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An in depth analysis of original antigenic sin in dengue virus infection

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ABSTRACT

The evolution of dengue viruses has resulted in four antigenically similar yet distinct serotypes. Infection with one serotype likely elicits life-long immunity to that serotype, but not generally against the other three. Secondary or sequential infections are common as multiple viral serotypes frequently co-circulate. Dengue infection, although frequently mild, can lead to dengue haemorrhagic fever (DHF) which can be life threatening. DHF is more common in secondary dengue infections implying a role for the adaptive immune response in the disease. There is currently much effort towards the design and implementation of a dengue vaccine but these efforts are made more difficult by the challenge of inducing durable neutralizing immunity to all four viruses. Domain 3 of the dengue virus envelope protein (ED3) has been suggested as one such candidate because it contains neutralizing epitopes and it was originally thought that relatively few cross-reactive antibodies are directed to this domain. In this report we performed a detailed analysis of the anti-ED3 response in a cohort of patients suffering either primary or secondary dengue infections. The results show dramatic evidence of original antigenic sin in secondary infections both in terms of binding and enhancement activity. This has important implications for dengue vaccine design because heterologous boosting is likely to maintain the immunological footprint of the first vaccination. Based on these findings we propose a simple *in vitro* ELISA assay to diagnose the original dengue infection in secondary dengue cases.

INTRODUCTION

Dengue virus is an insect-borne Flavivirus transmitted to man by the bite of an infected mosquito, usually *Aedes aegypti* (20). There are four circulating serotypes of dengue (Den1-4) that show up to 70 % sequence homology across their genomes (4, 17), and it is common for multiple viral serotypes to co-circulate in endemic countries. Most dengue infections are either asymptomatic or lead to uncomplicated dengue fever (DF). However, in 1-5% of cases, symptoms can be more severe with the development of plasma leakage and haemorrhage. Such dengue haemorrhagic fever (DHF) can lead to circulatory collapse, resulting in a mortality rate of around 20 % if left untreated.

The more frequent occurrence of DHF in secondary dengue infections in children and adults suggests a role for the acquired immune system in disease pathogenesis, and there has been considerable research into both the B and T cell responses. Antibody-dependent enhancement (ADE) of infection, proposed by Halstead in 1977 (24, 25), is one hypothesis for this increase in severity in secondary infections (23, 36). During a primary infection, antibodies that cross-react with the remaining 3 serotypes are induced. After a few months, when heterologous protection is no longer observed (54), it is hypothesized that these cross-reactive antibodies decline to sub-neutralizing levels, meaning that a heterologous infecting serotype is not controlled. Antibody made to the primary infecting virus may not be of sufficient avidity to neutralize a second serotype. Instead, these poorly neutralizing, low avidity cross-reactive antibodies bind the secondary virus and target it to Fcγ receptor bearing cells such as macrophage/monocytes (24, 25), leading to internalization and increased virus replication. *In vivo*, ADE has been shown to induce lethal disease in mice (2) and to drive high virus loads in primates (16, 22). ADE has also

been invoked to explain a peak in disease severity in primary cases during the first year of life, as titers of passively transferred maternal antibody fall (23, 35, 41, 56).

Dengue incidence increased sharply in the middle of the last century and is still increasing at an alarming rate (20). There are estimated to be around 3.6 billion people living in the tropics and subtropics who are at risk from dengue, with up to 50 million predicted infections per annum. To date, however, there are no specific treatments for dengue barring careful attention to fluid replacement. The scale of the problem posed by dengue has spawned much interest in the development of a dengue vaccine, with some candidates in phase II trials (67, 69), and also in anti-dengue drugs that have not yet reached clinical trials.

A number of vaccine candidates, ranging from live attenuated viruses to subunit vaccines, are currently being pursued. The Envelope (E) protein of dengue is a major target of neutralizing (48, 53, 62) and protective antibodies (32) and, as such, should be a key component for any subunit vaccines. The envelope protein consists of three domains: ED1, ED2 and ED3 (38, 47, 48). ED3 is proposed to be the binding domain for the virus (7, 9, 28), attaching to as yet poorly characterized cellular receptor(s); although heparan sulphate has been implicated in the interaction (29). Indeed, in mice, anti-ED3 monoclonal antibodies are potent neutralizers of dengue virus (5, 18, 19, 27, 43, 52, 55, 60, 61); often neutralising to greater levels than those targeting ED1 or 2 (60). ED3 is a target of both serotype-specific (5, 18, 42, 45, 53, 55, 60, 61) and cross-reactive (19, 43, 46, 52, 55, 60, 61) neutralizing antibodies. Of the anti-ED3 antibodies that are strongly neutralizing, however, the majority are usually serotype-specific (5, 55, 60), and cross-

reactive antibodies are generally weaker neutralizers (19, 61). There have been a number of vaccination studies investigating ED3 as a potential immunogen (3, 30, 57-59).

In this study, we have performed a detailed analysis of the anti-ED3 antibody responses in humans, using a cohort of patients suffering either primary or secondary dengue infections. We demonstrate that low level dengue cross-reactive anti-ED3 responses are induced upon primary infection and boosted dramatically during a secondary heterotypic infection. We have developed a competition ELISA to measure the relative avidities of these cross-reactive antibodies and show that the response is dominated by original antigenic sin. We propose this ELISA as a simple diagnostic test for determining the serotype of the original dengue infection in secondary dengue cases. Furthermore, we go on to show an inverse relationship between the avidity of the antibody response and enhancing activity.

MATERIALS AND METHODS

Plasma samples

Blood samples were collected, following written parental consent and the approval of the ethical committee, at the Khon Kaen Hospital in Thailand, and the Riverside Ethics Committee in the UK. Plasma samples were separated, transported to the UK and stored at -80 °C prior to use. Individuals diagnosed with Dengue Fever (DF, *n*=19) or different stages of Dengue Haemorrhagic Fever (DHF, *n*=52), following infection with different serotypes, were analysed. Individuals were aged 4 to 14 years, with approximately 57 % male and 43 % female. During the course of this study, we acquired 4 additional samples

from patients in Thailand (K1 to K4), for whom we had both pre- and post- secondary exposure samples. Of these, 2 individuals were experiencing DF and 2 DHF. Plasma samples (*n*=34) were also analysed from Vietnamese paediatric dengue patients experiencing DF; collected following written parental consent as part of an ongoing prospective study of dengue at two primary health care clinics in Ho Chi Minh City, Viet Nam. The study protocol was approved by the Hospital for Tropical Diseases and the Oxford Tropical Research Ethical Committee. The current infecting serotype was determined by RT-PCR-based gene identification (71). Secondary dengue infections were defined by a dengue-specific IgM:IgG ratio of < 1.8, by IgM and IgG capture ELISA.

ED3 expression and purification

ED3 (aa 295-401) of the E protein of each of the four dengue serotypes was expressed and purified for use in ELISA. Prototype strains of each serotype were used; Den1 strain Hawaii, Den2 strain 16681, Den3 strain H87 and Den4 strain H241. The PCR-generated ED3 inserts were cloned into a bacterial expression vector, pET3c (Novagen), for expression under IPTG-induction. ED3 of serotype 1 was expressed in Rosetta competent cells (Novagen), 2 in Origami competent cells (Novagen) and 3 and 4 in BL21 (DE3) competent cells (Novagen). ED3 proteins are expressed in inclusion bodies (IB), which were washed 5 times by dounce homogenization in 20 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% Triton-X-100). Between each wash, the suspension was clarified by centrifugation (10,000 x g, 30 min, 4 °C, Heraeus Biofuge JA-20R). IBs were denatured in 100 mM Tris HCl, 6 M guanidine-HCl, 2 mM DTT and left overnight at 4 °C. The lysate was clarified by centrifugation (15,000 x *g*, 25 min, 4 °C, Heraeus Biofuge 15R) and the supernatant collected for refolding. The denatured protein (50 mg) was then added to 500 ml of refolding buffer (100 mM Tris HCl, 500 mM L-arginine-HCl, 0.2 mM

EDTA, 3.7 mM Cystamine, 100 µM PMSF, 6.6 mM β-mercaptoethylamine) over a period of 2 h via a drop wise method and left spinning at 4°C for 48 h. Refolded protein was concentrated, buffer exchanged into PBS and purified in PBS through a size exclusion column (26/60 Superdex 75, GE healthcare). Protein was quantified by calculation with extinction coefficient, by BCA protein kit (Pierce, UK) and by Bio-Rad protein assay.

ED3 direct ELISA

Purified ED3(1, 2, 3 and 4) and BSA were used as coating antigen and negative control antigen, respectively. Ninety-six-well plates were coated overnight at 4 °C with 150 ng protein, in 50 µl of coating buffer (50 mM $NaHCO_3$, pH 9.6). Plates were rinsed 3 times with PBS containing 0.1 % Tween 20, using an automated 96-well plate washer, and were blocked for 2 h at room temperature (RT) with 200 µl of blocking buffer (1.5 % BSA in PBS). Plates were incubated for 90 min with 50 µl of 3-fold serial dilutions of sample plasma in dilution buffer (0.5 % BSA in PBS/0.1 % tween) and then for 90 min at RT with 50 µl of anti-human IgG-alkaline phosphatase conjugated secondary antibody (Sigma-Aldrich, 1:10,000 in dilution buffer). The activity was observed with p-nitrophenyl phosphate substrate (SIGMA FAST, Sigma-Aldrich, OD_{405}) and measured with Magellan Plate reader software. ELISA IgG end point titers (EPT) were defined as reciprocal plasma dilutions corresponding to 2 times the average OD values obtained with an irrelevant antigen (BSA). Pooled convalescent serum (PCS), obtained from recovering dengue patients, was used to standardize EPTs between plates and assays. To adjust for antigen loading on the plates, EPTs were also normalized to the saturation plateaus of mAb 2H12 in the ED3 ELISA. These were OD_{405} values of 2.09, 2.03, 1.87 and 1.95 for ED3(1), ED3(2), ED3(3) and ED3(4), respectively. The seropositivity cut-off values for

these 4 serotype-specific assays were defined as 3 x GMT of dengue-naïve serum (n=20). These values were 1282, 938, 907 and 837 for ED3(1), ED3(2), ED3(3) and ED3(4), respectively, and each gave 100 % specificity. Any EPT less than the cut-off value were subsequently given a 'negative' value of half the cut-off value.

Dissociation constants of monoclonal antibody 2H12

An ED3 direct ELISA was carried out with mAb 2H12, ensuring saturation plateaus were reached. The assay was carried out as described above except an anti-mouse IgG-alkaline phosphatase conjugated secondary antibody (Sigma-Aldrich, 1:10,000 in dilution buffer) was used. For each antigen, the % maximum OD was calculated and the dissociation constants (K_d) values were determined (Prism Software).

ED3 competition ELISA

Purified ED3(1, 2, 3 and 4) and BSA were used as coating antigen and negative control antigen, respectively. Ninety-six-well plates were coated overnight at 4 °C with 150 ng protein, in 50 µl of coating buffer (50 mM $NaHCO_3$, pH 9.6). Plates were rinsed 3 times with PBS containing 0.1 % Tween 20, using an automated 96-well plate washer, and were blocked for 60 min at 37 °C with 200 µl of blocking buffer (1.5 % BSA in PBS). During this incubation step, plasma samples were incubated with 10-fold serial dilutions of competing antigen, in a separate 96-well plate, for 60 min at 37 °C. All dilutions were made in dilution buffer (0.5 % BSA in PBS/0.1 % tween). For each antigen coated, one concentration of plasma was chosen (1 in 300 to 2000). This was determined by the anti-ED3(coated antigen) EPT, such that a similar OD was observed for all 4 ED3 serotypes, in the absence of competing antigen. Each plasma sample was competed with each of the 5 competing antigens separately: ED3(1, 2, 3 and 4) and FasD1, as the negative control.

ELISA plates were then incubated for 60 min with 50 µl of the plasma:competing antigen mixture and for a further 60 min at 37 °C with 50 µl of anti-human IgG-alkaline phosphatase conjugated secondary antibody (Sigma-Aldrich, 1:5,000 in dilution buffer). The activity was observed with p-nitrophenyl phosphate substrate (SIGMA FAST, Sigma-Aldrich, OD_{405}) and measured with Magellan Plate reader software. The background response against BSA, in the absence of competing antigen, was deducted from each of the responses obtained. The % max OD for each coated antigen was then calculated, using the response in the absence of competing antigen.

Focus reduction neutralization test (FRNT)

Micro-FRNTs were conducted on Vero cells as described previously (31). Den1 strain Hawaii, Den2 strain 16681, Den3 strain H87 and Den4 strain H241 were used. The $FRNT_{50}$ titer was defined as the reciprocal plasma dilution that reduced the number of foci by 50 %.

Enhancement assay on U937 cells

Den1 strain Hawaii and Den2 strain 16681 were used in these assays. Supernatants from Den1- or Den2-infected cells, resulting in an MOI of 1 upon infection, were incubated with 3-fold serial dilutions of plasma, in a 96-well U bottom plate, for 30 to 60 min at 37 °C. Plasma were heat-inactivated at 56 °C before use. The 2 virus stocks were equated for this assay by choosing the concentrations of virus that gave overlapping enhancement peaks with PCS. Supernatant from mock-infected cells was used as a negative control. All dilutions were made in R2: RPMI 1640 (Gibco) supplemented with 2 % heat inactivated foetal bovine serum (FBS, Gibco), 2 mM L-glutamine (Invitrogen), 50 units/ml penicillin and 50 µg/ml streptomycin (Invitrogen). Meanwhile, U937 cells were

plated in 96-well U bottom plates (150, 000/well in 30 µl). Fifty µl of the virus:plasma mixture were then added to the cells and incubated at 37 °C. After 2h, an additional 120 µl of R10 were added and the cells were left to incubate for a further 18 h. Cells were then stained intracellularly (see section below) for analysis by flow cytometry. The %-infected cells were recorded and the % infection observed for each virus in the absence of antibody (< 2 %) was deducted.

Monocyte infection assay

Supernatant from Den2 strain 16681 virus-infected cells, resulting in an MOI of 4 upon infection, was incubated with 2-fold serial dilutions of PCS, in a 96-well U bottom plate, for 30 to 60 min at 37 °C. The PCS was heat-inactivated at 56 °C before use. Supernatant from mock-infected cells was used as a negative control. All dilutions were made in R10: RPMI 1640 (Gibco) supplemented with 10 % heat inactivated foetal bovine serum (FBS, Gibco), 2 mM L-glutamine (Invitrogen), 50 units/ml penicillin and 50 µg/ml streptomycin (Invitrogen). Meanwhile, freshly isolated PBMCs were plated in 96-well U bottom plates (150,000/well) and centrifuged (400 x g, 5 min). Fifty µl of the virus:plasma mixture were then added to the PBMC pellet and incubated at 37 °C. After 2h, an additional 150 µl of R10 was added and the cells were left to incubate for a further 18 h. Cells were then stained intracellularly (see section below) for analysis by flow cytometry. Monocytes were gated by forward and side scatter and the % infected cells recorded.

Intracellular staining of infected cells

Infected U937 cells or PBMCs were transferred to a 96-well V bottomed plate and washed twice with PBS, by centrifuging at 700 x g, 2 min, 4 °C. The pellet was then fixed (10 min, RT, 100 µl 5 % formaldehyde, 10 min, RT), washed, permeabilised (10

min, RT, 100 µl FACS wash: PBS containing 0.5 % saponin, 0.5 % BSA, 1 % FBS, 0.5 % human serum, 0.1 % sodium azide) and washed again in FACS wash. The cells were then incubated with 50 µl 4G2 (10 µg/ml in FACS wash) for 45 min at 4 °C. Cells were washed twice and the pellet was subsequently incubated in 50 µl α-mouse Igs PE-conjugated (5 µg/ml in FACS wash, Dako) for 30 to 45 min at 4 °C. Cells were then washed twice more in FACS wash and a third time in PBS, before analysis with flow cytometry (FACSCalibur, Beckton Dickinson).

Depletion of anti-ED3 antibodies from plasma samples

Nickel charged agarose beads (Ni-NTA, Qiagen) were washed 5 times in PBS^+ (PBS containing an additional 150 mM NaCl and 50 mM Imidazole) and then incubated overnight, rotating at 4 °C, with C-terminally His-tagged ED3(1) and ED3(2) mixed together. Ten µg of each protein was added to every 50 µl of beads used, in a final incubation volume twice the bead volume. Some beads were incubated in the absence of ED3 antigen, as a beads only, mock control. Beads were then washed 5 times with PBS^+ and pre-cleared for 2 h at RT with a dengue-naïve human serum sample, at a dilution of 1 in 100, in an incubation volume 5 times the bead volume. Beads were then washed 3 times in PBS^+ and incubated with the human plasma samples of interest (50 µl beads per 800 µl sample) for 2 h at 4 °C. The convalescent samples (days 15-30 post-defervescence) were diluted in PBS^+ (primary infections: 1 in 250, secondary infections: 1 in 400) before mixing with the beads. The remaining depleted samples were collected, filter-sterilized and tested for complete depletion by ED3 direct ELISA.

RESULTS

The anti-ED3 response following primary infections

With much of the recent interest in ED3 as a possible dengue vaccine immunogen, we initially set out to determine the anti-ED3 response in humans. Convalescent blood samples were collected from individuals 2-4 weeks following their presentation to hospital with dengue infections. We first examined anti-ED3 IgG binding responses, by direct ELISA against bacterially expressed recombinant ED3, in individuals convalescing from a primary dengue infection (Fig. 1). To account for the amount of each serotype antigen coated on the ELISA plates, we normalized our responses with an anti-ED3 monoclonal antibody, 2H12, generated in our laboratory. This is highly cross-reactive between the four serotypes, exhibiting mean (n=4) K_d of 0.4496, 0.4248, 0.4655 and 0.4215 nM for ED3(1), (2), (3) and (4), respectively. Saturation binding of 2H12 to the coated ELISA plates varied by less than 11 % and titres were normalized against these saturation OD values, to allow for a fair comparison between serotypes. We also have confidence in the refolding of these antigens, as the same material was used to obtain crystal structures of ED3's from Den1, Den2 and Den3 complexed with the Fab of three separate anti-ED3 monoclonal antibodies, showing the correct ED3 conformation (our unpublished data).

In the primary dengue cases, the strongest anti-ED3 responses were elicited during Den1 infections, and the responses following Den2 and Den3 infections were lower (Fig. 1a). Across all infections, however, the highest response detected in most individuals was against the infecting serotype. This is consistent with previous reports, whereby a serotype-specific ED3 response was described after primary infection (44, 66). However,

we also detected low yet positive responses against the remaining 3 serotypes, demonstrating a degree of cross-reactivity. We established a competition assay to investigate this cross-reactivity in more detail (Fig. 1b and c). Immune plasma was incubated with an increasing concentration of recombinant ED3 from the four viral serotypes or with a control protein. Following incubation, the mix was added to ELISA plates coated with ED3 and the level of bound anti-ED3 antibodies, representing those not competed, was determined. Responses could be ranked in terms of avidity, with the highest avidity being displayed by the antigen that was able to compete at the lowest concentration.

Taking an individual with a primary Den1 infection (Fig. 1b), the ED3(2)-specific response (panel 2) could be completely blocked by the presence of competing ED3(1) protein. Conversely, when the plate was coated with ED3(1), ED3(2) was a poor competitor compared with ED3(1). This shows very clearly that the ED3(2)-specific response was indeed present and furthermore was completely cross-reactive with dengue serotype 1. Importantly, ED3(1) protein could completely block the response with 100-fold less antigen than competing with ED3(2) protein, indicating a higher avidity to the infecting serotype. The same effect is illustrated with a second patient (Fig. 1c). Following a primary infection with Den2, an ED3(4)-specific response was detected and could be completely inhibited by the presence of ED3(2) antigen. Again, the plasma avidity was strongest against the infecting serotype; a trend seen with the primary samples tested (n=10).

Complete cross-reactivity in secondary infections.

Anti-ED3 analyses of convalescent plasma from individuals experiencing secondary infections revealed that both the serotype-specific and cross-reactive antibody responses were higher than following primary infection, with endpoint titers reaching 480,000 (Fig. 2). Most individuals were seropositive for all 4 ED3 antigens but, unlike in primary infections, the titers gave no clear indication as to what the current or previous infecting serotypes may have been. In fact, titers against ED3(1) seemed to be slightly higher than the other serotypes, regardless of the infecting serotype.

Next, we went on to perform the competition ELISA in secondary infection; two representative cases are shown (Fig. 2b and 2c). Following a secondary infection with Den1, the ED3(1) response could be completely inhibited by competition with ED3(2) (Fig. 2b, Panel 1). Similarly cross-reactive responses to the other 3 serotypes were also completely inhibited by ED3(2). A similar pattern is seen with a patient suffering a secondary Den 2 infection (Fig. 2c). In this case, ED3(1) could completely inhibit binding to all four virus serotypes. The anti-ED3 response in these secondary infections was 100 % cross-reactive, and this was true for all samples tested (n=30). It was also always the case, barring one sample, that antigen from a serotype that was not that of the current infecting serotype provided the best competition. Up to 10,000-fold less antigen was required to out-compete the response against the infecting serotype, indicating a significantly stronger avidity to this other serotype. Interestingly, the strong binding responses seen against ED3(1) were not reflected in antibody avidity and instead all serotypes were, at least once, identified as “winners”.

Dramatic original antigenic sin in secondary dengue infections

Original antigenic sin was first described in the antibody responses following sequential influenza infections (10, 13-15, 68). Rather than effective *de novo* neutralizing responses being elicited against the current infecting serotype, memory responses to the primary infecting serotype were induced. Following this, Halstead showed that original antigenic sin was also present in the neutralizing antibody responses in dengue (26). In line with this, our competition results, in which 100 % of antibody produced to the secondary infecting virus showed cross-reaction and higher avidity to a different serotype, suggested the presence of original antigenic sin in these secondary anti-ED3 responses.

In order to test this hypothesis, we investigated samples from secondary infections in which the primary exposure was known. Pre- and post-exposure plasma samples were available from individuals experiencing a secondary dengue infection. In cases where a clear primary dengue serotype could be identified from neutralization analyses of the pre-exposure sample, a competition assay was performed on the post-exposure convalescent samples (Fig. 3). In each case, the serotype with the highest avidity in the competition assay matched the primary exposure identified by neutralization analyses. For example, with patient K1, the $FRNT_{50}$ of the pre-exposure plasma strongly indicated that Den1 had been the primary infection (Fig. 3a). In agreement with this, when we tested the post-exposure plasma in the competition assay, protein from serotype 1 was able to completely outcompete all responses at the lowest concentrations (Fig. 3b).

In 1983, Halstead proposed that original antigenic sin in the post-exposure neutralization response could help to deduce the serotype of the primary infecting virus, in that the strongest neutralizing activity is generally directed towards the primary infecting serotype

(26). It is often thought that in either an acute or convalescent sample, the serotype with the highest ND_{50} is indicative of the primary infecting serotype. There are different interpretations for this type of diagnosis (12, 26, 39) but a titer of at least 4-fold that of other serotypes would provide a highly stringent diagnosis.

We performed focus reduction neutralization tests (FRNT) on a selection of paired immune plasma from acute and convalescent time points, and the $FRNT_{50}$ were compared to the results from the competition ELISA (Table I). For individuals in whom one serotype exhibited a 4-fold higher $FRNT_{50}$, and thus the primary exposure was clear (*n*=10; Table I), the competition assay did indeed predict the primary infecting serotype. Unfortunately, it is often the case that neutralizing responses are complex, with neutralization of multiple viruses occurring. This does not always allow for the clear determination of the primary infecting serotype (1, 39). In our study, in individuals in whom one serotype did not exhibit a 4-fold higher $FRNT_{50}$ than the others (*n*=5, Table I), the competition ELISA was successful in indicating a probable primary serotype. As a result, this quick, easy, virus-free competition assay could provide an alternative to the time-consuming neutralization assay in current use.

Antibody-dependent enhancement in primary and secondary dengue infections

Original antigenic sin in the antibody response to dengue has two potential adverse outcomes for the infected host. Firstly, by driving the production of low avidity antibody, viral clearance may be delayed. In addition, original antigenic sin has the potential to affect ADE. At present, ADE is proposed to be driven predominantly by pre-existing antibody. However, as there are substantial increases in antibody during the early stages

of secondary infection, boosting of poorly neutralizing, low avidity responses during the course of the secondary infection may amplify this effect still further.

We therefore examined ADE in patients following either primary or secondary infection. ADE assays were performed on U937 cells comparing infection with either Den1 or Den2, and the %-infected cells was recorded (Fig. 4). Months after a primary infection with Den1, at a point when individuals would be expected to become susceptible to infections with heterologous serotypes, the peak of enhancement for Den2 occurred at higher concentrations of plasma than Den1 (Fig. 4b), indicating that enhancement of Den2 was more likely. We next examined two scenarios of secondary infection: Den1 followed by Den2 (Fig. 4c) and vice versa Den2 followed by Den1 (Fig. 4d). In each case, the concentration of immune serum required to enhance the second virus was greater than the concentration required to enhance the first; a pattern similar to that observed with the primary immune sera. This indicates that an individual would likely be more susceptible to the secondary virus, as has been reported previously in epidemiological studies (23). Furthermore, if we follow the time course of a secondary Den2 infection, the plasma continues to enhance Den2 more readily throughout (Fig. 4e). These data demonstrate that there is an inverse relationship between the avidity of the antibody response and enhancing activity, suggesting that original antigenic sin may also influence ADE.

Anti-ED3 responses contribute little to neutralizing and enhancing activity

In mice, ED3 is a known target of neutralizing antibodies and so has been proposed as a target of immunization studies. We were therefore interested to investigate the contribution of anti-ED3 antibodies to neutralizing and enhancing activity in the human

response. PCS and a selection of convalescent primary and secondary plasma samples were depleted of ED3 binding antibodies by incubation with sepharose beads coated with ED3(1+2) or no protein as a control. To ensure that the His-tagged protein had completely depleted all anti-ED3 antibodies, we performed an ELISA with the untagged protein.

Following depletion, the neutralizing and enhancing activity of the plasma was determined. There was no reduction in neutralizing activity on Vero or 293T cells (data not shown), and this is in agreement with Wahala *et al*, who showed that anti-ED3 antibodies did not contribute to neutralization in human sera (66). Interestingly, considering the highly cross-reactive nature of anti-ED3 antibodies in humans, depletion of anti-ED3 antibodies also had no effect on the ability of plasma samples to enhance Den2 infection in U937 cells (Fig. 5a-c). Furthermore, depletion from PCS did not affect activity on primary monocytes (Fig. 5d). Such measurements on monocytes are informative because they can be infected in the presence or absence of antibody, meaning that the full scope of both neutralization and enhancement is observed in the one assay.

DISCUSSION

Original antigenic sin was first described in 1953 from observations on immune responses to influenza viruses (10, 15). Following on from this, in his “disquisitions on original antigenic sin”, Fazekas made detailed observations on children vaccinated with two strains of influenza, who by virtue of their age could have only been naturally exposed to one of these strains (13). Upon heterologous vaccination, fully cross-reactive

antibodies were generated against the vaccinating strain and previously encountered strain. Despite recent reports corroborating these findings (34), there have been some studies with influenza vaccination that have questioned the significance or even the presence of original antigenic sin (21, 70).

The exact mechanisms of original antigenic sin are not fully understood. The degree of antigen similarity will clearly influence the ability of a single antibody to cross-react. It has been suggested that a difference of less than approximately 33-42 % will result in original antigenic sin (6, 11). Interestingly, dengue antigens show approximately 30 % amino acid difference between the serotypes, leaving them in a prime position for such a phenomenon. It is also suggested that cross-reactive memory responses are preferentially activated over naïve cells, as memory cells require a lower level of activation to proliferate (34). Furthermore, memory cells may outnumber naïve cells meaning that, when antigen levels are low, they could outcompete naïve cells for antigen. In addition, it appears that the vaccine formulation may also have a bearing on the effect (34).

Original antigenic sin in dengue was first described by Halstead in 1983 (26) and forms the basis for serological efforts to untangle the sequence of infection in secondary cases. More recently, original antigenic sin has also been described in dengue-specific T cell responses (49). In this study, we have taken a close look at antibody responses to ED3 in both primary and secondary infection. Dengue seems to be an extreme example of original antigenic sin, where the secondary response is entirely constructed from antibody that cross-reacts with previously encountered virus. Furthermore, these antibodies can be competed by 20- to 10,000-fold less antigen from the putative primary infecting serotype, indicating higher avidity for these antigens than for the secondary infecting virus.

Original antigenic sin has the advantage that a response can be rapidly mobilized from memory. However, the downside is that in some cases, such as dengue, the response is dominated by inferior quality antibody. In influenza, original antigenic sin has been shown to reduce the effectiveness of vaccination (13, 34, 51). In dengue, the effect of original antigenic sin has considerable bearing on vaccine strategies. Once a response has been established, it is unlikely that repeat boosting will be able to change its scope, meaning that balanced responses against the four virus serotypes will need to be established with the first vaccine dose.

In this report we have also shown that the primary infecting serotype, and thus antibody avidity, influences antibody dependent enhancement in subsequent infections. The balance between neutralization and enhancement will likely determine the susceptibility of an individual to an infection and, by driving higher virus replication, may expose some individuals to the risk of developing DHF. An association between higher virus loads and DHF has been demonstrated (63) and we believe that the mobilization of a poor quality antibody response in the early stages of secondary dengue infection may allow further amplification of virus replication via ADE.

In mice, some antibodies generated following infection with flaviviruses react to ED3 and show potent neutralization of infection (18, 19, 27, 42, 43, 52, 61). These observations have generated interest in the use of ED3 as an immunogen for subunit dengue vaccines, and several flavivirus anti-ED3 antibodies have been suggested as potential therapeutics (5, 33, 50, 55, 60). It has recently been described that some anti-ED3 neutralizing monoclonal antibodies do not neutralize all strains within a given serotype to equal levels (5, 55, 60, 65), meaning that therapeutic Abs will need to be carefully chosen.

In the experiments described here we used prototypic dengue strains for the four serotypes, and did not attempt to match to the virus causing each secondary infection. Nevertheless, the results on polyclonal human sera were clear, showing very high-level cross-reactivity during secondary infection. It remains possible that comparing a panel of ED3 strains from a given serotype in competition assays may allow a more precise description of the primary infecting virus beyond just its serotype.

ED3 has two potential attractions as an immunogen: firstly, antibodies directed to this domain are frequently of high affinity and show high neutralizing capacity for dengue virus compared to antibodies directed to other regions of the envelope protein (60). Secondly, ED3 is a target of serotype-specific mouse monoclonal antibodies (5, 18, 42, 45, 53, 55, 60, 61). Initially, it was thought that antibodies against this domain had limited cross-reactivity between the 4 serotypes, suggesting a reduced risk of ADE following vaccination. Recent reports in macaques (30) and humans (44, 66) also suggest that the primary ED3 response is relatively serotype-specific. However, it has since been recognised that there are indeed cross-reactive mouse monoclonal antibodies against this domain (19, 43, 46, 52, 55, 60, 61), even though neither the binding avidities nor the critical binding amino acids need be the same between serotypes (46). Furthermore, whilst cross-reactive ED3 antibodies can be strong neutralizers (52, 55, 60), they are generally weaker neutralizers compared to their serotype-specific counterparts (19, 61), meaning that the potential for ADE remains significant.

In this study of the human serological response to dengue, we demonstrate a low to moderate level of cross-reactivity in the ED3 response following primary infection, although the highest response was mostly indicative of the infecting serotype.

Subsequently, however, during a heterotypic secondary infection, this low level cross-reactivity was dramatically boosted to the extent that 100 % of antibody was cross-reactive.

Cross-reactive mouse monoclonals have been shown to bind with highest avidity to ED3 serotype 1 (19, 64) and, following ED3 immunization in mice, anti-ED3(1) polyclonal responses are also often the strongest (37). Whilst these data correspond well with the high ED3(1) binding titres that we observed during secondary infections, we did not see a bias towards this serotype in our polyclonal competition avidity ELISA. It has also been reported that cross-reactive monoclonals bind poorly to ED3 from serotype 4 (19, 64). Consistent with this, following primary dengue infections in humans, we found reduced levels of seropositivity against ED3(4) compared to the remaining serotypes. This may be a consequence of the slight structural differences in the ED3 of this serotype (64).

In humans, it has recently been described that anti-ED3 antibodies constitute a relatively minor proportion of the response against dengue (8, 66) and it appears that a major part of the anti-envelope antibody response targets the fusion loop of envelope (8, 40). Corresponding with this, the anti-ED3 response seems to contribute little or nothing to neutralization in humans, as we demonstrate here in monocytes and as reported by Wahala et al. (66). The limited contribution of the anti-ED3 response to neutralization in a natural infection does not however mean that inducing a high level response by vaccination would fail, and such vaccination strategies should not be discounted.

Finally, the ELISA competition assay we describe here may well provide an alternative to neutralization assays to predict the sequence of dengue infection. The technique is fast,

does not require infection-containment facilities and yields comparable results to the neutralization assays. Indeed, in a number of cases, neutralization results are complex and do not allow the diagnosis of the primary infection, whereas the competition ELISA usually gives clear definition of the avidity response.

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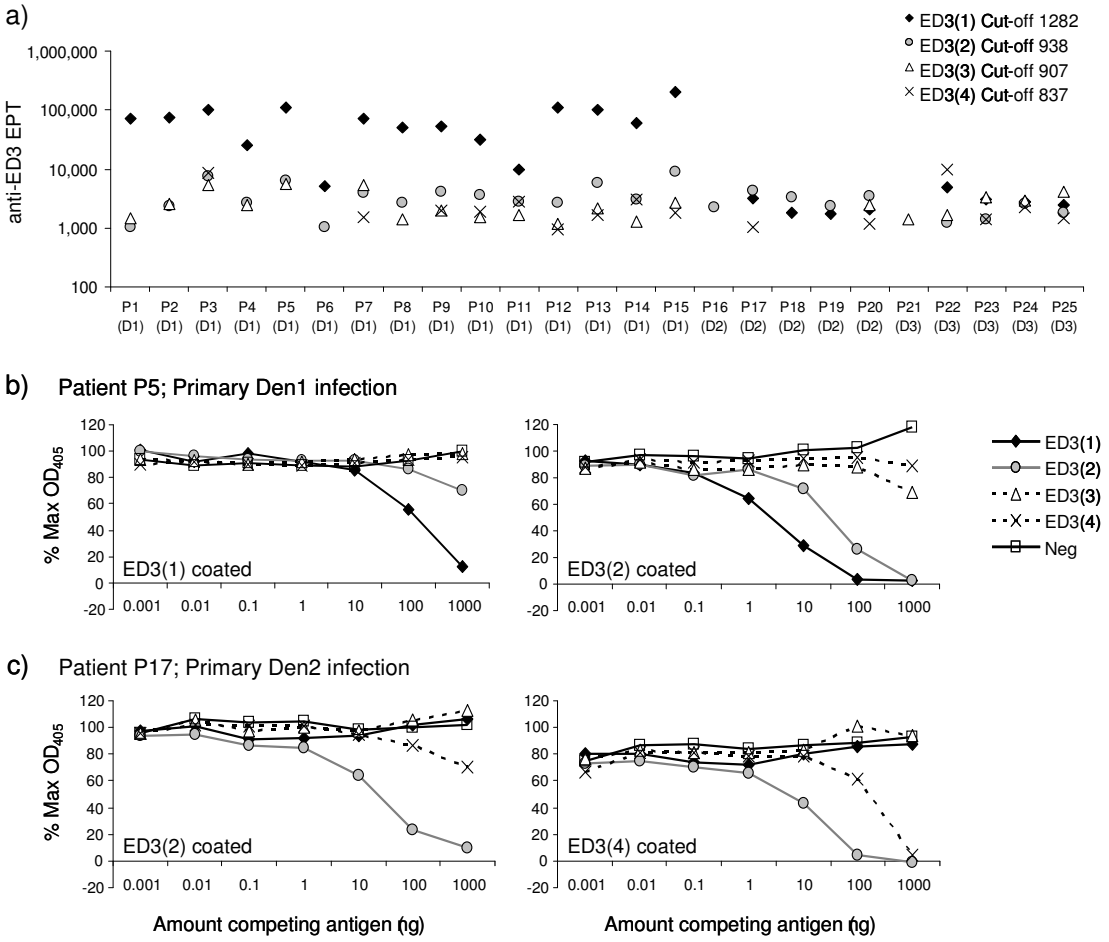

Fig. 1: Serotype cross-reactive anti-ED3 antibodies are induced after primary dengue infections in humans. a) Anti-ED3 end point titers (EPT) were determined for all 4 serotypes by direct ELISA against bacterially expressed recombinant ED3. All plasma samples taken 15 to 30 days after defervescence from individuals with primary dengue infections, are shown (*n*=25). Cut-off levels of seropositivity for each assay are indicated in the key. Assay values below these cut-offs are not depicted, meaning that for some individuals not all 4 titers are shown. P: primary; (D1-4): current infecting serotype, as determined by PCR. b) Competition ELISA for an individual with primary Den1 infection. The anti-ED3(1) and anti-ED3(2) responses, for which D1 and D2 are coated on the ELISA plate respectively, are shown. c) Competition assay for an individual with primary Den2 infection. The anti-ED3(2) and anti-ED3(4) responses are shown.

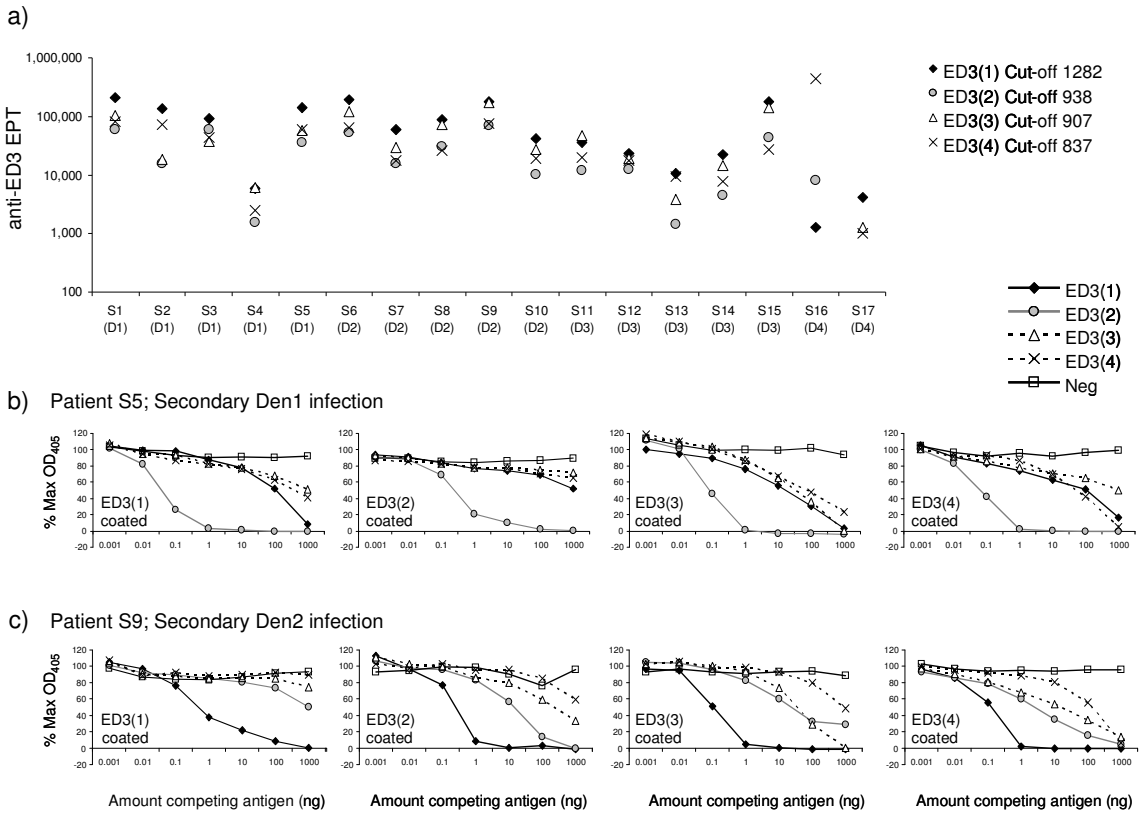

Fig. 2: Anti-ED3 antibodies are completely cross-reactive in secondary dengue infections in humans. a) Anti-ED3 end point titers (EPT) were determined for all 4 serotypes by direct ELISA against bacterially expressed recombinant ED3. A representative selection of plasma samples, taken 15 to 30 days after defervescence from individuals with secondary dengue infections, is shown (*n*=17 out of 76 analysed). Cut-off levels of seropositivity for each assay are indicated in the key. Assay values below these cut-offs are not depicted, meaning that for some individuals not all 4 titers are shown. S: secondary; (D1-4): current infecting serotype, as determined by PCR. b) Competition ELISA for an individual with secondary Den1 infection. The responses against all 4 ED3 serotypes are shown. c) Competition assay for an individual with secondary Den2 infection. The responses against all 4 ED3 serotypes are shown.

a)

	Serotype PCR	Time	Den1 FRNT$_{50}$	Den2 FRNT$_{50}$	Den3 FRNT$_{50}$	Den4 FRNT$_{50}$	Comp ELISA
K1	4	Pre Post	**7,077** 225,805	1,947 55,916	2,678 26,598	254 17,973	- 1
K2	4	Pre Post	228 8,671	135 7,517	**516** 17,281	< 40 5,020	- 3
K3	4	Pre Post	102 79,247	**2,901** 95,443	< 40 43,765	50 32,102	- 2
K4	1	Pre Post	< 40 20,080	< 40 13,292	< 40 48,343	**163** 49,697	- 4

b)

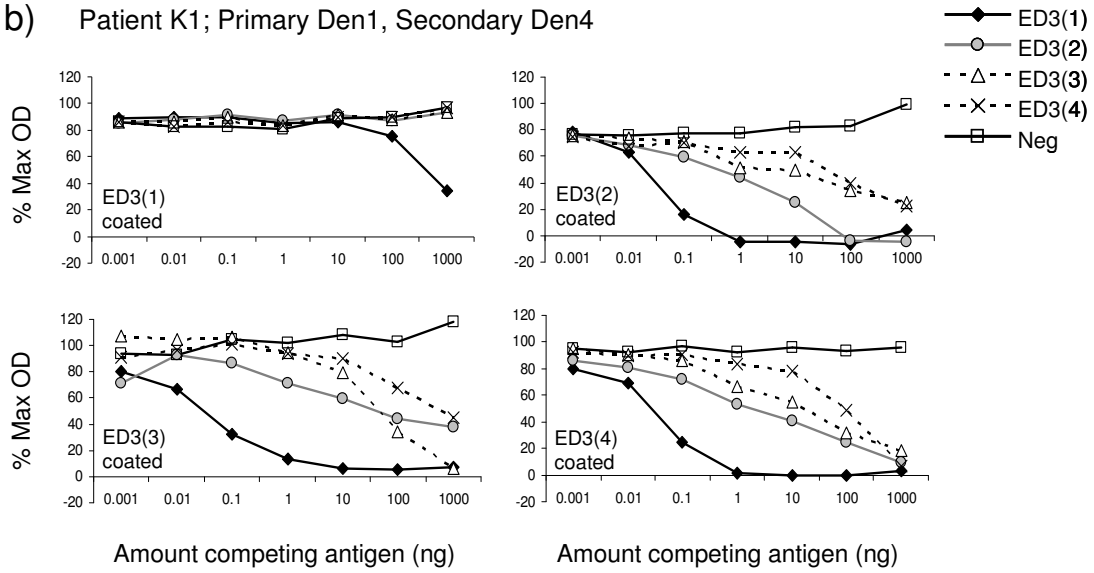

Fig. 3: ED3 competition assay predicts the primary infecting serotype. a) The pre- and post-exposure plasma samples from a selection of individuals with secondary infections (*n*=4) were analysed for neutralising activity and this information was used to determine the primary infecting serotype (bold). A competition assay was then carried out on the corresponding 2-4 wk post-exposure plasma samples. b) Competition ELISA for a single individual (K1) with a secondary Den4 infection, who had a primary Den1 exposure. The responses against all ED3 serotypes are shown.

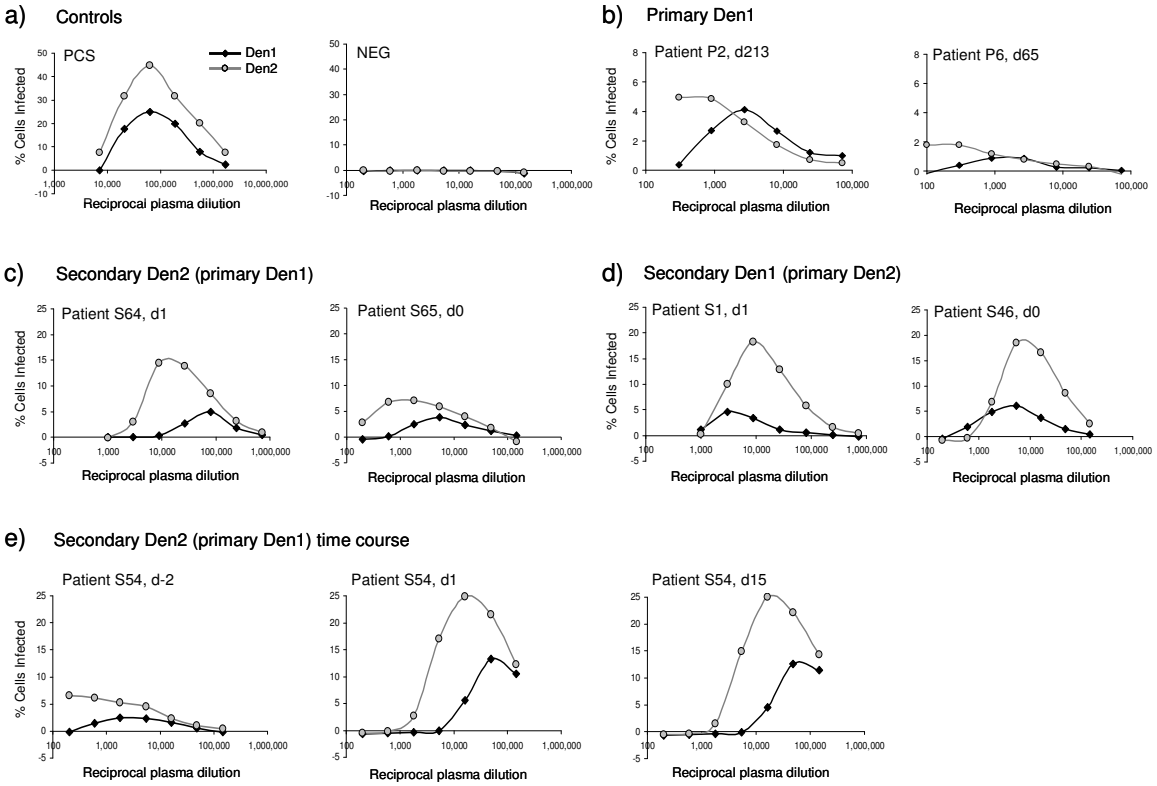

Fig. 4: ADE in primary and secondary dengue infections
Legend overleaf

Fig. 4: ADE in primary and secondary dengue infections. Enhancement assays of Den1 and Den2 were performed on U937 cells. The two virus stocks were equated by aligning the peaks of PCS enhancement (a). Virus was incubated for 1 h at 37 ℃ with decreasing concentrations of serum/plasma and then added to U937 cells for a further 24 h. Cells were stained intracellularly with the dengue cross-reactive monoclonal antibody 4G2. The % infected cells was determined by flow cytometry and the background levels (in the absence of plasma) were deducted. Enhancement assays were carried out using plasma from b) individuals who had a primary Den1 infection several months ago (*n*=5, 2 representative samples depicted); c) individuals with an acute secondary infection with Den2, and known to have had a primary infection with Den1 (*n*=5, 2 representative samples depicted); d) individuals with an acute secondary infection with Den1, and known to have had a primary infection with Den2 (*n*=4, 2 representative samples depicted). e) Enhancement assays were performed over the acute time course of an individual with Den2 and known to have had primary Den1 exposure. Days since defervescence are indicated.

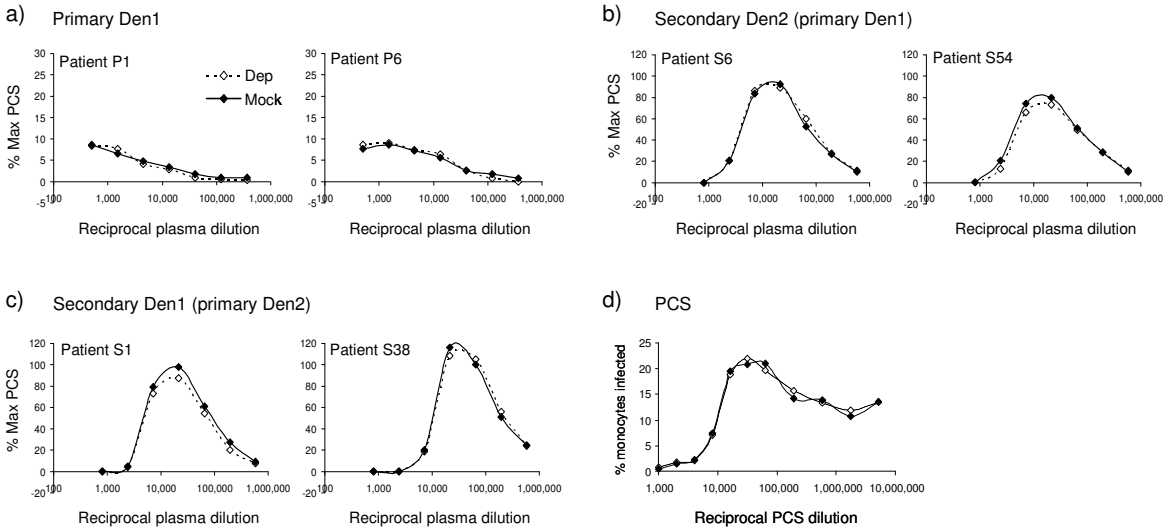

Fig. 5: Depletion of anti-ED3 antibodies from human plasma has no effect on enhancing activity. PCS and a selection of convalescent plasma samples were depleted completely of anti-ED3 antibodies. Samples were diluted and incubated with either His-tagged recombinant ED3(1+2), enabling pull-down with Ni2+-coated agarose beads (Dep), or with no protein (beads only, mock control). The depleted samples were then tested for enhancement of Den2 infection on a-c) U937 cells or d) primary monocytes. Plasma were tested from individuals with a) primary Den1 infection (*n*=5, 2 representative samples depicted); b) a secondary Den2 infection and known to have had a primary Den1 infection (*n*=5, 2 representative samples depicted); c) a secondary Den1 infection and known to have had a primary infection with Den2 (*n*=4, 2 representative samples depicted). The % cells infected were normalised to the maximum level of infection observed with a non-depleted PCS control. d) PCS, depleted of anti-ED3 antibodies, was tested for its ability to both neutralize and enhance Den2 infection on primary monocytes. In the absence of serum, 13.7 % infection was observed.

Table I: $FRNT_{50}$ titres and competition ELISA results from secondary infections. Bold: titres that are > 4-fold that of other serotypes. A: acute; C: convalescent. The serotype with the highest avidity in the competition ELISA is listed.

Patient ID	Clinical Diagnosis	Serotype PCR	Time	Den1 $FRNT_{50}$	Den2 $FRNT_{50}$	Den3 $FRNT_{50}$	Den4 $FRNT_{50}$	Comp ELISA
S1	DHF1	1	A	43,174	**465,764**	7,868	14,974	-
			C	16,542	**147,704**	1,950	5,806	2
S4	DHF2	1	A	267	95	**1,551**	10	-
			C	215	103	**2,238**	10	3
S5	DHF2	1	A	980	**13,240**	528	112	-
			C	13,482	**110,122**	6,084	1,128	2
S6	DHF1	2	A	**2,010**	201	135	10	-
			C	2,560	2,560	2,560	167	1
S9	DHF1	2	A	1,639,400	494,864	356,369	109,436	-
			C	65,804	84,338	24,658	28,336	1
S28	DHF1	1	A	904	457	2,167	13	-
			C	1,087	1,001	2,560	28	3
S38	DHF2	1	A	1,318	**6,728**	1,095	6,718	-
			C	3,921	**55,032**	3,944	8,003	2
S39	DHF2	1	A	43,880	110,262	3,530	9,854	-
			C	24,900	47,524	2,044	3,194	2
S41	DHF3	1	A	128,691	502,032	74,129	251,381	-
			C	25,120	61,196	8,861	37,808	2
S44	DHF3	1	A	482	202	**2,227**	100	-
			C	734	446	2,560	74	3
S45	DHF1	1	A	20,702	**98,716**	3,466	2,476	-
			C	29,754	22,868	8,212	2,714	2
S46	DHF3	1	A	5,252	**56,650**	902	2,202	-
			C	2,984	**18,976**	494	516	2
S54	DHF1	2	A	17,182	6,476	548	712	-
			C	107,270	47,328	2,554	3,302	1
S64	DHF3	2	A	**5,769,072**	615,852	426,661	183,843	-
			C	399,288	295,067	36,070	85,400	1
S65	DHF1	2	A	**2,560**	531	1,132	38	-
			C	**2,560**	537	1,261	54	1

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ERRATUM

An In-Depth Analysis of Original Antigenic Sin in Dengue Virus Infection

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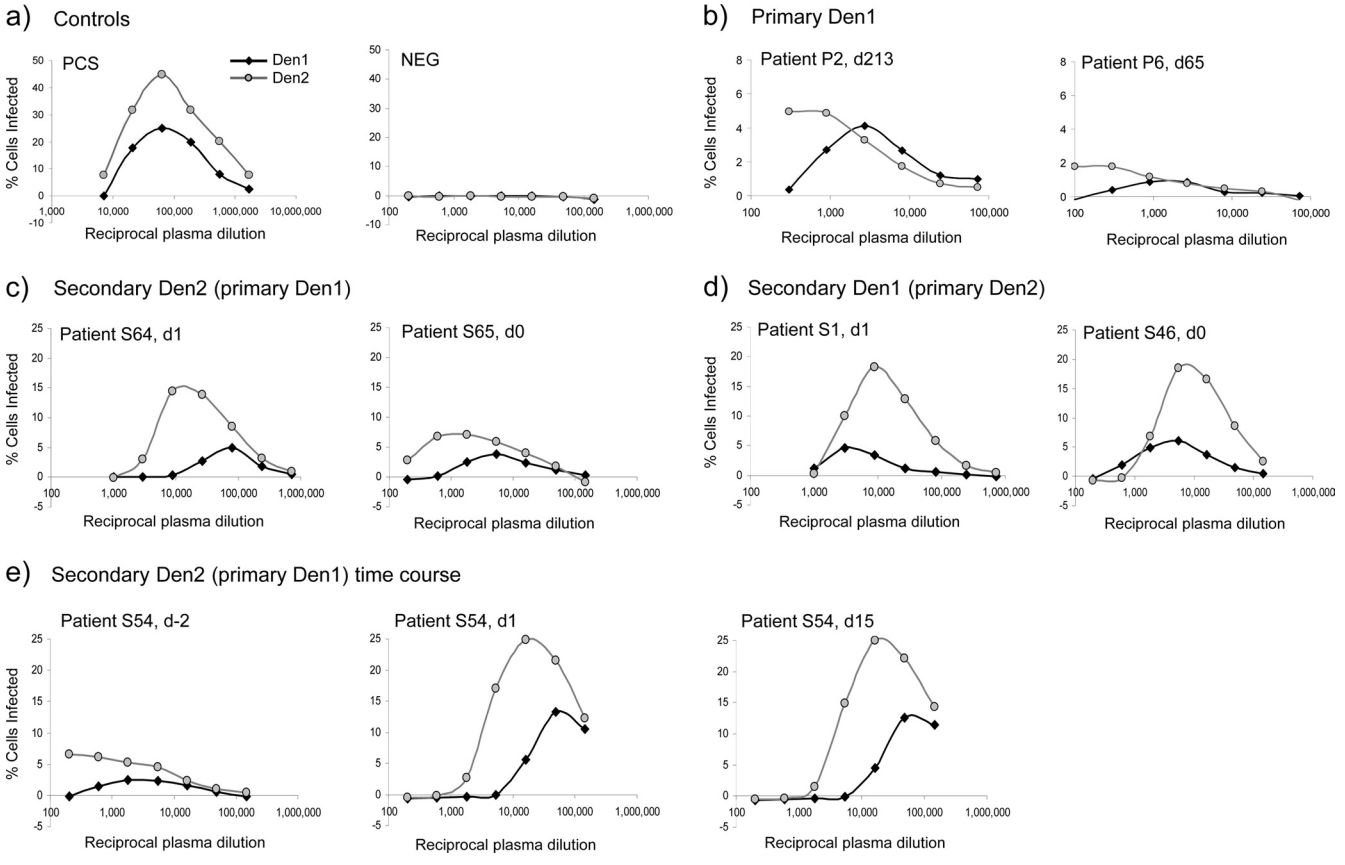
Volume 85, no. 1, p. 410–421, 2011. Page 410: The byline should appear as shown above.
Page 417, Fig. 4 should appear as shown below.



Page 417, legend to Fig. 4: The last three lines should read "representative samples depicted) (d). The number of days since defervescence from day 0 (d0) to day 213 (d213) are indicated after the patient identification. (e) Enhancement assays were performed over the acute time course of an individual with Den2 and known to have had primary Den1 exposure."