficacy of the vaccine will depend on sufficient match between the vaccine E protein and the infecting virus, similar to the current situation for influenza vaccines, and the lower efficacy for serotypes 1 and 2 may be due to differences between vaccine and circulating strains. The above also implies that suboptimal protection to other serotype variants may occur in other areas or in time. Moreover, escape from the vaccine strain may develop over time.

Based on EDiP, it is highly conceivable that inclusion of a limited number of variants for each serotype’s E-protein will focus the immune response on shared epitopes of the E protein, preferably on a single polyvalent VLP. Combination of four of these polyvalent VLPs will yield, according to the EDiP, a highly protective, cross-serotype dengue vaccine, much less likely to cause ADE-like phenomena. A single-particle VLP with at least three variants for each of the dengue serotypes will be preferred, for the same reasons as explained for malaria and influenza. Moreover, intact virus particles are preferred because it was recently established that intact VLPs have the so-called ‘E protein dimer epitope’ [60], and antibodies against this epitope bind strongly and are broadly (inter-serotype) cross-reactive for the four dengue serotypes. Efforts to produce such VLPs are currently ongoing.

HIV’s envelope protein (Env), consisting of two glycoprotein subunits (viz. gp41 and gp120), represents another important vaccine candidate antigen that is highly variable, and induction of broadly neutralizing antibodies against HIV has been shown to be extremely difficult. About 20% of HIV-infected people develop broadly neutralizing antibodies (bnAb) after 2–3 years of infection. The induction of these bnAb is the result of extensive affinity maturation, driven by cycles of new viral variant formation through escape mutations and further evolution of the Ab response [61–64]. Besides variability, further complexity is added by the high level of glycosylation, conformational flexibility, and exposure of non-neutralizing epitopes by shed gp120 subunits, which all divert the immune response from generating a virus-neutralizing Ab response [65]. Furthermore, the very restricted conserved parts of the molecule, i.e. the CD4-binding site (CD4bs) and the co-receptor-binding site, are well hidden inside the conformationally intact Env trimer and covered by variable loops that protect them from Ab recognition [66]. Broadly neutralizing antibodies that arise in some infected patients are characterized by extensive somatic hypermutation, requiring multiple rounds of antibody affinity maturation in germinal center reactions. Furthermore, these Ab tend to show polyreactivity for host antigen, indicating that many potential Env-specific B-cell clones will already have been deleted during B-cell selection in the bone
marron. Recent developments of sequencing Ab genes from isolated single-antigen-specific B-cells and computational analysis have made it possible to infer the germline common ancestral Ab sequence from the swarm of bnAb produced in time. It was shown that for both CD4bs and V1V2 loop-targeting bnAb, this ancestral Ab did not recognize the Env protein [61,67–69]. Therefore, sequential immunization strategies are needed, starting with an engineered HIV Env antigen that is recognized by the ancestral bnAb precursor [70–72]. The subsequent maturation of the B-cell response towards bnAb formation is however dependent on exposure to viral variants, that in turn develop under the continued selective pressure of the developing Ab response [73]. Both in silico modelling and experiments in mice seem to indicate that for HIV Env, sequential immunization of these viral variants instead of coadministration is needed for induction of bnAb [74]. However, in the final stages of the immunization cascade, a further broadening of the immune response may require an approach in which multiple variant Env trimers are presented on a single particle, to invoke faster selection of the desired bnAb response through the EDiP described above. Given previous observations for malaria AMA1 and influenza HA, it would be interesting to test an HIV vaccine comprised of several Env proteins from different clades, preferably the native (SOSIP conformation [75]) expressed on a polyvalent VLP. Indeed, mixtures of five native-like SOSIPs differing in the variable loops have recently been used as a final boost for the induction of PGT121 like bnAb [76]. However, the full potential of this strategy, especially the expression of multiple variants on a single particle, has not yet been explored.

6. Expert commentary

In order to exploit EDiP, broadly cross-neutralizing epitopes have to be accessible on the target antigen. This is the case for AMA1, dengue Envelope, HIV Env protein, and many other vaccine candidate antigens. Meningococcal factor H-binding protein appears to be an exception for which an alternative strategy has been used [77]. Previously published data on AMA1 suggest that EDiP requires at least three antigens [28,34,35]. The composition of the variants in the immunization mixture can differ, yet achieve similar results provided, strain-specific epitopes are efficiently diluted (e.g. AMA1 Natural [31] versus DiCo [16], influenza HA Historical versus Modern H1N1 [41]). This is supported by influenza studies where exposure to only three components yielded broadly neutralizing responses within an influenza subtype [41,43]. Of note here is that the immune dominance of the HA head epitopes differs significantly following infection or vaccination [78], which may have repercussions for the number of vaccine components required to induce broadly neutralizing responses.

In addition, the superiority of polyvalent (all Ag on a single particle) over multivalent (mixture of monovalent) VLPs needs to be demonstrated. Preliminary results obtained with influenza HA in mice suggest that polyvalent VLPs yield an increased breadth as compared to mixtures. Novel VLP technologies (AP205, HPV-Biotin VLP) currently emanating facilitate the production of polyvalent VLPs of defined composition [79,80]. These technologies allow for the production of VLPs with multiple antigenic variants on the surface. The fact that VLP and surface antigen do not require the same expression system adds to the versatility of these technologies. These systems also allow an evaluation of the effect of antigen density in relation to broadening. Another factor that may potentially influence EDiP is VLP particle size (and associated antigen density) with some VLPs having up to 400 HAs displayed on the surface, while others may have as few as 8 [81].

Recent technical advances in rearranged antibody sequencing can be used to demonstrate epitope dilution next to competition ELISA and epitope scaffolds. It is expected that epitope dilution would decrease diversity and move towards specificities recognized conserved epitopes.

EDiP may also apply to T-cell epitopes: one published paper suggests that T-cell responses to conserved regions increase following exposure to variant antigens [82]. Besides variable antigens, EDiP can also be applied to non-variable antigens; by mutating and diluting immunodominant but non-neutralizing sites, the immune response can be redirected towards neutralizing epitopes.

Key issues

- EDiP requires that broadly cross-neutralizing epitopes are accessible on the target antigen
- Broadening can be achieved with as few as three vaccine components
- Several combinations of 3 antigens can yield broadened antibody responses

Funding

The manuscript was funded by the Biomedical Primate Research Centre.

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

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• First paper describing epitope dilution. When the immune system is confronted with multiple variants, the response focusses on conserved epitopes.


Elegant confirmation of Epitope Dilution Phenomenon by the use of orthologue AMA1 protein scaffolds.


**Paper demonstrating that sequential infection with three H1N1 variants (either historical or modern) can confer protection against H1N1pdm challenge in ferrets.**

**Paper demonstrating that 20 years of H3N2 vaccination can be covered by immunization with three H3N2 variants.**


**Paper demonstrating that three MVA-vectorised H5 sequences can induce broadly protective H5 antibodies.**


**Paper demonstrating that 20 years of H3N2 vaccination can be covered by immunization with three H3N2 variants.**


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