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The live-attenuated yellow fever vaccine 17D induces broad and potent T cell responses against several viral proteins in Indian rhesus macaques – implications for recombinant vaccine design

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Abstract

The yellow fever vaccine 17D (YF17D) is one of the most effective vaccines. Its wide use and favorable safety profile make it a prime candidate for recombinant vaccines. It is believed that neutralizing antibodies account for a large measure of the protection afforded to YF17D-vaccinated individuals, however cytotoxic T lymphocyte (CTL) responses have been described in the setting of YF17D vaccination. YF17D is an ssRNA flavivirus that is translated as a full-length polyprotein, several domains of which pass into the lumen of the endoplasmic reticulum (ER). The processing and presentation machinery for MHC class I-restricted CTL responses favor cytoplasmic peptides that are transported into the ER by the transporter associated with antigen presentation (TAP) proteins. In order to inform recombinant vaccine design, we sought to determine if YF17D-induced CTL responses preferentially targeted viral domains that remain within the cytoplasm. We performed whole YF17D proteome mapping of CTL responses in 6 Indian rhesus macaques vaccinated with YF17D using overlapping YF17D peptides. We found that the ER luminal E protein was the most immunogenic viral protein followed closely by the cytoplasmic NS3 and NS5 proteins. These results suggest that antigen processing and presentation in this model system is not preferentially affected by the subcellular location of the viral proteins that are the source of CTL epitopes. The data also suggest potential immunogenic regions of YF17D that could serve as the focus of recombinant T cell vaccine development.

Keywords

Yellow Fever 17D; Vaccine; Cytotoxic T Lymphocyte; Indian Rhesus Macaque

Introduction

More than 95% of YF17D vaccinees mount vaccine-specific immune responses within 10 days of receiving the vaccine (Fields et al. 2001). YF17D has been used in over 500 million individuals with minimal severe side effects (Pulendran et al. 2008; Pugachev et al. 2005). Max Theiler developed YF17D in the 1930's by serial passage of the virulent Asibi strain of yellow fever until an attenuated, avirulent strain was obtained. YF17D, specifically the sub-strain 17D-204, differs from the pathogenic Asibi strain by a mere 32 amino acids (Hahn et al. 1987). Comparisons with other avirulent yellow fever vaccine sub-strains (17DD, 17D-213 and 17D-204) have found commonalities in 22 amino acid changes from wild-type yellow fever virus sequence, indicating that only a few amino acid changes are required for YF17D attenuation (Galler et al. 1998).

The principle mechanism by which YF17D induces immunity is thought to be the generation of potent and long-lived antibody responses. Indeed, patients have been described that still have high neutralizing antibody titers more than 35 years after vaccination (Poland et al. 1981). CTL responses are also induced by vaccination with YF17D (Querec et al. 2009; Bonaldo et al. 2010; Akondy et al. 2009). While the functional implications of these CTL responses with regards to YF17D clearance and disease prevention are largely unknown, their induction has led to the development of YF17D as a T cell vaccine vector (Bonaldo et al. 2010; Bonaldo et al. 2005; Tao et al. 2005; McAllister et al. 2000).

Several insertion sites for exogenous antigens have been developed in YF17D. Two sites allow entire proteins or large domains of proteins to be inserted into YF17D. The first utilizes an artificial domain created between the E and NS1 proteins (Bonaldo et al. 2007). We have introduced a portion of the simian immunodeficiency virus Gag protein into this location and established the immunogenicity of this recombinant YF17D virus in vaccinated rhesus macaques (Bonaldo et al. 2010). More recently, Stoyanov and colleagues have used a second site to insert the circumsporozoite protein from *Plasmodium yoelii* into the junction between the YF17D C and prM proteins and demonstrated immunogenicity and protection from challenge in a murine malaria model (Stoyanov et al. 2010). Other recombinant YF17D approaches have introduced smaller peptide regions into the C/prM junction (McAllister et al. 2000), into the E protein (Bonaldo et al. 2002), into the NS2b/NS3 junction (Tao et al. 2005; McAllister et al. 2000) or replaced the entire E protein with E proteins from other flaviviruses (Mateu et al. 2007; van Der Most et al. 2000; Lai and Monath 2003). Whether or not any of these insertion sites have an immunologic advantage is currently unknown. It is also not clear if the location of YF17D viral protein domains or recombinant inserts on the cytoplasmic or luminal aspects of the endoplasmic reticulum membrane (Figure 1) affects immunogenicity. Therefore, we comprehensively mapped all of the CD8⁺ T cell responses and some of the CD4⁺ T cell responses in a group of Indian rhesus macaques vaccinated with YF17D. We found that certain sites appear more immunogenic than others, but location in the cytoplasm or ER lumen did not appear to affect immunogenicity.

Materials and Methods

Animals

Indian rhesus macaques (*Macaca mulatta*) from the Wisconsin National Primate Research Center were selected for these studies based upon expression of Mamu-A*02 or Mamu-B*17 as determined by sequence-specific PCR (Knapp et al. 1997). Mamu-A*02+ and Mamu-B*17+ animals were selected because of the extensive characterization of these alleles in Indian rhesus macaques and because of the availability of an online epitope prediction algorithm for both alleles (Peters et al. 2005). All animals were housed and cared

for according to the regulations set forth in the Guide for the Care and Use of Laboratory Animals (1996) and all animal experiments were approved by the University of Wisconsin Institutional Animal Care and Use Committee. Six macaques (Table 1) were vaccinated subcutaneously with 100,000 plaque-forming units of YF17D.

Quantitative PCR

Quantification of YF17D virus in plasma from vaccinated animals was determined seven days after YF17D vaccination, using a previously employed assay (Bonardo et al. 2010). Viral RNA was isolated from EDTA-anticoagulated plasma by proteinase K digestion of the nucleocapsid in the presence of guanidine hydrochloride. Viral RNA was reverse transcribed and amplified using the SuperScript III Platinum® One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA, USA) in a Roche LightCycler® 480. The final reaction mixtures (100- μ L total volume) contained 0.2mM (each) deoxynucleoside triphosphates, 3.5mM MgSO₄, 750 ng of random hexamer primers (Promega, Madison, WI, USA), 4.0 μ L of SuperScript III reverse transcriptase and Platinum Taq DNA Polymerase in a single enzyme mix, 600 nM (each) amplification primers (forward primer YF-17D 10188 [5'-GCGGATCACTGATTGGAATGAC-3'], reverse primer YF-17D 10264 [5'-CGTTCGGATACGATGGATGACTA-3']), and 100 nM probe (6-carboxyfluorescein [6FAM]-5'-AATAGGGCCACCTGGGCCTCCC-3'-6-carboxytetramethylrhodamine [TetraM]). The reverse transcription reaction was performed at 37°C for 15 minutes and then 50°C for 30 minutes. An activation temperature of 95°C for 2 minutes was followed by 50 amplification cycles of 95°C for 15 seconds and 57°C for one minute with ramp times set to 2.2 °C per second. 10-fold serial dilutions of an YF-17D *in vitro* transcript served as the internal standard curve for each run. The transcript was made using a MEGAscript® (Ambion, Austin, TX, USA) high yield transcription kit. A plasmid, pCR-Blunt, containing a PCR amplicon with a sequence identical to nucleotides 8,621-10,354 of GenBank accession number U17066, served as the template for transcription.

Peptides

All peptides used in these studies were synthesized to >70% purity by GenScript (Piscataway, NJ, USA). 163 peptides predicted to bind *Mamu-A*02* by a published online algorithm (Peters et al. 2005) were pooled into 21 separate groups. The 21 peptides with the highest predicted *Mamu-A*02* binding affinity measurements (the lowest predicted IC₅₀ values) were split into each of the pools so that no single pool contained more than one peptide with high predicted *Mamu-A*02* binding affinity. For whole proteome mapping, peptides 15 amino acids in length overlapping by 11 amino acids encompassing the YF17D proteome were also synthesized. These peptides were pooled into groups of 10 contiguous peptides for initial response screening.

Whole Proteome Epitope Mapping

Fresh PBMC isolated from EDTA-anticoagulated blood were used in IFN- γ ELISPOT assays as previously described (Loffredo et al. 2007), however the determination of positive responses was made using a new technique which we have found in control experiments to be more sensitive and reproducible than our previously used method. Test wells were run with two replicates while positive and negative control wells were run in replicates of 2, 4 or 6 depending on the assay. Responses containing < 50 spot forming units per 1×10^6 cells were considered negative and not tested statistically. Positive responses were determined using a one-tailed t-test and $\alpha = 0.05$, where the null hypothesis (H_0): background level $\geq$ treatment level. If determined to be positive statistically, the reported values equal the average of the test wells minus the average of all negative control wells. In the indicated experiments, PBMC were first depleted of CD8⁺ cells using a non-human primate magnetic

bead separation protocol (Miltenyi Biotec, Auburn, CA, USA). Greater than 99% of CD8⁺ cells were successfully depleted in all experiments and verified by FACS analysis.

Results

To evaluate the relative immunogenicity of various regions of the YF17D proteome in Indian rhesus macaques, we vaccinated four *Mamu-A1*00201*⁺ (*Mamu-A*02*⁺) animals and two *Mamu-B*01701*⁺ (*Mamu-B*17*⁺) animals with 100,000 plaque-forming units of YF17D. A list of the animals included in this study along with their MHC class I alleles as defined by sequence-specific PCR is provided in Table 1. We detected viral RNA amplification in four of five animals seven days after vaccination using a previously described quantitative PCR assay (Bonaldi et al. 2010), indicating that the animals were indeed infected with replicating YF17D (Table 2). The single animal without detectable viremia made anti-YF17D-directed T cell responses post-vaccination, indicating that it was likely infected with replicating YF17D.

To detect Mamu-A*02-restricted epitopes in the YF17D proteome, we used a published, online algorithm (Peters et al. 2005) to generate a list of predicted Mamu-A*02-binding peptides derived from the entire YF17D proteome. We then selected 163 peptides with predicted binding affinities (IC₅₀) < 50 nM for synthesis. We pooled the synthesized peptides into 21 different groups. We then mapped IFN-gamma ELISPOT responses using PBMC in the four Mamu-A*02⁺ animals on day 17 post-vaccination using the Mamu-A*02-predicted peptide pools. To our surprise, only one of these pools elicited a positive response in two of the four Mamu-A*02⁺ animals. We broke down the positive pool on day 20 post-vaccination and found that both animals responded to the same E protein peptide, E₆₉₆₋₇₀₅ (Table 3). In spite of the robust responses directed towards this epitope in two of the Mamu-A*02⁺ animals, the lack of response in the other two Mamu-A*02⁺ animals suggests that this response is not recognized by high frequencies of CD8⁺ T cells in all Mamu-A*02⁺ animals.

We proceeded to map T cell responses directed against the YF17D proteome in vaccinated macaques using a set of 849 peptides that were 15 amino acids long and overlapped by 11 amino acids. This peptide set covered the entire YF17D polyprotein. Our primary goals were to determine the relative immunogenicity of the various viral proteins, to detect Mamu-B*17-restricted responses and to see if preferential CD8⁺ T cell targeting of YF17D protein domains might have occurred based upon their subcellular location in relation to the luminal or cytoplasmic side of the endoplasmic reticulum. We analyzed responses from all 6 vaccinated animals to this peptide set on the day of vaccination and found no responses above background (data not shown). On day 17 post-vaccination, we mapped IFN-gamma ELISPOT responses directed against pools of 10 sequential overlapping peptides encompassing the entire viral proteome for both total PBMC and CD8-depleted PBMC (representing CD4⁺ T cell responses) from each animal. All positive responses detected in this initial screen on day 17 were then subsequently verified and broken down to the individual 15-mer peptides on day 20 post-vaccination. These results are summarized in Figure 2 and Table 3. We reliably detected 21 separate putative CD8⁺ T cell responses in seven of the ten viral proteins. We did not detect any responses to the C, prM or NS4b proteins in our vaccinated animals. The two Mamu-B*17⁺ animals, r00012 and r03031, did not share any responses indicating that we were unable to detect immunodominant Mamu-B*17-restricted YF17D epitopes in our experiment. We also could not detect any immunodominant Mamu-A*02-restricted YF17D epitopes since none of the responses in the four Mamu-A*02⁺ animals were found in more than two individual animals. However, we did detect the shared predicted Mamu-A*02-restricted E₆₉₆₋₇₀₅ AF10 epitope in our total proteome scan. The concordance of our overlapping 15-mer scan with our Mamu-A*02

predicted epitope results suggests that YF17D does not contain a large number of broadly immunogenic or immunodominant Mamu-A*02-restricted CTL epitopes. Interestingly, simian immunodeficiency virus contains a total of seventeen distinct Mamu-A*02-restricted CTL epitopes, three of which are immunodominant and targeted by CTL in almost all Mamu-A*02⁺ SIV-infected animals (Loffredo et al. 2004).

Two high magnitude CD8⁺ T cell responses were detected in r04131 and r03031, E₆₈₁₋₆₉₉ and NS3₂₀₂₁₋₂₀₃₈, suggesting these animals share one or two MHC class I alleles that restrict these responses (Table 3). Our SSP-PCR MHC class I typing of these two animals (Table 1) did not detect any shared alleles, therefore the restriction of these epitopes remains unknown.

CD4⁺ T cell epitopes were more difficult to detect in our experiments due to problems with high background on our CD8-depleted IFN-gamma ELISPOT plates. This phenomenon of high background on CD8-depleted IFN-gamma ELISPOT plates following YF17D vaccination had been noted in previous experiments and is currently being characterized in more depth (Costa Neves et al., manuscript in preparation). Nevertheless, we were able to detect two low-level CD4⁺ T cell responses that were shared in r02059 and r03031, E₃₂₉₋₃₄₃ and NS3₁₈₃₇₋₁₈₅₁ (Figure 3). Once again, the shared responses suggest these animals have an MHC class II allele in common that restricts these responses. Due to the high background and other potential confounding issues, we cannot be certain that we were able to map the entire CD4⁺ T cell repertoire against YF17D. However, these two responses were reproducibly observed in both of these animals.

Finally, we compiled our comprehensive CD8⁺ T cell response mapping data to assess the relative immunogenicity of the various viral proteins (Figure 1). The most immunogenic protein in our 6 animals was the E protein followed closely by the NS3 and NS5 proteins. Our data may be biased slightly towards E since the Mamu-A*02⁺ animals, two of which target the E₆₉₆₋₇₀₅ AF10 epitope, clearly have a preference for the E protein when compared with the Mamu-B*17⁺ animals. Nevertheless, the most immunogenic regions of YF17D in our cohort of macaques were clearly the E, NS3 and NS5 proteins. All of these proteins were targeted in at least 3 of the 6 animals. The NS5 protein was targeted most frequently in 6 out of 6 animals followed by E in 5 out of 6 animals.

Discussion

YF17D is being developed as a recombinant T cell vaccine vector (Bonaldo et al. 2010; Bonaldo et al. 2005; Tao et al. 2005; McAllister et al. 2000). The relative immunogenicity and efficacy of the various recombinant insertion sites remains unknown. Therefore, we evaluated the immunogenicity of the YF17D viral proteins to determine if certain regions of the viral proteome might be more immunogenic than others. This work was premised on the mechanism of MHC class I antigen processing and presentation which is widely believed to favor the transport of antigenic proteins from the cytoplasm. We postulated that perhaps the location of several viral proteins on the luminal side of the endoplasmic reticulum membrane (Figure 1) might diminish their immunogenicity. However, our data do not support this conclusion. In light of work describing the generation of immunogenic MHC class I-restricted peptides from defective ribosomal products (DRiPs) (Lev et al. 2010; Yewdell et al. 1996), it is reasonable to conclude that the irrelevance of the final subcellular location of our antigenic YF17D regions to their immunogenicity is likely due to processing and presentation occurring upstream of this final localization. Because ER translocation occurs in tandem with translation, we presume that antigen processing and presentation of these regions might occur within its own immunologic translation compartment (Lev et al. 2010). Our YF17D immunogenicity data most closely fit the defective ribosomal products

model. We did not detect preferential targeting of cytoplasmic polyprotein domains. In fact, the region of YF17D with the highest magnitude of CD8⁺ T cell responses in our experiments was the ER luminal E protein. Any concerns about using insertion sites in or near the E protein because of its ER luminal location are therefore unjustified.

We also determined that the NS5 protein followed by the E protein and the NS3 protein were the most frequently targeted antigens in YF17D. The NS5 protein was targeted by CD8⁺ T cell responses of variable magnitudes in all six of our vaccinated animals. NS5 was also a regular target of T cell responses in YF17D vaccinated humans (Akondy et al. 2009). Our work suggests that a strategy to introduce recombinant YF17D insertion sites in or immediately adjacent to the NS5 protein might be desirable for a recombinant T cell vaccine. Recombinant insertions in this region would likely induce insert-directed responses in most individuals.

Finally, we were surprised by the small number of detected Mamu-A*02- or Mamu-B*17-restricted YF17D epitopes. Mamu-A*02 and Mamu-B*17 are relatively frequent MHC class I alleles in captive populations of rhesus macaques (frequencies in all animals typed to date [N>8,200] are 20.78% and 10.35% for Mamu-A*02 and Mamu-B*17, respectively). We did not detect any immunodominant Mamu-A*02- or Mamu-B*17-restricted epitopes in our vaccinated cohort. We found a single putative Mamu-A*02-restricted response and were unable to define any Mamu-B*17-restricted responses. It is possible that the transient viremia seen in YF17D vaccinated humans and macaques is insufficient to induce high frequency immunodominant responses in all animals, however the description of one such response in HLA-A0201⁺ YF17D vaccinated humans (Akondy et al. 2009) suggests that this is not always the case. Interestingly, we detected two responses of large magnitude in two of our animals suggesting that they both recognized the same immunodominant epitopes.

Perhaps the discrepancy between Mamu-A*02-restricted responses in acute and chronic SIV infection (Loffredo et al. 2004) and acute YF17D infection is a function both of MHC class I allele specificity for the two viruses and the kinetics of antigen exposure. Notably, the HLA-A0201 allele, which restricts a single immunodominant response in YF17D vaccinated humans, restricts several immunodominant epitopes in HIV-infected humans (Yusim et al. 2009). It seems reasonable that the detection of only a single immunodominant HLA-A0201-restricted YF17D response in vaccinated humans might indicate a lower antigenic stimulatory capacity of YF17D when compared with HIV infection. In spite of this apparent reduction in YF17D's capacity to induce responses of both high frequency and high magnitude, it is worth noting that the induction of T cell vaccine responses of high frequency and high magnitude in SIV-challenged macaques has not been correlated with improved outcome after infection. Hence, the quality and efficacy of CD8⁺ T cell responses engendered by a T cell vaccine does not correlate with the induction of such responses. These qualitative aspects of successful antiviral T cell responses are not accurately measured by our current assays. Therefore, we should continue to move forward with several vaccine vectors in efficacy trials in our search for a successful T cell-based vaccine.

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Figure 1. Depiction of membrane topology of a generic flavivirus adapted from Fields Virology, Fourth Edition, page 998 (Fields et al. 2001). Membrane topology was predicted from the calculated hydrophobicity of the viral proteins.

Figure 2.

Cellular immune responses directed against YF17D peptides in PBMC. Six rhesus macaques (four Mamu-A*02⁺, left column; two Mamu-B*17⁺, right column) were vaccinated with 100,000 plaque-forming units of YF17D. Positive IFN gamma ELISPOT responses to pools of overlapping YF17D 15-mer peptides were broken down to individual 15-mers on day 20 post-vaccination. Locations of positive individual 15-mer peptides and the corresponding magnitude of the indicated responses in IFN gamma ELISPOT are mapped for each animal. The proportion of each animal's total positive response directed towards individual viral protein domains is diagrammed with a pie chart. The total proportions for all six animals and for both groups of MHC-matched animals are also included. Pie charts represent the total magnitude for the individual responses within the indicated viral domain compared to the total magnitude of all responses made.

Figure 3.

CD4⁺ T cell responses reliably detected above background in two YF17D vaccinees. Responses were mapped to individual YF17D 15-mers using magnetically CD8-depleted PBMC. Background responses were higher in CD8-depleted ELISPOT experiments; therefore the statistical test we used to determine positive responses was less sensitive. We could only reliably detect the indicated responses in two of the macaques with the lowest level of background. The fact that these two animals have two responses in common indicates they share one or more MHC class II alleles.

Table 1

SSP-PCR MHC Typing of Vaccinated Animals

Animal	A*02	B*17	B*01	B*22	B*30
r02059	X			X	X
r97101	X				X
r00058	X				
r04131	X		X		
r00012		X		X	X
r03031		X			

YF17D Quantitative PCR**Table 2**

Animal	Day 0	Day 7
r02059	0 copies/mL	129 copies/mL
r97101	0 copies/mL	115 copies/mL
r00058	0 copies/mL	0 copies/mL
r04131	0 copies/mL	314 copies/mL
r00012	0 copies/mL	18 copies/mL
r03031	Not Tested ^a	Not Tested ^a

^a r03031 did not have sufficient quantities of blood available for testing on Day 7 post-vaccination. All values are listed as viral RNA copies per mL of plasma. This assay can reliably detect values ≥ 15 copies/mL.

Table 3
YF17D CTL responses

Epitope	Peptide Sequence	Responding Animal(s)	Magnitude (SFU/10 ⁶)
E ₃₄₁₋₃₅₅	ARKVCYNAVLTHVKI	r02059	75
E ₆₈₁₋₆₉₉	IGKLFTQTMKGVERLAVMG	r04131	1,187
		r03031	822
E ₆₉₆₋₇₀₅	AVMGDTAWDF	r97101	387
		r00058	150
E ₇₄₅₋₇₅₉	VLIWVGINTRNMTMS	r97101	98
NS1 ₇₆₉₋₇₈₃	MMFLSLGVGADQGCA	r97101	183
NS1 ₈₅₃₋₈₇₁	INAI FEENEVDISVVVQDP	r97101	153
NS1 ₈₈₉₋₉₀₃	LQYGWKTWGNLVS	r97101	103
NS1 ₉₄₁₋₉₅₅	FTTRVYMDAVFEYTI	r97101	53
NS1 ₉₅₃₋₉₆₇	YTIDCDGSILGAAVN	r97101	68
NS2a ₁₁₈₅₋₁₁₉₉	TVAVGLHFHEMNNGG	r97101	143
NS2a ₁₃₀₅₋₁₃₁₉	IIGVLHQNFKDTSMQ	r97101	185
NS2b ₁₄₂₉₋₁₄₄₃	DVASEQGEFKLLSE	r03031	70
NS3 ₁₉₂₅₋₁₉₄₃	EGRKVAIKGPLRISASSAA	r00058	83
NS3 ₂₀₂₁₋₂₀₃₈	FRELVRNCDLPVWLSWQV	r04131	347
		r03031	1,057
NS4a ₂₁₈₉₋₂₂₀₂	MAMGT MAGCGYLMF	r03031	117
NS5 ₂₅₆₁₋₂₅₇₅	VSRGTAKLRWFHERG	r04131	78
		r03031	77
NS5 ₂₈₂₅₋₂₈₃₉	ASMVNGVIKILTYPW	r02059	125
		r00058	78
NS5 ₃₁₀₅₋₃₁₁₉	DQRGSGQVVITYALNT	r04131	88
NS5 ₃₁₆₅₋₃₁₇₉	LKRMAVSGDDCVVRP	r97101	135
NS5 ₃₁₇₇₋₃₁₉₁	VRPIDDRFGLALSHL	r00012	388
NS5 ₃₁₉₃₋₃₂₀₇	AMSKVRKDI SEWQPS	r00058	75