Design of a subunit vaccine that could work against all four dengue serotypes

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Dengue virus infection is a mosquito-borne illness worldwide, affecting up to 390 million people annually. Endemic in more than 120 countries, nearly four billion people are at risk of infection. Most clinical cases self-resolve but approximately 5% experience severe dengue. Antibody-dependent enhancement has been hypothesized as a mechanism to explain severe dengue upon secondary infection. Many investigators are working on dengue vaccines with one currently licensed vaccine on the market and several candidate vaccines are in clinical trials. In an earlier study, we analyzed the structures and sequences of the viral envelope glycoprotein of the dengue serotypes and have located a putative epitope that is shared by the various serotypes. Judicious amino acid replacements to enhance the antigenicity of this epitope relative to the rest of the molecule could produce a possible universal subunit vaccine against dengue without antibody-dependent enhancement. Here, we present our design of such a vaccine.

KEYWORDS

dengue serotypes, common epitope, universal vaccine, antibody-dependent enhancement

INTRODUCTION

Dengue is a global mosquito-borne illness with nearly four billion people living in at risk areas around the world. Approximately 5% of cases experience life-threatening severe dengue and a small percentage of cases are fatal. With four known infectious serotypes, patients who are infected with dengue virus will only develop immunity to the serotype with which they are infected. They do not develop long-term cross-protective immunity to infection with other serotypes. Severe dengue infection is associated with a secondary heterotypic infection, younger age, longer duration between infections, and certain serotypes (Halstead and O'Rourke 1977, Halstead 2003). Antibody-dependent enhancement (ADE) has been hypothesized as the mechanism to explain severe dengue upon secondary infection.

Peptide-based vaccines are currently under development for various clinical conditions including infectious diseases, cancer, allergy, autoimmune diseases, as well as dengue (for a review, see Reginald et al. 2018). In vitro-synthesized, highly immunogenic peptides of 20-30 amino acids can be developed to elicit a robust, long lasting B and T cell immune response. However, peptide vaccines are poorly immunogenic and are susceptible to enzymatic degradation, and therefore require the addition of carriers and adjuvants. A subunit vaccine that includes most if not all of the entire molecule would be more stable and would more closely mimic the structure of the native molecule than a 20-30 amino-acid vaccine, and thus elicit a more realistic antibody response.

In an earlier study (Mikita and Padlan 2019), we analyzed the structures and sequences of the viral envelope glycoprotein (ENV) of the dengue serotypes and have located a putative epitope that is shared by the various serotypes. Judicious amino acid replacements to enhance the antigenicity of this epitope relative to the rest of the molecule could produce a possible universal subunit vaccine against dengue while de-emphasizing the other epitopes, including the one that likely elicits antibody-dependent enhancement. Here, we report our attempt to design a subunit vaccine that is based on the whole ENV molecule and which may be effective against all pathogenic dengue serotypes.

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Table 1: Representative DEN1-4 ENV sequences used in this study

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         10         20         30         40         50         60
          |          |          |          |          |          |
MRCVGIGSRDFVEGLSGATWVDVVLEHGSCVTTMAKDKPTLDIELLKTEVTNPAVLRKLC DEN1
MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC DEN2
MRCVGVGNRDFVEGLSGATWVDVVLEHGGCVTTMAKNKPTLDIELQKTEATQLATLRKLC DEN3
MRCVGVGNRDFVEGVSGGAWVDLVLEHGGCVTTMAQGKPTLDFELTKTTAKEVALLRTYC DEN4
         70         80         90        100        110        120
          |          |          |          |          |          |
IEAKISNTTTDSRCPTQGEATLVEEQDANFVCRRTFVDRGWGNGCGLFGKGSLITCAKFK DEN1
IEAKLTNTTTASRCPTQGEPSLNEEQDKRFVCKHSMVDRGWGNGCGLFGKGGIVTCAMFT DEN2
IEGKITNITTDSRCPTQGEAVLPEEQDQNYVCKHTYVDRGWGNGCGLFGKGSLVTCAKFQ DEN3
IEASISNITTATRCPTQGEPYLKEEQDQQYICRRDVVDRGWGNGCGLFGKGGVVTCAKFS DEN4
         130        140        150        160        170        180
          |          |          |          |          |          |
CVTKLEGKIVQYENLKYSVIVTVHTGDQHQVGNESTEHGTTATITPQAPTTEIQLTDYGA DEN1
CKKNMEGKIVQPENLEYTIVITPHSGEENAVGNDTGKHGKEIKVTPQSSITEAELTGYGT DEN2
CLEPIEGKVVQYENLKYTVIITVHTGDQHQVGNET-Q-GVTAEITPQASTTEAILPEYGT DEN3
CSGKITGNLVQIENLEYTVVVTVHNGDTHAVGNDTSNHGVTAMITPRSPSVEVKLPDYGE DEN4
         190        200        210        220        230        240
          |          |          |          |          |          |
LTLDCSPRTGLDFNEMVLLTMKEKSWLVHKQWFLDLPLPWTSGASTSQETWNRQDLLVTF DEN1
VTMECSPRTGLDFNEMVLLQMENKAWLVHRQWFLDLPLPWLPGADTQGSNWIQKETLVTF DEN2
LGLECSPRTGLDFNEMILLTMKNKAWMVHRQWFFDLPLPWTSGATTETPTWNRKELLVTF DEN3
LTLDCEPRSGIDFNEMILMKMKKKTWLVHKQWFLDLPLPWTAGADTSEVHWNYKERMVTF DEN4
         250        260        270        280        290        300
          |          |          |          |          |          |
KTAHAKKQEVVVLGSQEGAMHTALTGATEIQTSGTTTIFAGHLKCRLKMDKLTLKGMSYV DEN1
KNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSGNLLFTGHLKCRLRMDKLQLKGMSYS DEN2
KNAHAKKQEVVVLGSQEGAMHTALTGATEIQNSGGTSIFAGHLKCRLKMDKLELKGMSYA DEN3
KVPHAKRQDVTVLGSQEGAMHSALAGATEVDSGDGNHMFAGHLKCKVRMEKLRIKGMSYT DEN4
         310        320        330        340        350        360
          |          |          |          |          |          |
MCTGSFKLEKEVAETQHGTVLVQIKYEGTDAPCKIPFSTQDEKGVTQNGRLITANPIVTD DEN1
MCTGKFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMDLEKRHVLGRLITVNPIVTE DEN2
MCTNTFVLKKEVSETQHGTILIKVEYKGEDAPCKIPFSTEDGQGKAHNGRLITANPVVTK DEN3
MCSGKFSIDKEMAETQHGTTVVKVKYEGAGAPCKVPIEIRDVNKEKVVGRIISSTPLAEN DEN4
         370        380        390
          |          |          |
KEKPVNIEAEPPFGESYIVIGAGEKALKLSWFKK                           DEN1
KDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKK                           DEN2
KEEPVNIEAEPPFGESNIVIGIGDNALKINWYKK                           DEN3
TNSVTNIELEPPFGDSYIVIGVGNSALTLHWFRK                           DEN4
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Representative ENV sequences for the four dengue serotypes are shown. The residues that are in the putative His317 epitope are shown **bold**; the residues in the epitope that are in the second molecule of the dimer are shown bold and **red**. Underlined are two regions, 256-264 and 368-377, that contain peptides which could serve as T-cell epitopes (see text).

MATERIALS AND METHODS

Sequence analyses

The dengue virus envelope protein (ENV) sequences that we used here were those that we had collected for our previous study (Mikita and Padlan 2019). The ENV sequences were retrieved from the National Center for Biotechnology Information (NCBI) database. Only those sequences in which all 495 residues, or 493 in the case of serotype 3, were identified were included in our study; those with insertions or deletions were excluded. The total number of ENV sequences compared was 3,900 for serotype 1 (hereinafter referred to simply as DEN1), 3,450 for serotype 2 (DEN2), 2,101 for serotype 3 (DEN3), and 969 for serotype 4 (DEN4). For each serotype, the sequence that is most similar to the other sequences of the serotype was chosen to represent the serotype. The sequences that we chose to represent the various serotypes are GenBank Entries ABW35430 (DEN1), AFI71739 (DEN2), Q6YMS3 (DEN3), and P09866 (DEN4). These representative ENV sequences are shown in Table 1.

Analysis of the structure and variation of the envelope glycoprotein of dengue virus

Prior to infection, ENV exists as a dimer on the surface of the virus, with close association of the two ENV molecules of the dimer. There is no known structure for the whole dimeric ENV and the best available dimeric ENV structures extend only to residue 394 in the case of serotype 2, or to 392 in the case of serotype 3. The high-resolution dimeric ENV structure that we analyzed is that for serotype 2 (identified by its Protein Data bank (PDB) Entry code 1OAN (Modis et al. 2003)).

Calculation of antigenicities and identification of putative epitopes

The method developed by one of us (Padlan 2008) was used for the characterization of putative antibody epitopes on ENV.

Table 2: The 1OAN (DEN2) sequence after de-antigenization of the residues that were 3/4-, 1/2-, 1/3-, and 1/4-buried

```
         10         20         30         40         50         60
          |          |          |          |          |          |
MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKYC       1OAN
MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMATGKPTLDFELIKTEAKQPATLRKYC       3/4
MRCIGISNRDFVEGVSGGSWVTIVLEHGSCVTTMATGKPTLDFELIKTEATAPATLRKYC       1/2
MRCIGISNRDFVEGVSGGSTVTIVLAHGSCVTTMATGKPTLDFELITTEATAPATLRTYC       1/3
MRCIGISNRDFVEGVSGGSTVTIVLAHGSCVTTMATGKPTLDFELITTTATAPATLRTYC       1/4
         70         80         90        100        110        120
          |          |          |          |          |          |
IEAKLTNTTTESRCPTQGEPTLNEEQDKRFVCKHSMVDRGWGNGCGLFGKGGIVTCAMFT       1OAN
IEAKLTNTTTESRCPTQGEPTLAEEQDKRFVCKHSMVDRGWGNGCGLFGKGGIVTCAMFT       3/4
IEAKLTTTTTESRCPTTGAPTLAEEADTRFVCKHSMVDRGWGAGCGLFGKGGIVTCAMFT       1/2
ITATLTTTTTESRCPTTGAPTLAEAASTAFVCTTSMVDRGWGAGCGLFGKGGIVTCAMFT       1/3
ITATLTTTTTSACPTTGAPTLAAAASTAFVCTTSMVDRGWGAGCGLFGKGGIVTCAMFT       1/4
         130        140        150        160        170        180
          |          |          |          |          |          |
CKKNMEGKIVQPENLEYTVVITPHSGEEHAVGNDTGKHGKEVKITPQSSITEAELTGYGT       1OAN
CKKNMEGKIVQPENLEYTVVITPHSGEEHAVGNDTGKHGKEVKITPQSSITEAELTGYGT       3/4
CTTTMEGKIVAPAALEYTVVITPHSGEETAVGNSTGTHGKTVTITPTSSITTATLTGYGT       1/2
CTTTMAGKIVAPAALEYTVVITPHSGAETAVGSSTGTTGTTVTITPTSSITTATLTGYGT       1/3
CTTTMAGTIVAPAALTYTVVITPHSGATTAVGSSTGTTGTTVTITPTSSITTATLTGTGT       1/4
         190        200        210        220        230        240
          |          |          |          |          |          |
VTMECSPRTGLDFNEMVLLQMKDKAWLVHRQWFLDLPLPWLPGADTQGSNWIQKETLVTF       1OAN
VTMECSPRTGLDFGEMVLLQMTGKAWLVHRQWFLDLPLPWLPGADTQGSNWIQKETLVTF       3/4
VTMECSPRTGLSFGTMVLLQMTGTAWLVTRAWFLGLPLPWLPGADTTGSSWIQKATLVTF       1/2
VTMTCSPATGLSFGTMVLLQMTGTAWLVTAAAFLGLPLPWLPGASTTGSSWITKATLVTF       1/3
VTMTCSPATGLSAGTMVLLTMTGTAWLVTAAAFLGLPLPWLPGASTTGSSWITKATLVTF       1/4
         250        260        270        280        290        300
          |          |          |          |          |          |
KNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSGNLLFTGHLKCRLRMDKLQLKGMSYS       1OAN
TNPHAKKQTVVVLGSQEGAMHTALTGATEIAMSSGNLLFTGTLKCRLRMDKLQLKGMSYS       3/4
TNPHATTQTVVVLGSQEGAMHTALTGATAIAMSSGTLLFTGTLTCRLRMGTLALTGMSYS       1/2
TNPHATTQTVVVLGSQEGAMHTALTGATAIAMSSGTLLFTGTLTCTLTMGTLALTGMSTS       1/3
TSPHATTQTVVVLGSQAGAMATALTGATAIAMSSGTLLATGTLTCTLTMGTLALTGMSTS       1/4
         310        320        330        340        350        360
          |          |          |          |          |          |
MCTGKFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMDLEKRHVLGRLITVNPIVTE       1OAN
MCTGKFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMDLEKRTVLGRLITVNPIVTE       3/4
MCTGKFKVVKTIAETQHGTIVIRVQYEGGGSPCKIPFTIMDLTKATVLGRLITVNPIVTA       1/2
MCTGTFTVVTTIAETQHGTIVIRVQYAGGGSPCKIPFTIMDLTTATVLGRLITVGPIVTA       1/3
MCTGTFTVVTTIAETQHGTIVIRVTYAGGGSPCTIPFTIMSLTTATVLGRLITVGPIVTA       1/4
         370        380        390
          |          |          |
KDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKK                                  1OAN
KDSPVNIEAEPPFGDSYIIIGVEPGQLKLTWFKK                                  3/4
TGSPVNIEAEPPFGDSYIIIGVTPGQLKLTWFKK                                  1/2
TGSPVNIEAEPPFGDSYIIIGVTPGQLTLTWFKK                                  1/3
TGSPVNIEAEPPFGDSTIIIGVTPGALTLTWFKK                                  1/4
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The residues in the 1OAN sequence that are in the putative His317 epitope are shown bold and **red**. The replacement residues during de-antigenization are shown bold and **green**. The total number of 1OAN residues that were de-antigenized were 12 for those whose sidechains are at least 3/4-, 59 for 1/2-, 93 for 1/3-, and 112 for 1/4-buried, i.e., not exposed to solvent.

Briefly, the method identifies all the residues within a certain radius of each Cα (alpha-carbon) position in a molecule and assigns an antigenicity value for that location based on the summed reactivities of those residues weighted by their exposure to solvent. The reactivities that we used were those based on the values compiled by De Gentz et al. (2002). We calculated the exposures to solvent by the method of Connolly (1983) using programs developed by Sheriff et al. (1985).

In view of the great variety of antigenic structures and of antibody combining sites (see, for example, Padlan 1994), the exact extent and shape of antibody epitopes cannot be guessed. We could, nonetheless, make estimates from available structures of antibody-ENV complexes. There are several structures in the PDB of antibodies bound to the ENV of the four serotypes from which we could get estimates of the size of ENV epitopes. For simplicity, we define the size of an antibody epitope as the radius of the circle that encloses all the residues in the epitope. In order to include as many potentially contributing residues as possible, we chose a radius of 17 A (Angstroms) as the size of the putative epitopes in this study. This size is larger than what we measured for all the PDB antibody-ENV structures that were available.

Table 3: Antigenicity of the His317 and Gln211 epitopes after various degrees of de-antigenization

Residue		Antigenicity value at various exposure levels			
	(in 1OAN)	3/4	1/2	1/3	1/4
317	**His -0.27**	**His 0.70**	**His 2.98**	**His 3.31**	**His 3.31**
211	Gln -1.41	Gln -0.62	Ala -0.46	Ala -0.21	Ala -0.16

His317 (shown in **red**) is the center of the putative epitope common to all dengue serotypes (Mikita and Padlan 2019). Antigenicity values were calculated using the method developed by one of us (Padlan 2008). The values shown are the standard deviations above the mean for all the putative epitopes in the dimer. No other putative epitopes show standard deviations above the mean of 3.00, or higher.

The antigenicity of the putative epitope centered at Gln211, which has been hypothesized as the possible cause of ADE (Mikita and Padlan 2016), is shown for comparison. Gln211 is not within the His317 epitope and had been replaced by Ala at the later three stages of de-antigenization (shown on the right). Note that the antigenicity of the Gln/Ala211 epitope remains below the mean in all five instances.

Comparison of sequences and identification of a common antibody epitope

The sequences of the four serotypes are sufficiently similar so that they are readily aligned (see Table 1). The alignment allowed us to identify the putative epitopes with residues that are the most common among the four serotypes. We found that the putative epitope centered at His317 of the 1OAN dimer contains the greatest number of residues that are common among the four serotypes (Mikita and Padlan 2019). The residues that are within 17 A of the Cα of His317 are marked in Table 1. This high degree of overall similarity of the residues contributing to the putative His317 epitope in the four dengue ENV sequences led us to predict that an antibody directed against the His317 epitope of one serotype will probably also bind to the corresponding epitope in the other serotypes (Mikita and Padlan 2019).

Design of a subunit vaccine that could be effective against all dengue serotypes

A subunit vaccine that directs antibodies to the His317 epitope could be designed using the method proposed by one of us (Padlan 2010). Briefly, the method aims to lower the antigenicity of all but the His317 epitope, while maintaining the three-dimensional structure of the molecule. This would enhance the antigenicity of the His317 epitope relative to the rest of the molecule and could produce a possible universal subunit vaccine against dengue. The reduction in antigenicity of the rest of the molecule would also result in de-emphasizing the epitope that probably causes antibody-dependent enhancement whose location is around Gln211 (Mikita and Padlan 2016).

We did trial calculations on the degree of de-antigenization to try to find the one that is probably best for the design of the desired subunit vaccine. We calculated the effect of lowering the reactivity of all the residues in the molecule except for those in the His317 epitope in the four cases wherein the side-chains are three-fourths (3/4) buried, half buried (1/2), one-third (1/3) buried, and one-fourth (1/4) buried. Lowering the reactivity of individual residues was done based on the replacement rules developed by one of us (Padlan 2008). The replacement rules were developed to preserve the local, as well as the overall, structure of the molecule. The resulting "de-antigenized" sequences are shown in Table 2. The number of residue replacements is 12 for the trial calculation based on the 3/4-buried criterion, 59 for 1/2-buried, 93 for 1/3-buried, and 112 for 1/4-buried (Table 2).

RESULTS AND DISCUSSION

The effect of the various de-antigenization calculations on all the possible epitopes in the ENV cannot be easily presented here in view of the large number of residues (394) in the molecule. For brevity, we present in Table 3 the effect of the various degrees of de-antigenization only on the His317 epitope. The fractional values shown in Table 3 represent the significance of the relative contribution of the epitope (as standard deviations above the mean) to the antigenicity of the whole dimer.

As expected, the antigenicity of the putative His317 epitope is high. Moreover, the highest contribution is already achieved when the replacements were performed on the 1/3-buried residues. In our judgment, de-antigenization of the 1/3-buried residues is sufficient to emphasize the antigenicity of the His317 epitope over all others in the ENV molecule. While de-antigenization of the 1/4-buried residues achieves the same emphasis, the number of residues de-antigenized (112) is larger than those for the 1/3-buried case (93).

Also presented in Table 3 is the effect of the various de-antigenization calculations on the Gln211 epitope, which we believe could be responsible for antibody-dependent enhancement (Mikita and Padlan 2016). Note that emphasizing the antigenicity of His317 also results in de-emphasizing the antigenicity of the Gln211 epitope. On the basis of these results, we believe that a subunit vaccine based on the 1/3-buried-replaced sequence would be useful in the fight against dengue.

Another vaccine possibility is a virus transformed to express on its surface this de-antigenized sequence plus the residues not present in the available three-dimensional structures (Modis et al. 2003).

Further analysis of the 1/3-buried de-antigenized ENV sequence

We have also considered other properties of the 1/3-buried de-antigenized ENV. One important property is its ability to elicit a T-call response. Another is its stability in solution.

We referred to the immune-epitope-database (IEDB), maintained by the National Institute of Allergy and Infectious Diseases [https://www.iedb.org/], which contains the known dengue T-cell epitopes. We found two regions: residues 253-267 and 368-380, that contain peptides that could serve as T-cell epitopes in the 1/3-buried de-antigenized ENV. There are other regions in the de-antigenized ENV that contain potential T-cell epitopes, but the 253-267 and 368-380 regions, in our view, are the best candidates. Two peptides in these regions, QEGAMHTAL (residues 256-264) and EAEPPFGDSY (residues 368-377) both CD8+ T-cell epitopes, are listed in IEDB. These sequences in DEN2 are underlined in Table 1.

Since the His317 epitope is made up of residues from both monomers in the dimer, we think that a disulfide linkage

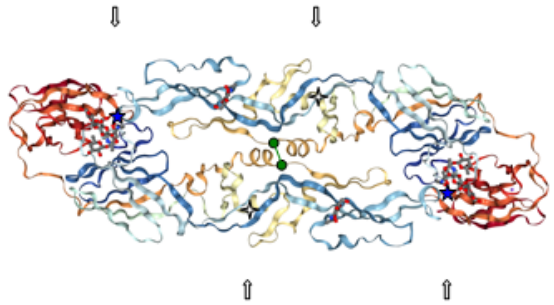

Figure 1: Ribbon diagram of the ENV structure in PDB Entry 1OAN showing the two molecules constituting the dimer. The two molecules are side-by-side in an anti-parallel fashion. The approximate locations in both molecules of Ser255 (small circles connected by a green line), Gln211 (small 4-pointed stars), and His317 (small 5-pointed stars) are shown.

between the two monomers in the ENV dimer would increase the stability of the dimeric structure and, likewise, of the His317 epitope. Calculating the Cα-to-Cα distances between residues of one monomer from those of the other, we found that a disulfide bridge between the residues at position 255 (currently occupied by serine and to be replaced by cysteine) in both monomers might be feasible. The separation of the Cα of the Ser255 of the two monomers is 6.67 A. This is comparable to the Cα-to-Cα distances: 6.49, 6.57, 6.63, 6.64 A, in the inter-domain disulfide bridges in the PDB Entry 4NZU (Grover et al. 2014) which, as of this writing, is the Fab structure in the PDB that has been determined at the highest resolution (1.20 A). The location of the possible Ser255-Ser255 disulfide bridge in DEN2 ENV is shown in Figure 1, as well as the approximate locations of His317 and Gln211.

CONCLUSION

The ENV protein of the dengue virus recognizes a receptor molecule on the surface of a target cell. ENV binding to the receptor allows the viral and cellular membranes to fuse, followed by viral entry into the cell (Modis et al. 2003). In the prefusion form, ENV is a dimer of identical protein units. Upon binding to the receptor molecule on the surface of the target cell, ENV undergoes a major structural change that results in a trimeric structure for ENV. The dimeric prefusion form of ENV is the target of our proposed subunit vaccine. The immune response against dengue virus is complicated by the possibility of ADE. Here, we try not only to develop a vaccine against all four serotypes, but also to try to eliminate ADE. We feel that we have succeeded in doing that.

We propose that the sequence shown for the 1/3-buried version (Table 2) would be a good starting point for a subunit vaccine, or as the basis for a transformed virus for use as a vaccine, that could be useful against all four dengue serotypes without the added burden of ADE.

CONTRIBUTIONS OF THE AUTHORS

C. P. Mikita provided the medical significance of the work and researched the early literature. E. A. Padlan did the sequence analyses.

CONFLICT OF INTEREST

There are no conflicts of interest. The views expressed in this article are those of the authors and do not reflect the official policy of the Department of Army/Navy/Air Force, Department of Defense, or U.S. Government.

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