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Yellow fever 17D-vectored vaccines expressing Lassa virus GP1 and GP2 glycoproteins provide protection against fatal disease in guinea pigs

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Abstract

Yellow Fever (YF) and Lassa Fever (LF) are two prevalent hemorrhagic fevers co-circulating in West Africa and responsible for thousands of deaths annually. The YF vaccine 17D has been used as a vector for the Lassa virus glycoprotein precursor (LASV-GPC) or their subunits, GP1 (attachment glycoprotein) and GP2 (fusion glycoprotein). Cloning shorter inserts, LASV GP1 and GP2, between YF17D E and NS1 genes enhanced genetic stability of recombinant viruses, YF17D/LASV-GP1 and –GP2, in comparison with YF17D/LASV-GPC recombinant. The recombinant viruses were replication competent and properly processed YF and LASV GP proteins in infected cells. YF17D/LASV-GP1&GP2 induced specific CD8+ T cell responses in mice and protected strain 13 guinea pigs against fatal LF. Unlike immunization with live attenuated reassortant vaccine ML29, immunization with YF17D/LASV-GP1&GP2 did not provide sterilizing immunity. This study demonstrates the feasibility of YF17D-based vaccine to control LF in West Africa.

Keywords

Yellow Fever; Lassa Fever; YF17D vaccine; recombinant vaccines

1. Introduction

Yellow Fever 17D is one of the most efficacious and safe vaccines ever developed with a highly favorable benefit-risk profile and excellent cost-effectiveness. In YF endemic areas a vaccinated child is fully protected over a 50-year life time against different genotypes of YF for an investment of a few cents per year [1,2]. Since 1937 more than 540 million doses

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have been delivered world-wide and no reversion to wild-type YF virus has been reported. Rare observed adverse effects seem to be associated with host factors and are not due to virus reversion [3–5].

After sub-cutaneous inoculation, YF17D minimally replicates in local DCs and stimulates DC subsets via multiple Toll-like-receptors (TLRs) to elicit pro-inflammatory cytokines [6–8]. Cytokine-activated and mature dendritic cells (DCs) become protected against YF-induced cytopathogenicity and migrate to regional lymph nodes to elicit a broad spectrum of innate and adaptive immune responses [9–13]. A systems biology approach confirmed that YF17D induces integrated multi-lineage and poly-functional immune responses including activation of effector mechanisms of innate immunity (complement, multiple TLRs, cytokines/cytokine receptors, and interferons), as well as adaptive immune responses through an early T cell activation followed by robust B cell responses [14,15]. Computational analyses identified a gene signature, B cell growth factor TNFRSF17, predicting with up to 100% accuracy the neutralizing antibody response, a well-established correlate of protection [15].

The introduction of active immunization programs and vector control measures in 1937–1938, transformed YF from a major human plague to a medical curiosity by the end of World War II [1,16,17]. However, during the past 20 years the absence of effective public health policy, social-ecological factors and vaccine shortage resulted in a resurgence of YF (~ 200,000 cases and 30,000 deaths annually) in South America and Africa. Thirty-two African countries with a total population of 610 million people including more than 219 million urbanites are now considered at risk for YF. The vast majority of cases and deaths take place in sub-Saharan Africa, where YF is a major public health problem occurring in epidemic patterns [1]. In 2007 WHO–UNICEF recommended including YF17D vaccine in routine infant immunization programs and initiated mass vaccination campaigns to rapidly increase population immunity in high-risk areas [18].

Based on outstanding YF17D safety records and recent success in molecular biology of flaviviruses, the genetic backbone of YF17D has been used for construction of chimeric YF17D-based viruses expressing prM and E proteins of closely-related flaviviruses, Japanese encephalitis, Dengue, and West Nile virus. ChimeriVax™-based vaccines against these flaviviruses are currently undergoing Phase II–III clinical testing (reviewed by [19]). Insertion of short immunogenic peptides into E surface glycoprotein protein did not compromise the morphological structure and attenuated phenotype of YF17D. Recombinant YF17D-based vectors have also been used in the development of promising new vaccines carrying immunogenic epitopes of flavivirus-unrelated pathogens (influenza, malaria, oncogenes) [6,20–23].

We recently developed a novel strategy to express relatively large foreign genes using the YF17D backbone. We used this strategy to design a bivalent YF17D-based recombinant vaccine against Lassa Fever (LF) caused by Lassa virus (LASV). LASV circulates in YF endemic areas in West Africa and is responsible for thousands of deaths annually [24,25]. Prospective serological and epidemiological studies performed in Sierra-Leone, Guinea, and Nigeria revealed the population at risk to be 59 million with an estimated annual incidence of illness of 3 million [25–28]. The sizeable disease burden makes a strong case for LASV vaccine development [29–31].

Like other members of the *Arenaviridae*, LASV has a bi-segmented (L and S) ambisense RNA genome [32,33]. The L RNA encodes a large protein (L, or RNA-dependent RNA polymerase) [34], and a small zinc-binding Z protein [35]. The S RNA encodes the major structural proteins, nucleoprotein (NP) and glycoprotein precursor (GPC), cleaved into GP1,

GP2, and a stable signal peptide, SSP [36–38]. In contrast to YF, CD8⁺ T cell responses play a major role in protection against LASV. Live replication-competent vaccine candidates based on vaccinia [31], vesicular stomatitis virus (VSV) [39], and Mopeia virus (MOPV), a non-pathogenic arenavirus relative of LASV [40,41], have been proposed as potential vaccine candidates. However, vector safety concerns remain a major obstacle for further development of these vaccines. We designed a recombinant LASV vaccine based on the YF17D vector which, as it has been mentioned, was extensively tested in humans.

A LASV truncated GPC gene (with SSP deletion) was cloned between the E and NS1 genes of YF17D. The YF17D/LASVΔGPC recombinant virus was replication-competent, deeply attenuated, induced immune responses against both pathogens, YFV and LASV, and protected guinea pigs against fatal LF in proof-of-concept challenge experiments [42]. However, our further attempts to scale-up this technology were complicated by instability of the YF17D/LASVΔGPC recombinant viruses. The LASVΔGPC insert within a monocistronic YF17D vector was stable only during the first 5 passages and the insert was randomly deleted after passage no. 5. In this paper, we describe our approaches to increase stability of LASV inserts cloned into the YF17D vector. The YF17D-based recombinant LASV vaccines with enhanced genetic stability have been tested in challenge experiments in strain 13 guinea pigs.

2. Materials and methods

2.1. Viruses and cells

LASV, strain Josiah/SL, was obtained from the Centers for Disease Control (Atlanta, GA). All work with LASV-infectious samples was performed within the maximum containment laboratory at the Southwest Foundation for Biomedical Research in San Antonio, Texas. YF17D-204 was purchased from ATCC (Manassas, VA). The viruses were grown on Vero E6 cells cultured in Dulbecco's modified minimum Eagle's medium (DMEM, GIBCO-BRL) with 2% fetal calf serum (FCS, GIBCO-BRL), 1% penicillin-streptomycin, and L-glutamine (2 mM) at 37° C in 5% CO₂ [41] by using a multiplicity of infection of 0.01. Supernatants were collected at 72 hours post-infection (LASV) or when cytopathic effect was clearly developed (YF17D), titrated on Vero cells as previously described [42], and virus stocks ($1-5 \times 10^7$ PFU/ml) were stored at -70° C.

2.2. Construction of YF17D/LASV recombinant viruses

The YF17D/LASV-GPC plasmid was constructed in the background of the full-length YFV17D cDNA clone by fusion PCR mutagenesis [42]. The recombinant plasmid was linearized by XhoI and used for in vitro RNA transcription. In vitro RNA transcription, electroporation of the BHK-21J cells, in vivo RNA labeling, preparation of virus stocks, immunofluorescence and plaque assays were previously described [42,43]. LASV-GPC subunits, GP1 (aa 59–259) and GP2 (aa 260–491), were cloned between E and NS1 genes using similar techniques. Recombinant YF17D/LASV-GP1 viruses were replication competent and grew to titers ($7-8 \log^{10}$ PFU/ml) comparable with the YF17D virus. The initial attempts to construct YF17D/LASV-GP2 did not result in production of infectious virus despite detectable viral RNA synthesis and positive immunofluorescence staining for YF NS1. This was probably due to the lack of cleavage between the YF17D E and LASV GP2 protein, which was supposed to be mediated by cellular signalase. To solve this problem, two new YF17D/LASV-GP2 recombinants with modified YF17D E – LASV GP2 fusion sequences were designed. In the first set of recombinant, YFV17D/LASV-GP2BgIII, the naturally occurring YF17D E – NS1 cleavage site was restored by mutating the first amino acid of GP2 from Leu to Asp, which resulted in the production of a properly sized LASV GP2 and created a BgIII site as indicated by the name of the construct. In the second

sets of recombinants, YFV17D/GP2fus, the sequence encoding the first 14 aa of LASV GP1 was inserted between YF17D E and LASV GP2. This resulted in the production of a GP2 with a small N terminal GP1 extension. Both approaches gave infections recombinant viruses with high titer ($1.0\text{--}2.0 \times 10^7$ PFU/ml) and stable LASV GP2 expression.

2.3. Analysis of genetic stability of recombinant viruses

To test the genetic stability of the recombinant viruses, virus stocks recovered from YF17D/LASV-GP1 or -GP2-electroporated cells were passed 10 times in BHK-21J cells [43]. Supernatants of the infected cells were routinely harvested 24 hours post infection, diluted to 1:10 with phosphate buffered saline (PBS) and used to inoculate fresh cells. Two independent passage experiments were performed for each recombinant virus, YF17D/LASV-GP1 and -GP2. Electroporated and/or infected cells were labeled with $^3\text{[H]}$ -Uridine in the presence of actinomycin D, intracellular RNA was isolated and analyzed by electrophoresis as previously described [42].

2.4. Detection of YF17D-specific and LASV-specific proteins in infected cells

To determine whether YF17D proteins were properly processed and released from the recombinant polyprotein-precursor containing LASV-GP1 and -GP2 protein-inserts, infected cells were incubated with radioactively labeled amino acids and processing of YF-specific proteins was monitored in pulse-chase experiments. The virus-specific proteins were immunoprecipitated with mouse polyclonal hyperimmune ascitic fluid to YF (ATCC), and subjected to SDS-PAGE analysis. For detection of LASV-GP1 and -GP2, transfected or infected cell lysates were subjected to SDS-PAGE separation and proteins were electroblotted to PVDF membranes (Hybond-P, Amersham, Piscataway, NJ). Monoclonal anti-GP1 and polyclonal rabbit anti-GP2 antibodies (gift from W. Garten, the Institut für Virologie der Phillips-Universität Marburg, Germany) were used to identify the LASV glycoproteins after incubation with an appropriate secondary goat IgG conjugated to alkaline phosphatase using NBT/BCIP (Invitrogen, Carlsbad, CA) as chromogenic substrate.

2.5. Immunogenicity studies in CBA/J mice

Four-week-old female CBA/J mice from Jackson Laboratories were used for immunogenicity studies. Previously, we showed that CBA/J mice are a useful small animal model to evaluate immunogenicity of a LASV vaccine candidate, the reassortant ML29 [41,44]. In general, this reassortant encodes major immunogenic proteins, GPC and NP, from LASV and RNA polymerase and Z protein from MOPV. It also contains 5 non-conserved amino acid substitutions, mostly in L protein, that distinguished ML29 from the parental viruses. A single-shot immunization with ML29 induces broad CD8⁺ T cell-mediated sterilizing immunity in all tested animal models including non-human primates (review in [45–47]). In this study, ML29 was used as a positive vaccination control and for mapping immunodominant LASV GPC H2^k-restricted CD8⁺ T cell epitopes. CBA/J mice were immunized intraperitoneally (i.p.) or subcutaneously (s.c.) with 1000 PFU of ML29 and euthanized on a weekly basis. Bulk splenocytes were used for flow cytometry to evaluate the activation status of populations of CD3⁺CD8⁺ T lymphocytes using an antibody to CD11b. For detection of virus-specific T cells and mapping GPC H2^k-restricted epitopes, immune splenocytes or in some experiments purified CD8⁺ T cells (MACS, Miltenyi Biotec Inc, Auburn, CA) were stimulated with a peptide library consisting of overlapping 21-mer peptides (MIMOTOPES, Australia) derived from LASV GPC, GP1, and GP2. Purified splenocytes from immunized or naïve mice were plated in a PVDF membrane-bottomed 96-well plate and stimulated with GPC-, GP1-, GP2-derived peptides (100 ng), ConA (10 µg) or RPMI medium. Cells were incubated overnight and processed the next day according to the protocol for mouse IFN-γ ELISPOT (U-CyTech biosciences, Utrecht, The Netherlands), as provided by the manufacturer. In brief, after the stimulation,

the cells were washed, re-suspended in the same medium, and $0.3 - 0.4 \times 10^6$ cells per well were added to ELISPOT 96-well plates pre-coated with antibodies specific to mouse IFN- γ . The plates were incubated at 37°C for 5 h and the cells were washed away. Biotinylated detection antibodies were then added and the plates were incubated for 1 h at 37°C. Plates were washed, incubated with anti-biotin antibody labeled with gold particles. The spot-forming cells (SFC) secreting IFN- γ were detected and enumerated with C.T.L. Ltd Immunospot® S5 Micro-analyzer and Immunospot® V 4.0 software.

For an immunogenicity study, CBA/J mice (4 animals per group) were immunized s.c. or i.p. with YF17D, YF17D/LAS-GPC, or YF17D/LAS-GP2 (1×10^6 PFU/mice in 0.1 ml) and boosted on day 14 with the same amount of the vaccines. Immunized animals were sacrificed on day 14 after the boost and secreted IFN- γ was evaluated by ELISPOT using bulk splenocytes stimulated with GPC-, GP1-, and GP2- derived peptides.

2.6. Vaccination-challenge experiments in strain 13 guinea pigs

Strain 13 guinea pigs (300–500 g, female) were purchased from USAMRIID (Fort Detrick, Frederick, MD). Animals (four to six animals per group) were vaccinated with YF17D/LASV-GP1&GP2 s.c. Both recombinant vaccines expressing GP1 and GP2 with infectious titers of 5×10^6 PFU/ml were equally mixed in PBS and injected in 0.5 ml in two separate sites on the back of animals. As a positive vaccination control 1,000 PFU of reassortant ML29 vaccine was inoculated s.c. As a negative vaccination control, animals were inoculated with PBS in 0.5 ml. On day 14, animals from the experimentally vaccinated group were boosted with the same dose of YF17D/LASV-GP1&GP2. On day 44, animals were challenged s.c. with LASV (Josiah), 1,000 LD₅₀/ml.

The animals were observed twice daily for clinical manifestations according to an approved scoring sheet (temperature, weight, decreased activity, ruffled fur, loss of weight, labored breathing, hunched posture). Temperature was monitored by using implanted chips. Death or survival past 25 days was defined as an endpoint [40,48]. Previously we showed that animals past this time point did not change survival outcome during extended follow-up period [41]. Animals which survived to day 25 or animals which met euthanasia criteria (fever, weakness, labored breathing, >25% loss in weight) were euthanized and sacrificed for histological studies as previously described [41]. For detection of viral RNA, tissue samples from vaccinated and challenged animals were submerged in RNAlater and cryopreserved. RNA was extracted later on using RNeasy mini kit (Qiagen, cat. no. 75142). RNA was converted into cDNA and amplified with 36E2 and 80F2 primers targeting LASV-GPC gene [49]. Standards used in qRT-PCR were generated from RNA isolated from serial 10-fold dilutions (10^1 - 10^{-7} PFU/ml) of LASV Josiah/SL viral stocks that were enumerated in triplicate by conventional plaque assay as previously described [41]. Sensitivity of qRT-PCR was around 100–300 viral RNA copies per g of tissue.

2.7. Data analysis

Statistical analyses (mean, SD, T-test) and graphics were performed using the Origin 6.0 package (Microcal Software, Inc., Northampton, MA).

3. Results

3.1. Further development and improvement of YF17D recombinant technology based on foreign gene insertion between E and NS1 genes

Foreign gene inserts between the YF17D E and NS1 genes must be stable for at least 10 passages to consider this cloning strategy for further scale-up development. Ten passages will be enough to produce master-seed-lots and manufacture non-GMP bulk lots for pre-

clinical studies [19]. As mentioned, our recombinant YF17D/ LASVΔGPC viruses were reasonably stable for at least 5 passages [42]. However, some virus stocks lost the inserted cassettes when the passages were extended. Similar observations were made by Bonaldo et al. [50]. To track the LASVΔGPC cassette integrity in Vero cells, a cassette region containing the insert and flanking sequences (Fig. 1A) was amplified (sense: AGGAAAGTTGTTCACTCAGACCATG; antisense: CAAGCTTCACAGGATCTTCTGGATA) and analyzed by gel electrophoresis. The size of the cassette with the LASVΔGPC insert was 1.8 kb. The 1.8 kb amplicon was detected in passage 3–5 viruses (P3–P5). However, P6 viruses displayed a diverse electrophoresis profile indicating the loss of the LASVΔGPC inserts as confirmed by further sequence analysis (not shown).

This loss of the insert during multiple cycles of viral RNA replication was originally attributed to homologous recombination, due to the duplication of the transmembrane region of YF17D E coding sequence flanking the LASVΔGPC insert [42]. In an attempt to reduce recombination and enhance the stability of LASVΔGPC insert, the downstream transmembrane E sequence was substituted with a heterologous sequence with the similar function ensuring insertion of the YF17D into the endoplasmic reticulum. We suggested that the lower homology of the ΔGPC-NS1 fusion sequence to the C-terminal part of E protein can enhance the stability of the recombinant virus. Two new linkage sequences were tested (Fig. 1). The 1st fusion sequence was derived from West Nile virus (WNV). The 2nd one was an artificial heterologous sequence encoding a signal sequence motif for flavivirus NS1 protein.

Insertion of these sequences between ΔGPC and NS1 genes improved stability of the GPC cassette, but did not completely resolve the instability problem. As seen in Fig. 1B, the YF17D/LASVΔGPC viruses made according to this strategy had enhanced stability. At least some P10 clones with WNV fusion sequence contained the full ΔGPC insert as was judged by RT/PCR analysis (not shown). Nevertheless, P10 recombinant viruses were still not fully stable in terms of preservation of integrity of LASVΔGPC sequences. The observed heterogeneity of the P10 virus population suggested that the loss of the LASV ΔGPC insert was a gradual process and not the result of the recombinant event. Apparently RNA with a length close to the parental YF17D virus has an advantage in replication and/or packaging over recombinant RNA containing large inserts like the LASV ΔGPC gene.

In further experiments, we decided to reduce the size of LASV inserts and separately clone LASV GP1 and GP2 between E and NS1 genes as described in Materials and Methods. Based on our previous observations, we have reasonably assumed that LASV GP1 and GP2 maturation does not interfere with proteosomal degradation and binding with MHC-I molecules. At least LASV GPC, with deleted SSP [42] or with genetically modified GP1–GP2 cleavage site and deleted GP2 transmembrane domain (Lukashevich et al., unpublished), induced protective virus-specific CD8⁺ T cell responses in guinea pigs and in mice. We do see any concerns associated with the fusion sequences derived from WNV. Nevertheless, in further experiments we used an artificial fusion sequence between GP1 or GP2 and NS1.

3.2. Genetic stability of a new generation of YF17D/LASV-GP recombinant vaccines

As seen in Fig. 1, size reduction of cloning inserts combined with introduction of modified fusion sequences between the GP insert and NS1 gene resulted in recovery of YF17D-based recombinant viruses with enhanced genetic stability. To test the genetic stability of the recombinant viruses, virus stocks recovered from YF17D/LASV-GP1 or – GP2-electroporated cells were passed 10 times in BHK-21J cells. Supernatants of the infected cells were routinely harvested 24 hours post infection, diluted and used to inoculate fresh

cells. Two independent passage experiments were performed for each recombinant virus, YF17D/LASV-GP1 and -GP2. Electroporated and/or infected cells were labeled with ^3H -Uridine in the presence of actinomycin D, intracellular RNA was isolated and analyzed by electrophoresis. A single RNA band was detected in passage 10 (P10) cells as well as in transfected cells. This RNA band migrated slightly slower than those from YF17D-infected cells and contained LASV GP1 and GP2 inserts as was confirmed by RT/PCR analysis. Interestingly, radioactive labeling revealed reduced RNA content in cells infected with YF17D/LASV-GP1 virus vs. -GP2 virus. Variations in infectivity during the passages and/or accumulation of defective interference particles can be possibly responsible for this observation.

3.3. Phenotypic features and stability in vitro

Recombinant YF17D/LASV-GP1 and -GP2 viruses induced pronounced cytopathogenic effects in BHK-21J and Vero cells and virus yields in these cells were around $1-2 \times 10^7$ PFU/ml. The recombinant viruses produced clear plaques, which appeared to be smaller than those produced by the parental virus, YF17D (Fig. 2), in line with our previous observation [42]. This plaque phenotype was stable for P1–P10 viruses.

Both YF17D/LASV-GP1 and -GP2 RNA-transfected cells, as well as cells infected with recombinant viruses, passage 1 (P1) and passage 10 (P10) viruses, were positively stained with anti-YF17D NS3 antibody, showing typical perinuclear staining. Also, LASV GP1 and YFV NS3 were found to be co-localized when double labeling was performed on YF17D/LASV-GP1-transfected cells (Fig. 3). Staining of cells transfected and/or infected with YF17D/LASV-GP with polyclonal antibodies against GP-derived peptide gave similar results [42].

Cells infected with recombinant YF17D/LASV-GP1 and -GP2 viruses were capable of expressing properly processed YFV antigens. As seen in Fig. 4, YF17D-specific antibodies precipitated from infected cells YF NS1, NS2A, NS2B, NS3, NS4B, NS5, PrM and E proteins. These results indicate that monocistronic recombinant mRNA translated into a polyprotein-precursor, and the processing of YF17D polyprotein-precursor was not affected by the LASV GP1 and GP2 inserts.

Finally, LASV-specific proteins, GP1 and GP2, were detected in YF17D/LASV-GP-infected cells by Western blot using GP1 monoclonal and GP2 polyclonal antibodies as previously described [42]. These results (Fig. 5) demonstrate that both LASV glycoproteins, GP1 and GP2, were translated from recombinant monocistronic mRNA and properly processed and released from the recombinant YF17D/LASV-GP1 and -GP2 polyprotein.

3.4. YF17D/LASV-GP1 and -GP2 recombinant viruses induce specific CD8⁺ T cell responses in CBA/J mice

It is important to evaluate the immunogenicity of experimental vaccines in small animal models before testing putative vaccine candidates in expensive vaccination-challenge experiments. Currently, there is no mouse model of human LF. The outcome of LASV infection in immunocompetent mice depends on H2 genotype, age, routes of infection, and resembles LCMV-like immunopathology (review in [46]). In contrast, virulence of LASV in man and in non-human primates is directly related to virus load [25,40,51] and is not associated with immunopathology. We previously showed that young adult CBA/J mice inoculated s.c. or i.p. with the MOP/LAS (ML29) reassortant induced protective CD8⁺ T cell responses [41,45,47] and can be used as an economical assay for vaccine immunogenicity to access capacity to elicit specific CD8⁺ T cell responses that play a major role in LASV protective immunity [45,47]. After ML29-immunization, activation of CD8⁺

T cells peaked on day 14 and this time-point was used for collection of splenocytes for mapping immunodominant epitopes. Immune splenocytes stimulation with pools of peptides (Fig. 6B) or with individual peptides (not shown) revealed that LASV GPC H2^k-restricted epitopes were located within GP1 glycoprotein at aa positions 57–83, 218–244 and within GP2 glycoprotein at positions 400–426.

Immunization of CBA/J mice with recombinant YF17D/LASV-GP1 or -GP2 vaccines, but not with parental YF17D vaccine, induced LASV antigen-specific CD8⁺ T cell immune responses detected in IFN- γ ELISPOT (Fig. 6C). Extracellular secretion of IFN- γ was detected after stimulation of immune GP1- or GP2-specific splenocytes either with peptide cocktails derived from LASV GPC or from GP1 and GP2, respectively. The level of extracellular IFN- γ production after stimulation of immune splenocytes with LASV GP-specific peptides was approximately 6-fold lower after immunization of mice with YF17D-based recombinant GP1 and GP2 vaccines in comparison with ML29 reassortant expressing naturally processed LASV GPC.

3.5. Recombinant YF17D/LASV-GP1 and -GP2 vaccines protected strain 13 guinea pigs against fatal LF disease but did not prevent LASV infection

Individually expressed in vaccinia virus, LASV GPC subunits, GP1 and GP2, did not protect non-human primates against fatal LF [30]. Based on this observation, recombinant YF17D-based viruses expressing GP1 and GP2 were equally mixed and used for immunization of strain 13 guinea pigs. These animals are extremely sensitive to LASV with LD₅₀ < 1 PFU/ml [40,52] and all non-vaccinated control animals met euthanasia criteria 2 weeks after challenge. The most prominent manifestations of the disease included fever, weight loss, and elevated liver enzymes; pathohistological findings included intestinal pneumonia with septal and alveolar edema, and hepatitis with hepatocytic vacuolization and lipidosis [46,53]. Vaccination control group animals were fully protected against LASV challenge and had no signs of the disease. In these animals LASV was only marginally detected by RT-PCR in peritoneal lymph nodes in good confirmation with our previous results [41,53]. Five of six guinea pigs vaccinated with YF17D/LASV-GP1&GP2 were also protected against fatal LF. However, surviving animals expressed clinical symptoms of LF, had an elevated body temperature, lost body weight, and LASV was detected in tested tissues. Still, viral titers as well as clinical symptoms were lower than in non-vaccinated control group and animals with disease signs recovered at the end of the observation period (Fig. 7). These results indicate that immunization with recombinant YF17D/LASV-GP1&GP2 vaccine protected guinea pigs against fatal LASV disease but did not prevent LASV infection.

4. Discussion

Due to its outstanding safety record, YF17D has been used as a promising vector for the development of vaccines against arthropod-borne flaviviruses and against non-flavivirus-related pathogens [6,22,54–57]. The YF17D-based ChimeriVax™ technology [58] replaces the prM and E genes of YF17D with the corresponding genes of the closely-related flaviviruses, Dengue, WNV, and Japanese encephalitis. These vaccines are in advanced stages of development and ChimeriVax™-WN vaccine (PreveNile) was licensed in the U.S. for veterinary applications (rev. [19]).

Various insertion locations in structural and non-structural genes and in the 3'UTR of YF17D were used to clone and express flavivirus-unrelated foreign inserts. The E protein is the major flavivirus antigen inducing neutralizing antibodies and represents an attractive site for insertion of foreign epitopes. However, the structural limitations of the flavivirus E protein allowed expression of relatively short inserts, ~ 30–35 aa [21]. Importantly, these inserts resulted in additional attenuation of recombinant YF17D viruses concomitant with

enhanced safety profiles of chimeric YF17D-based vaccines (rev. [19]). The NS1 protein also induces antibody responses contributing to protection against flaviviruses. In a recent study the M2e peptide (23 aa) of influenza virus was successfully cloned into the NS1 gene using transposon-mediated insertion [59].

Non-flavivirus CTL epitopes have been also cloned between the NS2B-NS3 cleavage site. The insertion of the H-2Kd-restricted CTL epitope of the circumsporozoite protein of *P. yoelii* at this site resulted in a recombinant virus which was replication competent, stable, and diminished the parasite burden in the liver by ~70% [22]. Immunization of mice with recombinant YF17D carrying ova-specific T-cell epitope, SIINFELK, between NS2B and NS3, induced a specific CD8⁺ T-cell population and protected animals from lethal challenge with an aggressive lethal melanoma cell line [54].

We cloned a relatively large gene encoding LASV ΔGPC glycoprotein (433 aa) between YF17D E and NS1 genes in attempts to design bivalent YF17D-LASV vaccine to control both infections in co-endemic areas of Africa [42]. The recombinant virus induced humoral and cell-mediated immune responses against YF and LASV, respectively, and protected guinea pigs against fatal LF disease. In spite of these encouraging results, we [42] and others [50] faced instability problems when large inserts were cloned at the E-NS1 site. It has been suggested that duplication of flavivirus sequences flanking an inserted gene contributed to the genetic instability of the virus. In the current study two approaches have been used to address this problem: (i) insertion of modified fusion sequences between the C-terminal part of foreign inserts and NS1 gene to prevent possible recombination events resulting in deletion of the foreign gene; and (ii) reduction of insert size. We have shown that modification of fusion sequences improved stability but did not preserve the large ΔGPC insert from the deletion during 10 passages in tissue culture. When the size of the insert was reduced almost 2-fold, GP1 and GP2 sub-units of LASV GPC were successfully cloned at the E-NS1 site and these inserts were stable during 10 passages. The size of the LASV GP1 is 200 aa and the size of GP2 is 231 aa residues. In accord with our results, a similar sized insert, 224 aa residues, from SIVmac239 Gag was cloned between the genes encoding the viral proteins E and NS1. The resulting recombinant virus, the YF17D/SIVGag_{45–269}, was stable in vivo and able to induce SIV-specific CD8⁺ T cell responses in rhesus macaques [60].

The size limitation of foreign gene inserts in YF17D vector seems not to be unique for the E-NS1 site. Recently recombinant YF17D vaccine was engineered by cloning CSP (aa 57–344) from *P. yoelii* using a novel insertion site, the capsid gene. The insert was cloned in frame between the highly conserved cyclization sequences (nt 1–75) and FDMV 2A and Ubi genes which were inserted to ensure appropriate cleavage [61]. Notably, the CSP insert was intact up to 6 passages in tissue culture and became undetectable by RT/PCR by passage 7. We have used a similar strategy with limited success in attempts to clone and express overlapping fragments of LASV NP resembling in some ways cytoplasmic YF core protein (Bredenbeek, unpublished).

Taken together these results indicate that a monocistronic YF17D genome encoding a relatively large flavivirus-nonrelated gene inserts at the E-NS1 site or in the C gene can produce viable recombinant viruses in cell cultures. However, genetic stability of recombinant viruses is the major problem because these viruses lose genetic material after more than 10 passages. We have shown here that cloning of inserts of moderate size (200–230 aa residues) between the genes encoding the viral proteins E and NS1 resulted in genetically stable recombinant viruses during 10 passages in tissue culture. The YF17D genome itself is remarkably stable [62]. Ten passages of recombinant YF17D-based viruses

will be sufficient to manufacture non-GMP lots for toxicology and pre-clinical studies and for making Phase I lots during vaccine development [19].

Insertion of foreign genes into the YF17D genome resulted in additional attenuation of chimeric or recombinant YF17D-based viruses. In plasma and tissue of vaccinated guinea pigs [42] and marmosets (Lukashevich, unpublished) YF17D/LASV-GPC was not detectable by plaque assay and only transient and limited replication was confirmed by co-cultivation in Vero and/or by RT/PCR in line with previously published data [19,63,64]. Still, we were able to detect anti-YF antibodies in YF17D/LASVΔGPC-immunized animals. Nevertheless, for bivalent recombinant YF17D-based vaccines anti-YF efficacy must be additionally evaluated in challenge experiments with wild-type YF virus to confirm efficacy of the parental vaccine, YF17D.

In the recent review [19] theoretical safety (reversion to virulence and potential visceros- and neurotropism, recombination with different flaviviruses or distantly-related RNA viruses) and environmental concerns (transmission by arthropod vectors) of YF17D-based vaccines have been carefully addressed and evaluated in support of their further late development and registration. Importantly, YF17D is safe and effective in HIV-infected individuals with CD4 cell counts above 200 cell/mm³, even if they have previously developed AIDS or if their viral load is above 10,000 copies/ml [65,66]. Moreover, flaviviruses decrease CD4 expression and inhibit HIV-1 replication in human CD4⁺ T cells [67]. We have recently showed that our live attenuated reassortant ML29 expressing LASV GP and NP is safe and immunogenic in SIV-positive rhesus macaques [68]. Taken together these results suggest that YF17D-vectored LASV vaccines will be safe in HIV-infected individuals representing the most vulnerable group among immunocompromized populations in West Africa.

As mentioned above, low or marginal transient viremia in animals immunized with recombinant YF17D-based vaccines seems to be associated with relatively low immunogenicity. As a result, for recombinant YF17D-based vaccines, in contrast to parental YF17D, a prime-boost immunization protocol is often required to induce antigen-specific immune responses. To avoid anti-vector immunity and stimulate specific immunity the optional prime-boost strategy has to include different vectors. For example, priming with recombinant BCG expressing SIV antigens increased the frequency of the SIV-specific CD8⁺ T-cell responses after boosting of rhesus monkeys with recombinant rYF17D/SIVGag₄₅₋₂₆₉ [60]. However, we and others [61] showed that prime-boost immunization with YF17D-based recombinant vaccines does not necessarily induce specific responses against cloned foreign antigens and/or undesirable anti-vector immunity. In our experiments with YF17D/LASV-GPC [42] or –GP&GP2 vaccines (this study) we did not see differences in protective efficacy after single shot or prime-boost vaccination. Based on the nature of protective immune responses against certain pathogens, it is reasonable to consider other possible approaches to enhance specific immune responses against protective antigens cloned into YF17D vector. At least in the case of anti-LASV immunity, rational design of the GP antigen seems to be a promising approach. Preliminary experiments with structurally stable trimeric LASV GP showed that CD8⁺ T cell cross-priming can additionally contribute to effects of immunization based on conventional MHC type I antigen presentation.

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Fig. 1.

Construction of genetically stable YF17D/LASV-GP vaccines. (A) Schematic representation of recombinant YF17D-LASV virus carrying the Δ GPC insert. In the initial constructs [42] the insert (1,365nt) was flanked by homologous E-derived sequences (red box) to insure the proper processing of recombinant polyprotein. In a new set of recombinant viruses, the fusion sequence between GP2 and NS1 (pink box) was substituted with heterologous sequences derived either from WNV or artificially designed (Art) to reduce homology with YF17D. Arrows indicate the sequences designed for PCR amplification of LASV Δ GPC insert and flanking areas to track the insert size during passages in BHK-21J cells. (B) Genetic stability of recombinant YF17D/LASV- Δ GPC. Virus-specific RNA was labeled with 3 [H]-Uridine in the presence of actinomycin D and intracellular RNA at passage 1, 5 and 10 was analyzed by gel electrophoresis. The RNA from YF17D-infected cells was used as a control. Arrows indicate recombinant YF17D/LASV Δ GPC RNA, 12,227nt; and parental YF17D RNA, 10,862nt. (C), (D) Genetic structure of recombinant YF17D/LASV-GP1 and -GP2 viruses, respectively. Cleavage site between the YF17D E and GP2 protein was designed as described in Materials and Methods. (E) The size of LASV GP inserts after electroporation (Elpo) or after 10 passages in BHK-21J. Two independent passage experiments were performed, Exp1 and Exp2. Arrows indicate recombinant YF17D/LASV-GP1 RNA, 11,609nt; and parental YF17D RNA, 10,862nt. The size of YF17D/LASV-GP2 RNA is 11,672nt.

(A) YF17D/LASV-GP2 (B) YF17D

Fig. 2.

Plaque morphology of YF17D/LASV-GP viruses. YF17D/LASV-GP2 (A) and YF17D (B) viruses recovered after electroporation were plated on confluent monolayers of BHK-21J cells in 6-well plates and viruses were titrated by plaque assay as previously described [42]. The transfected cells were incubated for 96 hours, fixed with 8% formaldehyde and stained with crystal violet. The final titer was 1×10^8 and 2×10^7 PFU/ml for YF17D and YF17D/LASV-GP2, respectively.

Fig. 3.

Immunofluorescence staining of transfected cells. Cells transfected with YF17D/LAS-GP1 were fixed at 24 hours post electroporation, followed by staining for YF17D NS1 (green) and LASV GP1 (red). Nuclei were stained with Hoechst (blue). Magnification: $\times 40$.

Fig. 4.

Pulse-chase labeling of YF17D proteins. Cells infected with either YFV17D or the recombinant viruses were radioactively labeled for 4 hours, or pulsed 30 min, followed by chasing for either 1 hour or 3.5 hours. Cell lysates were subjected to immunoprecipitation with mouse hyperimmune ascetic fluid to YF17D (ATCC). The precipitates were separated on 12.5% SDS-PAGE gel, and analyzed by phosphor-imaging. 1, label 4 hours; 2, pulse-labeling, 30 minutes; 3, 1 hour chase; 4, 3.5 hours chase. Viral proteins: NS5 (103 kDa), NS3 (70 kDa), E (50 kDa), NS1 (46 kDa), NS4B (27 kDa), PrM (26 kDa), NS2A (22 kDa), and NS2B (14 kDa) could be seen. Molecular weight markers are indicated on the left side.

Fig. 5.

Western-blot analysis of LASV-specific glycoproteins in cells infected with recombinant YF17D/LASV-GP1 and -GP2 viruses. The analysis was carried out with a 1:2,000 dilution of anti-GP1 and anti-GP antibodies as previously described [42]. Markers in kilodaltons are indicated on the side. The arrow in (A) indicates GP1 and in (B) indicates GP2 glycoproteins. The recombinant YF17D/LASV-GP2BgIII contained natural YFV E – NS1 cleavage site with Leu/Asp mutation in the first amino acid of GP2. The recombinant YF17D/LASV-GP2fus contained a sequence encoding the first 14 aa of LASV GP1 inserted between YF17D E and LASV GP2 (see Materials and Methods for details).

Fig. 6.

Evaluation of immunogenicity of LASV vaccine candidates in CBA/J mice. (A) Vaccination control group: activated CD3+CD8+ cells peaked on day 14 after immunization. (B) Mapping of GPC H2^k-restricted epitopes: on day 14 after vaccination splenocytes were stimulated with a peptide library consisting of overlapping 21-mer peptides derived from LASV GPC. Stimulated splenocytes were incubated in wells of the ELISPOT plates pre-coated with Abs against mouse IFN- γ and washed away. Cytokine-secreting cells or spot-forming cells (SFC) cells were detected and enumerated according to the manufacturer's instructions for mouse IFN- γ ELISPOT (U-CyTech biosciences, Utrecht, The Netherlands). Responses to peptides derived from GP1 and GP2 are indicated by black bars. (C) CD8+ T cell responses in mice immunized with recombinant YF17D/LAS-GP1 and -GP2 viruses. Splenocytes from immunized mice were stimulated with peptide cocktails derived from full GPC (black boxes) or from GP1 or GP2 subunits (sparse boxes).

Fig. 7.

YF17D/LASV-GP1&GP2 vaccination protects guinea pigs from fatal outcome but does not prevent LASV infection. (A) Survival rate and clinical scores (insert). Clinical scoring was based on evaluation of responsiveness, edema, dehydration, rash, appearance, petechia, feed consumption, anorexia, stool, urine, fluid uptake (0, normal; 1, mild; 2, moderate; 3, severe. (B) Temperature (°F) was monitored by sensor-implants and recorded twice per day for each animal. (C) Weight of animals on day of challenge was assayed as 100%. Open circles, animals vaccinated with YF17D/LASV-GP1&GP2; open squares, animals vaccinated with ML29 (positive control); closed squares, negative control (animals inoculated with media). (D) Viral loads (LASV RNA copies) in vaccinated-challenged animals. Black boxes, animals challenged with LASV (no vaccine); white boxes, animals vaccinated with YF17D/LASV-GP1&GP2; spare boxes, animals vaccinated with ML29 (positive control).