Molecular and immunological characterization of a DNA-launched yellow fever virus 17D infectious clone

Xiaohong Jiang,1 Tim J. Dalebout,1 Igor S. Lukashevich,2 Peter J. Bredenbeek1 and David Franco3

1Department of Medical Microbiology, Center of Infectious Diseases, Leiden University Medical Center, P. O. Box 9600, 2300 RC Leiden, The Netherlands
2Department of Pharmacology and Toxicology, School of Medicine, Center for Predictive Medicine for Biodefense and Emerging Infectious Diseases, NIH Regional Bio-containment Laboratory, University of Louisville, KY, USA
3Aaron Diamond AIDS Research Center, Rockefeller University, 455 First Avenue, New York, NY 10016, USA

Yellow fever virus (YFV)-17D is an empirically developed, highly effective live-attenuated vaccine that has been administered to human beings for almost a century. YFV-17D has stood as a paradigm for a successful viral vaccine, and has been exploited as a potential virus vector for the development of recombinant vaccines against other diseases. In this study, a DNA-launched YFV-17D construct (pBeloBAC-FLYF) was explored as a new modality to the standard vaccine to combine the commendable features of both DNA vaccine and live-attenuated viral vaccine. The DNA-launched YFV-17D construct was characterized extensively both in cell culture and in mice. High titres of YFV-17D were generated upon transfection of the DNA into cells, whereas a mutant with deletion in the capsid-coding region (pBeloBAC-YF/DC) was restricted to a single round of infection, with no release of progeny virus. Homologous prime–boost immunization of AAD mice with both pBeloBAC-FLYF and pBeloBAC-YF/DC elicited specific dose-dependent cellular immune response against YFV-17D. Vaccination of A129 mice with pBeloBAC-FLYF resulted in the induction of YFV-specific neutralizing antibodies in all vaccinated subjects. These promising results underlined the potential of the DNA-launched YFV both as an alternative to standard YFV-17D vaccination and as a vaccine platform for the development of DNA-based recombinant YFV vaccines.

INTRODUCTION

Flaviviruses are a group of small enveloped viruses that contain a plus-strand RNA genome around 11 kb in length. The genome encompasses a 5’-untranslated region (5’-UTR), followed by a single large ORF and a highly structured 3’-UTR (Lindenbach & Rice, 2007). Many flaviviruses, are arthropod-borne pathogens (arboviruses) that are transmitted by either mosquitoes or ticks. Clinical presentation of human infection ranges from mild febrile illness to severe haemorrhagic fever or meningoencephalitis (Widman et al., 2008). Yellow fever virus (YFV) is the prototype of the Flavivirus genus and causes haemorrhagic fever featured by liver dysfunction and jaundice (WHO, 2009). There is no cure for yellow fever, and vaccination is the single most important preventive measure against the disease (WHO, 2009).

The YFV-17D vaccine is a live-attenuated vaccine empirically developed in the 1930s by Theiler & Smith (1937, 2000), for which Theiler garnered the 1951 Nobel Prize in medicine. Since its development, YFV-17D vaccine has been administered to over 540 million humans globally, and has stood as a paradigm for a successful vaccine with a great record of both safety and efficacy (Barrett et al., 2007). The vaccine is well tolerated, with only rare cases of severe adverse reactions (reviewed by Barrett & Teuwen, 2009; Pulendran, 2009; WHO, 2009). Over 95% of the vaccinees will develop protective immunity against all known WT YFV strains within 10 days after vaccination (Barrett & Teuwen, 2009), although the molecular determinants of its attenuation and immunogenicity are not yet fully understood. A single immunization induces a broad spectrum of immune responses, including cytotoxic T lymphocytes, a mixed T helper type I (Th1)–Th2 profile, and neutralizing antibodies that can persist for up to 30 years (Monath, 2005). These adaptive immune responses are preceded by the activation of several effector arms of innate immunity and the induction of a network of
antiviral genes, including various cytokines (IP-10, IL-1x) and molecules involved in sensing viruses (TLR-7, RIG-I and MDA-5), as well as transcription factors that regulate type I interferons (IRF7 and STAT1) (Gaucher et al., 2008; Querec et al., 2009).

The broad spectrum of the immune responses triggered by YFV vaccine renders it an interesting vector for the development of recombinant vaccines. Indeed, YFV-17D has been used as the backbone in chimeric vaccines against other flaviviruses of clinical significance, such as West Nile virus (Arroyo et al., 2004; Monath et al., 2006), Japanese encephalitis virus (Appaiahgari & Vrati, 2010; Chambers et al., 1999; Guirakhoo et al., 1999; Monath et al., 1999) and dengue virus (Galler et al., 2005; Guirakhoo et al., 2001; Guy et al., 2010). These YFV-based chimeric vaccines were found to be safe and highly immunogenic in both preclinical and clinical trials, with IMOJEV, a YFV-17D-based Japanese encephalitis vaccine, passing phase III clinical trials and being licensed for human use in Australia (Halstead & Thomas, 2011). YFV-17D has also been successfully exploited as a vector for the expression of foreign T-cell or B-cell epitopes, as potential vaccine candidates against malaria (Bonaldo et al., 2002; Tao et al., 2005) or as therapeutic anticancer vaccine (McAllister et al., 2000). Moreover, recombinant YFV-17D viruses expressing heterologous antigens were proposed as bivalent vaccine candidates against Lassa virus (Bredenbeek et al., 2006; Jiang et al., 2011) or as alternative vaccine vectors for malaria (Stoyanov et al., 2010) and HIV (Franco et al., 2010; Van Epps, 2005). In vitro characterization demonstrated that these recombinant viruses were viable and could express both YFV and foreign proteins. Experiments in small-animal models showed promising results in terms of immunogenicity and protection. Despite these encouraging results, genetic instability has been encountered for these recombinant viruses, especially with larger inserts, which is attributed to the limited packaging capacity of the icosahedral virion (Bonaldo et al., 2007; Jiang et al., 2011; Stoyanov et al., 2010).

Despite the availability of a highly effective YFV vaccine, the number of YF cases has increased over the past two decades, due to the declining immunity to YFV infection in the population, deforestation, urbanization, population movements, poor vector control and climate change (WHO, 2009). Until recently, the vaccine was still in short supply, and vaccine coverage is low in high-risk areas (WHO, 2013). Alternative strategies to facilitate vaccine production, transportation or storage would therefore help accommodate the increasing needs for YFV vaccine.

Compared with conventional live-attenuated vaccines, DNA vaccines are temperature-stable, easily stored and can be manufactured on a large scale (Kutzler & Weiner, 2008). Although the concept of DNA vaccination has generated a great deal of excitement since its introduction in the early 1990s, no DNA vaccine has been approved for human use. Significant challenges will need to be overcome before DNA vaccines achieve mainstream acceptance, especially regarding effective delivery and augmenting antigen expression level for a sufficiently potent immune response (Kutzler & Weiner, 2008; Weiner et al., 2010).

With the objective of combining the desirable qualities of DNA vaccines with those of a live-attenuated vaccine, a DNA-launched YFV-17D vaccine was constructed using a similar strategy as described for a Kunjin virus-based DNA vaccine (Hall et al., 2003). This infectious YFV-17D DNA was stable in the Escherichia coli host, and was extensively characterized both in cell culture and in mice to evaluate its potential as an alternative vaccine platform. In addition, deletions/mutations were introduced into the sequences encoding the capsid or RNA-dependent RNA polymerase (RdRP), rendering the virus deficient in either virus packaging or genome replication. Murine immune responses induced by these DNA-launched YFV mutants were determined and compared with those triggered by DNA-launched, replication- and packaging-competent YFV-17D.

**RESULTS**

**Construction of an infectious DNA clone of YFV-17D**

The characteristics of the DNA construct that was created to launch infection of YFV-17D from DNA are shown in Fig. 1. The 5′-UTR of the viral RNA was fused to a cytomegalovirus (CMV) promoter so that cellular RNA polymerase II would initiate transcription of the YFV-17D genome. The hepatitis delta virus ribozyme (HDVr) was engineered precisely after the last nucleotide of YFV genome to ensure the production of an authentic 3′-end of the transcribed viral RNA. Initially the CMV promoter and HDVr cassettes were cloned into the pACNR-FLYFx vector (Bredenbeek et al., 2003). However, the resulting plasmid pACNR-CMVp-FLYF-HDVr was found to be genetically unstable during propagation in E. coli DH5α. Attempts to stabilize the plasmid by using different bacteria strains or alternative culture conditions were unsuccessful. To further decrease the copy number for the stabilization of the construct, the bacterial artificial chromosome (BAC) vector pBeloBAC11 was evaluated as a vector for the DNA-launched YFV-17D cassette. The resulting BAC pBeloBAC-FLYF was shown to be genetically stable at least up to 20 passages in either E. coli DH5α or E. coli DH10B (data not shown).

To determine the contribution of viral replication and virus spread to the immunological characteristics of a DNA-launched YFV-17D vaccination regime, two other DNA-launched YFV BACs were constructed using pBeloBAC-FLYF as a template. In pBeloBAC-YF/GSA, the GDD motif of the viral RdRP was mutated to GSA, which inactivated the RdRP (Khromykh et al., 2001), whereas in pBeloBAC-YF/ΔC, nt 185–331 in the nucleocapsid gene were deleted (Seregin et al., 2006). The latter should result
in the CMV promoter-driven transcription of replication-competent YFV-17D RNA that is unable to produce infectious virus.

**Characterization of DNA-launched YFV-17D in cell culture**

The pBeloBAC-FLYF, pBeloBAC-YF/AΔ and pBeloBAC-YF/GSA DNA were transfected into BHK-21J cells by electroporation. The transfected cells were fixed at 24, 48 and 72 h post electroporation (p.e.) and the expression of YFV NS3 was analysed by indirect immunofluorescence. At 24 h p.e., a small percentage of NS3-positive cells were observed in the cells that were transfected with pBeloBAC-FLYF or pBeloBAC-YF/AΔ (Fig. 2a). As anticipated, the proportion of NS3-positive cells increased for the cells transfected with pBeloBAC-FLYF BAC, while remaining at a low percentage for the cells transfected with YFV capsid-deletion mutant (Fig. 2a). No positive signal for YFV NS3 was detected in the cells transfected with pBeloBAC-YF/GSA DNA (Fig. 2a). Medium of the DNA-transfected cells was sampled at 24 h intervals and used to determine virus production by plaque assays. As shown in Fig. 2(b), increasing amounts of YFV-17D were detected in the medium of the cells transfected with pBeloBAC-FLYF, reaching a titre of over 10^7 p.f.u. ml^{-1} at 72 h p.e., whereas no virus production was detected in the supernatants collected from cells transfected with pBeloBAC-YF/AΔ or pBeloBAC-YF/GSA DNA. The results of both the immunofluorescence assay and the plaque assay confirmed that, as expected, only the cells that were transfected with pBeloBAC-FLYF DNA were able to produce infectious virus capable of spreading to neighbouring cells. The RNA transcribed from pBeloBAC-YF/AΔ was able to replicate, as demonstrated by the readily detectable expression of YFV NS3, but was incapable of producing infectious progeny virus owing to the lack of a functional capsid protein.

In order to evaluate the growth properties of the virus generated from pBeloBAC-FLYF DNA transfection, BHK-21J cells were infected with virus stocks derived from DNA transfection or those from *in vitro* transcripts at m.o.i. 0.1 or 1. Medium of the infected cells was sampled at 6 h intervals and used to determine virus production by plaque assays. The two viruses were virtually identical in their growth kinetics, no matter what m.o.i. was used (Fig. 2c); besides, their plaque morphology was also indistinguishable (data not shown).

To further investigate the YFV RdRP-driven intracellular RNA synthesis, cells transfected with pBeloBAC-FLYF were labelled with [^3H]uridine in the presence of actinomycin D (Act D) with 6 h intervals. [^3H]-labelled viral RNA was first detected at 33 h p.e. and the amount of labelled RNA gradually increased over time, probably reflecting the spread of infection to cells that were initially not transfected (Fig. 3a). Careful inspection of the fluorogram revealed a faint and faster migrating RNA species in samples collected at later time points, suggesting the presence of a YFV replicon RNA. Flavivirus replicons are RNAs that are able to replicate autonomously, but cannot form virus particles due to an in-frame deletion within the region encoding the structural genes. Trans-complementation by functional structural proteins can lead to the formation of replicon-containing virus particles (Khromykh & Westaway, 1997).

To test for the presence of YFV replicon RNA in the virus stocks derived from the DNA-launched system, BHK-21J cells were infected with YFV-17D virus derived from *in vitro*-transcribed infectious RNA or from pBeloBAC-FLYF transfection. The infected cells were labelled with [^3H]uridine in the presence of Act D and the intracellular...
RNA was analysed by electrophoresis of denatured RNA. A band suggestive of YFV replicon RNA was easily detected in the cells infected with the virus stock obtained from DNA-transfected cells, whereas this band was not seen in cells infected with virus derived from in vitro transcripts (Fig. 3b).

Different from BHK-21J cells transfected with in vitro-transcribed YFV RNA, the infectious YFV DNA pBeloBAC-FLYF has to be transcribed in the nucleus of cells, exposing the viral genome RNA to spliceosomes. Computer-aided prediction revealed several potential splice donor and acceptor sites within the sequence of the YFV structural genes (Fig. 3c). A splicing event with the use of the predicted splice donor site at nt 708 (score 1) and splice acceptor site at nt 2440 (score 0.99) would result in an in-frame deletion of nt 708–2440, which could yield an RNA transcript that functioned as a YFV replicon.

RT-PCR (reverse transcription polymerase chain reaction) was performed on total intracellular RNA isolated from BHK-21J cells infected with the DNA-launched virus to determine whether splicing at these predicted, favourable splice donor and acceptor sites was the origin of the YFV replicon-like RNA. Based on the predicted splice donor and acceptor sites, a forward primer corresponding to nt 40–64 within the viral 5′-UTR and a reverse primer complementary to nt 2491–2513 in the NS1 gene were selected for this analysis. In addition to the approximately 2.5 kb product that was expected to be produced with this primer set from full-length viral RNA, a smaller fragment with a length of around 750 bp was amplified, among other less prominent bands (data not shown). The 750 bp product was gel purified and sequenced. Sequence analysis revealed an in-frame deletion of nt 708–2440 (Fig. 3d), strongly suggesting that the predicted splice donor and acceptor sites were indeed used on a fraction of the CMV promoter-driven transcripts in the nucleus, which resulted in the production of a YFV replicon RNA. Owing to the low percentage of replicon RNA (<5%, Fig. 3b) present in the population of viruses produced from the BAC, no efforts were made to determine the significance of this splicing event on the immunogenicity of the construct.

**In vivo characterization of DNA-launched YFV-17D constructs**

The induction of cellular and humoral immune responses against YFV-17D was investigated by delivering pBeloBAC-FLYF, pBeloBAC-YF/ΔC and pBeloBAC-YF/GSA with an electroporation device to transgenic AAD mice using a homologous prime–boost immunization regimen. Three doses were used: 10 ng, 100 ng or 1 μg. Two weeks after the boost immunization, serum was collected from the

---

Fig. 2. Characterization of DNA-launched YFV-17D clones in BHK-21J cells. BHK-21J cells were electroporated with pBeloBAC-FLYF, pBeloBAC-YF/ΔC and pBeloBAC-YF/GSA and analysed for the expression of YFV antigen (a) and virus production (b). (a) Indirect immunofluorescence staining of YFV-17D NS3 protein. Cells were fixed at the indicated times p.e. and stained as described in Methods. YFV NS3 shows up as green (Alexa488) and the nuclei of the cells were stained with Hoechst and show up as blue. (b) Kinetics of virus production in BHK-21J cells after transfection with pBeloBAC-FLYF (circle), pBeloBAC-YF/ΔC (inverted triangle) and pBeloBAC-YF/GSA (triangle) as determined by plaque assays of the supernatants collected at the indicated times p.e. (c) Growth curves of DNA-launched YFV-17D (i.e. virus derived from pBeloBAC-FLYF transfection) at m.o.i. 1 (purple square) or m.o.i. 0.1 (blue circle) were compared with RNA-launched YFV-17D (i.e. virus derived from in vitro transcripts), at m.o.i. 1 (green triangle) or m.o.i. 0.1 (inverted pink triangle). Plaque assay was done in duplicate. Each data point represents the mean of the two measurements.
DNA- and mock-electroporated animals and neutralizing antibody titres against YFV were determined by a plaque reduction neutralization test (PRNT). Mice were subsequently sacrificed and their spleens were collected and used to determine the frequency of IFN-γ-producing cells after exposure to YFV peptides identified as dominant HLA-A2-restricted CD8+ and CD4+ T-cell epitopes in an ELISpot assay.

A dose-dependent cellular immune response against YFV was clearly detectable in mice vaccinated with pBeloBAC-FLYF or pBeloBAC-YF/ΔC (Fig. 4a, b). Irrespective of the amount of DNA used, mice electroporated with pBeloBAC-YF/GSA were unable to produce significant response compared with PBS-treated mice. Incubation of the splenocytes with the control peptide induced no significant response (Fig. 4c), confirming the specificity of the T-cell response induced by DNA vaccination with pBeloBAC-FLYF or pBeloBAC-YF/ΔC. Both CD8+ T-cell and CD4+ T-cell responses were highest following a dose of 1 μg of priming and boosting, with levels comparable between the full-length construct and the capsid mutant (Fig. 4a, b).

Intracellular cytokine staining (ICCS) was performed on CD8+ T cells to evaluate the quality of cellular immune response against YFV upon DNA immunization. Again, a dose-dependent response was observed for YFV-specific CD8+ T cells producing IL-2 and/or IFN-γ (Fig. 5). For the dose range tested in this study, it was obvious that mice immunized with the highest dose (1 μg) of either pBeloBAC-FLYF or pBeloBAC-YF/ΔC produced the highest percentage of cytokine-secreting T cells (Fig. 5).

In contrast to the induction of specific and multifunctional T-cell responses, no production of neutralizing antibodies was detected in mouse sera obtained 2 weeks after the boost (data not shown). However, YFV-specific neutralizing antibodies were easily detected in A129 mice that are deficient in expressing the IFNα/β receptor. The A129 and the congenic 129 mice were either electroporated with 5 μg of pBeloBAC-FLYF or injected intramuscularly with 106 p.f.u. YFV-17D, respectively. Serum was drawn.

---

**Fig. 3.** Characterization of YFV-17D replicon RNA that is produced by RNA splicing in pBeloBAC-FLYF transfected BHK-21J cells. (a) Analysis of intracellular YFV RNA synthesis by [3H]uridine labelling and gel electrophoresis. BHK cells transfected with pBeloBAC-FLYF were labelled for 6 h in the presence of Act D and sampled at 6 h intervals from 15 h post transfection onwards. (b) Analysis of viral RNA synthesis in BHK-21J cells infected with YFV-17D derived from in vitro-transcribed RNA (lane 1) or viruses derived from pBeloBAC-FLYF DNA (lane 3). Lane 2 is RNA from mock-infected cells. (c) Prediction of splice donor and acceptor sites in the sequence of the YFV structural genes. The splice donor and acceptor sequences that were used based on the sequencing result shown in (d) are indicated in yellow and pink, respectively. (d) Part of the nucleotide sequence of the 750 bp PCR product demonstrating the in-frame deletion in the YFV structural genes resulting in the production of the replicon RNA. Nucleotides 701–707, upstream of the splice donor site at nt 708, are highlighted in yellow, while nt 2441–2450, downstream of the splice acceptor site at nt 2440, are highlighted in pink.
every other day for the analysis of viraemia and YFV antibody production. No virus was detected in the sera of 129 mice that were transfected with pBeloBAC-FLYF, or infected with YFV-17D at any time during the experiment. For the YFV-17D-injected A129 mice, only serum samples collected on day 3 showed low levels of viraemia ($10^3$ and $3 \times 10^3$ p.f.u. ml$^{-1}$). Despite the transient and low level of viraemia, YFV-17D vaccinated A129 mice produced significant amounts of neutralizing antibodies against YFV. The PRNT50 value of sera collected on day 14 was determined to be above 1280 and remained high till the end of the experiment (>1280 at day 29), as shown in Table 1. Despite the fact that no virus was detected at any time point in the sera of A129 mice that were immunized with pBeloBAC-FLYF, these animals produced specific YFV-17D-neutralizing antibodies. The PRNT50 values were 40 and 160 at day 14, and 80 and 320 at day 29, for the two subjects, respectively (Table 1), clearly lower than those observed for the YFV-17D-injected A129 mice.

**DISCUSSION**

Introduced in the early 1990s, the concept of vaccination using naked DNA has gained widespread recognition, due to its apparent advantages over conventional vaccines, such as ease of production and quality control as well as low cost of propagation. The low immunogenicity in humans, however, is a major obstacle that has stymied the field (Kutzler & Weiner, 2008; Webster & Robinson, 1997; Weiner et al., 2010). In contrast, live-attenuated virus vaccines induce strong humoral and/or cellular immune responses. YFV-17D, the attenuated YFV strain that is used as a vaccine to protect against potentially lethal YFV infection, is one of the most successful attenuated vaccines ever developed (reviewed by Pulendran, 2009). To explore the feasibility of combining the advantages of DNA vaccine and live-attenuated vaccine, a DNA-launched YFV-17D was constructed and characterized both in cell culture and in mice.

As also encountered during this study, a severe handicap in YFV research is the lack of readily affordable small-animal models that manifest clinical symptoms representative of those seen in primates without the necessity of host adaptation of the virus (Meier et al., 2009). Mice possessing functional innate immune systems are disease-resistant to both WT and vaccine strains of YFV after subcutaneous inoculation, with restricted viral dissemination. Neither morbidity nor mortality was exhibited (Meier et al., 2009). Intracerebral inoculation can cause encephalitic disease in mice (Barrett & Gould, 1986), which nonetheless lacks relevance to human infection, which exhibits viscerotropism with possible lethal outcomes. Despite these drawbacks, mice are the only affordable small-animal model for the initial testing of a DNA-launched YFV-17D as presented in this report.

To compensate for a possible underestimation of the immunogenicity of the YFV-17D infectious DNA in mice...
due to murine restriction on YFV replication and dissemination (Meier et al., 2009; Sangster et al., 1998), in vivo electroporation was employed to deliver DNA into HLA-A2.1 transgenic C57BL/6 mice, a method proven to exponentially increase antigen expression and the resulting immune responses in various animals (Gardiner et al., 2009; Khan et al., 2005; Luxembourg et al., 2006; Medi et al., 2005; Otten et al., 2004, 2006; Tollefsen et al., 2003; Widera et al., 2000). The same electroporation device was also successfully used in HIV-1 DNA vaccine human trials (Kopycinski et al., 2012; Vasan et al., 2011). Since previous studies (Bredenbeek et al., 2006; Guy et al., 2010; Jiang et al., 2011) with recombinant YFV-17D-based vaccines showed that anti-vector immunity was a minor concern during prime–boost immunization, a prime–boost vaccination regimen was adopted to further augment the immune responses. Nevertheless, in vivo delivery of YFV infectious DNA by electroporation failed to induce YFV-neutralizing antibodies in HLA-A2.1 transgenic C57BL/6 mice, despite readily detectable CD8\(^+\) T-cell responses, whereas YFV-17D vaccination of BALB/c mice was reported to yield a balanced Th1–Th2 immune response (Franco et al., 2010). The mouse strain used could be one of the reasons for the skewed immune responses observed, since C57BL/6 mice and BALB/c mice are genetically predisposed to develop Th1 or Th2 immune responses, respectively (Hsieh et al., 1995). Indeed, when this manuscript was in consideration, it was reported (Tretyakova et al., 2014) that DNA-launched YFV-17D vaccine induced virus-specific neutralizing antibodies in BALB/c mice in titres comparable to those induced by immunization with parental YFV-17D. The observation that the capsid mutant induced cellular immune responses of similar magnitude to those induced by the WT construct was suggestive of restricted virus propagation after electroporation of the infectious DNA construct into HLA-A2.1 transgenic C57BL/6 mice, which could also contribute to poor induction of neutralizing antibodies. Unfortunately, we did not analyse the bio-distribution of YFV antigens at the site of injection or the spread of virus to draining lymph nodes or other organs, which could have provided valuable information.

In order to study whether the Th1-biased immune response was somehow correlated with the restriction of YFV production and dissemination by murine innate immune responses and the inability of YFV to evade these antiviral countermeasures in a non-natural host (Meier et al., 2009), humoral immune responses produced in A129 mice were analysed in parallel with those produced in congenic 129 mice after YFV-17D or YFV infectious DNA vaccination. A129 mice, which have deficiencies in innate immunity, were proposed to represent a biologically more relevant animal model for studying viscerotropic infection and disease development following WT YFV inoculation, as well as mechanisms of 17D-204 vaccine attenuation (Meier et al., 2009). Consistent with previous findings, viraemia was detected in A129 mice inoculated with YFV-17D, whereas no viraemia was detected in serum samples at any time point in YFV-17D-vaccinated 129 mice, strongly indicating that the murine type I interferon signalling pathway is important in restricting YFV replication. Despite the lack of obvious viraemia, A129 mice were able...
Table 1. YFV-specific neutralizing antibodies in mice vaccinated with pBeloBAC-FLYF or YFV-17D

Sera drawn from A129 or 129 mice at 14 or 29 days post pBeloBAC-FLYF DNA or YFV-17D vaccination were heat inactivated and tested for YFV-specific neutralizing antibody. Results are presented as the reciprocal of the highest dilution that can reduce plaque number by at least 50% (PRNT50).

<table>
<thead>
<tr>
<th>Group</th>
<th>Mouse no.</th>
<th>PRNT50 at 14 days</th>
<th>PRNT50 at 29 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBeloBAC-FLYF A129</td>
<td>1</td>
<td>40</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>160</td>
<td>320</td>
</tr>
<tr>
<td>YFV-17D A129</td>
<td>3</td>
<td>1280</td>
<td>&gt;1280</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1280</td>
<td>&gt;1280</td>
</tr>
<tr>
<td>pBeloBAC-FLYF 129</td>
<td>5</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>YFV-17D 129</td>
<td>7</td>
<td>ND</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>ND</td>
<td>160</td>
</tr>
</tbody>
</table>

ND, Not determined.

to mount an efficient humoral immune response after YFV DNA vaccination, which provides justification for more extensive immunization studies with these and other small-animal models.

This publication and the recently published paper by Tretyakova et al. (2014) provide evidence for the successful launch of YFV-17D infection in vivo and the induction of YFV-specific cell-mediated immunity and neutralizing antibodies in experimental animals. Of course, to better evaluate the potential of this DNA-launched YFV vaccine platform and its possible applications in human beings, the immunogenicity and efficacy as well as the safety profile of the DNA-based YFV vaccine should be more appropriately assessed in non-human primates. Besides, vaccination dosage needs to be adjusted based on the size and the susceptibility to YFV infection of the vaccinated species. Furthermore, novel DNA formulations and delivery routes could be explored to achieve better immunogenicity and acceptability. Although the electroporation method used in this study has been widely tested in a broad range of animals and has shown promising results in human clinical trials with DNA vaccines (Kopycinski et al., 2012; Vasan et al., 2011), intradermal electroporation delivery of DNA vaccines has started to attract more attention, owing to the accessibility of the skin and the abundance of antigen-presenting cells in the epidermis and dermis (Bräve et al., 2010; Lin et al., 2012). With the advances in DNA formulation and delivery technologies, the DNA-launched YFV-17D vaccine may prove to be a stable, facile-to-produce and effective alternative for the current YFV live-attenuated vaccine, and further holds promise as a platform for the development of YFV-17D-based recombinant DNA vaccines.

**METHODS**

**Cell cultures and YFV-17D stocks.** BHK-21J cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) supplemented with 8% FCS. YFV-17D stocks were obtained by harvesting the medium of BHK-21J cells that were transfected with *in vitro*-transcribed RNA of an infectious YFV-17D cDNA clone (Bredenbeek et al., 2003). Virus titres were determined by plaque assays on BHK-21J cells, as described previously (Silva et al., 2010).

**Construction of infectious YFV-17D DNA clones.** General standard nucleic acid methodologies were used throughout this study (Sambrook et al., 1989), unless described in more detail. Chemically competent *E. coli* DH5α cells were used for cloning (Inoue et al., 1990), except for BAC construction, for which DH10B competent cells (Invitrogen) were used. Nucleotide numbering of the YFV clones was based on GenBank accession no. X03700 (Rice et al., 1985).

The CMV promoter and HDVr sequences were amplified from pcDNA3.0 (Invitrogen) and RluCep-HDVr (Tilgner & Shi, 2004) plasmids and fused to the penultimate nucleotides at the 5′- and 3′-UTRs of the viral genome by fusion PCR (Charlier et al., 2003). The resulting PCR products were cloned into pACNR-CMVp-FLYF-HDVr in which the 5′-end of the full-length YFV insert was directly fused to a CMV promoter and the HDVr was engineered to ensure the production of a YFV RNA with an authentic 3′-end. For the construction of pBeloBAC-FLYF, the complete insert of pACNR-CMVp-FLYF-HDVr encompassing the CMV promoter, YFV-17D cDNA and the HDVr was moved as a NotI–AflII fragment into a modified version of the BAC pBelocAC11 with unique NotI and AflII sites in the polylinker sequence.

A QuikChange mutagenesis-based strategy (Stratagene) was used to create two mutants of pBeloBAC-FLYF. The pBeloBAC-YF/ΔC mutant contains an in-frame deletion of 49 aa (nt 185–331) in the capsid gene of YFV-17D (Seregin et al., 2006). In pBeloBAC-YF/GSA, the GDD motif (aa 3171–3173) of the viral RdRP N55 was mutated to GSA (Khromykh et al., 2001; Seregin et al., 2006).

**Transfection of BHK-21J cells with the YFV-17D DNA-driven constructs.** BAC DNA was prepared using Nucleobond Xtra Maxi EF (Macherey-Nagel), followed by CsCl gradient purification. DNA purity and quality were routinely checked with electrophoresis, and the concentration was determined using a NanoDrop spectrophotometer. BHK-21J cells were transfected with 5 μg BAC DNA by electroporation as described previously (van Dinten et al., 1997). At various time points after electroporation, transfected cells were fixed with 3% paraformaldehyde for immunofluorescence assay, and supernatants were collected for virus titration by plaque assay, as described above.

http://vir.sgmjournals.org
Immunofluorescence assay. Transfected cells seeded on coverslips were fixed with 3% paraformaldehyde in PBS (pH 7.4) at 24, 48 and 72 h p.e. for at least 30 min and washed with PBS containing 10 mM glycine. Following permeabilization with 0.1% Triton X-100 in PBS, indirect immunofluorescence was carried out with rabbit anti-YFV NS3 antisera diluted 1:2000 in PBS/5% FCS, and visualized with a secondary Alexa488-conjugated goat anti-rabbit IgG (Invitrogen) antibody with a dilution of 1:300 in PBS/5% FCS.

Animals and immunization protocols. Female, 6–8 week old, transgenic AAd mice in the C57BL/6 background were obtained from Dr Ralph Steinman’s lab and were used for immunization studies in groups of five or six mice. Mice were immunized intramuscularly by electroporation (Gardiner et al., 2009) with 10 ng, 100 ng or 1 μg endotoxin-free pBeloBAC-FLYF or derivatives per mouse on days 0 and 14.

For additional studies on virulence and the production of YFV-neutralizing antibody titres, 6–8 week old A129 mice, which are deficient in IFN-α and -β receptors, and congenic 129 mice (obtained from Dr Charles Rice’s lab) were immunized intramuscularly with 106 p.f.u. of YFV-17D or 5 μg of pBeloBAC-FLYF. Blood was drawn every other day up until 29 days post immunization. Plaque assays and plaque reduction neutralization assays were used to determine the titres of YFV-17D and virus-neutralizing antibody levels in the serum. All animal work was in compliance with approved institutional protocols.

ELISPOT assays for enumeration of IFN-γ spot-forming colonies (SFC). Two weeks after the final immunization, AAd mice were sacrificed and their spleens were removed to prepare splenocytes as described previously (Franco et al., 2010). ELISPOT assays (BD Biosciences) were performed to determine the frequency of IFN-γ-excreting cells, according to the manufacturer’s protocol. Briefly, splenocytes were cultured in the presence of concanavalin A (2.5 μg ml⁻¹; Sigma) or YFV-specific CD8⁺ and CD4⁺ T-cell peptides (Maciel et al., 2008) at a concentration of 1 μg ml⁻¹. A YFV-17D-specific peptide (CD4/E151; ERWVFVRNPFAVTAL) with a greatly reduced capacity to stimulate YF17D-specific CD4⁺ T cells (Maciel et al., 2008) was used as a negative control. After 16 h of culture, the plates were processed to determine the frequency of IFN-γ SFC (Franco et al., 2010). Briefly, the plates were washed and incubated with biotinylated anti-IFN-γ for 2 h at room temperature, followed by HRP-conjugated avidin for 1 h at room temperature. Reactions were developed with 3-amino-9-ethyl carbazole (AEC) substrate (HRP-conjugated avidin for 1 h at room temperature) and visualized with a 3-amino-9-ethyl carbazole (AEC) substrate. Final enumeration of IFN-γ SFC was performed with the IMMUNOPOT software version 3.0 (Cellular Technologies). Data are presented as SFC (10⁵ cells)⁻¹. Results were considered positive if the number of SFCs was above 20 and higher than the background (culture with medium alone). The results are presented after subtraction of the background, which was consistently found to be 10–25 spots (10⁵ cells)⁻¹ throughout the experiments.

ICCS and flow cytometry. Splenocytes from immunized mice were analysed by ICCS for IL-2 and IFN-γ production after stimulation with YF17D-specific 15 aa peptides as described previously (Franco et al., 2010). Cultures without stimulation or those treated with concanavalin A served as negative or positive control, respectively.

Stained cells were analysed with the BD LSR-II flow cytometer using FACSDiva software (BD Biosciences). The data were processed with FlowJo 8.6.1 software (Tree Star).

PRNT. YFV-17D-specific neutralizing antibody titres were determined by 50% endpoint PRNT in six-well plates of BHK-21J cells using a standard PRNT protocol. Briefly, a twofold serial dilution of heat-inactivated mouse serum was prepared in PBS/2% FCS; 5 μl serum of each dilution was incubated with 5 μl PBS/2% FCS containing approximately 80 p.f.u. YFV-17D virus. After 1 h of incubation on ice, the serum–virus mixtures were diluted with 300 μl PBS/2% FCS and inoculated onto BHK-21J cells. After 1 h of incubation at 37 °C the inocula were removed, and an overlay of DMEM/2% FCS with 1.2% Avicel was added. Plaques were visualized by staining with 0.1% crystal violet after 4 days of incubation.

Acknowledgements

We thank Professor David Ho (Aaron Diamond AIDS Research Center, Rockefeller University) and Professor C. M. Rice (Laboratory of Virology and Infectious Disease, Rockefeller University) for facilities and helpful discussions.

References


