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Identification of three H-2K^d restricted CTL epitopes of NS4A and NS4B protein from Yellow fever 17D vaccine

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In this study, 12 nonamers derived from the nonstructural protein NS4A and NS4B of a Chinese attenuated Yellow fever 17D (YF-17D) vaccine strain were screened for their ability to elicit a recall interferon-γ (IFN-γ) response from the splenocytes of BALB/c mice following DNA vaccination and a booster vaccination with recombinant vaccinia virus expressing NS4A or NS4B protein. Three peptides (amino acid residues S_{106}YIMLIFFV from NS4A protein and V_{43}YVGIVTML and L_{174}YLLLALSL from NS4B protein) were identified as CD8$^+$ cytotoxic T lymphocyte (CTL) epitopes. These peptides elicited a response from a significant numbers of IFN-γ secreting cells compared with the other nonamers of NS4A and NS4B and compared to one irrelevant nonamer derived from infectious bronchitis virus (IBV) H52 strain. Bioinformatics analysis demonstrated that these three nonamers are H-2K^d-restricted CTL epitopes. The multiple amino acid sequence alignments among different YFV NS4A, and NS4B sequences submitted to GenBank indicated that these three CTL epitopes are strongly conserved; this novel finding will potentially aid further studies on cellular immunity against YF virus and YFV-based expression vectors.

1. Introduction

Yellow fever virus (YFV) is the prototype member of the family *Flaviviridae* and genus *Flavivirus* (Lindenbach et al., 2007) and was first isolated in West Africa in 1927 (Barrett and Higgs, 2007). This virus is both viscerotropic and neurotropic, and presents a major public health problem, causing epidemic and endemic disease in large parts of tropical Africa and South America; also, the virus poses a serious health risk to those who travel to areas with endemic disease (Barrett et al., 2007; Proutski et al., 1997). The World Health Organization (WHO) estimates that YFV causes 200,000 cases of clinical disease and 30,000 deaths each year (Staples et al., 2010).

The YFV genome consists of positive-stranded RNA, approximately 11.8 kb length, encoding all known *flavivirus* proteins as follows: 5′-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′ (Lindenbach et al., 2007; Rice et al., 1985). Viral structural proteins (capsid, prematrix, and envelope) are encoded at the 5′ end, followed by non-structural (NS) proteins encoded by the remaining gene segment (Rice et al., 1985).

Currently, there is no effective drug treatment for YF, and there has been a dramatic increase in the number of YF cases in recent years; YF is presently considered a re-emerging disease (Barrett et al., 2007). Public health efforts have concentrated on preventive measures that focus on the administration of the YFV vaccine to those who may be exposed to YFV through work, travel, or habitation in endemic areas (Staples et al., 2010). The YFV vaccine, a live attenuated vaccine (strain 17D) derived from the YF-Asibi strain, is one of the most effective and safe vaccines available (Barrett, 1997). It has been administered to over 500 million people for over 70 years, and only a minority of recipients experience local reactions such as redness or tenderness at the site; encephalitis has been very rarely reported in young infants (Barrett et al., 2007; Barrett and Teuwen, 2009). The effectiveness of this vaccine in humans and animals has been attributed to the development of complement fixing antibodies to the NS1 protein and neutralizing antibodies to the E structural protein; the vaccine exhibits duration of protection as long as 35 years (Brandriss et al., 1990; Co et al., 2002; Lobigs et al., 1987; Poland et al., 1981). In animal studies, active immunization with the YFV structural protein E, the nonstructural protein NS1, or passive immunization with monoclonal antibodies to these proteins has been demonstrated to protect mice and monkeys against lethal YFV infection (Brandriss et al., 1986; Co et al., 2002; Putnak and Schlesinger, 1990; Schlesinger et al., 1986, 1993). The excellent

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safety record and proven efficacy of the attenuated YF-17D vaccine virus suggests that YFV-17D is the obvious candidate for chimeric vaccine development (Brandler and Tangy, 2008; van der Most et al., 2002). Several recombinant chimeric viruses have been developed that express the prM and E genes from Japanese encephalitis virus (JEV) (Chambers et al., 1999) or dengue virus (Guirakhoo et al., 1999, 2000; van Der Most et al., 2000) in a genetic background similar to YFV-17D. Furthermore, immunization followed by challenge in mice (Guirakhoo et al., 1999; van Der Most et al., 2000) and rhesus monkeys (Guirakhoo et al., 2000; Monath et al., 2000) demonstrated that these chimeric vaccines are highly immunogenic and provide strong protective immunity (van der Most et al., 2002). Moreover, YF-17D based recombinant vaccine expressing genes or epitopes from unrelated pathogens, including immunodeficiency virus SIVmac239 Gag, Lassa virus, GP1 and GP2 glycoproteins, a *Trypanosoma cruzi* Amastigote Surface Protein-2 and glycoprotein G of vesicular stomatitis virus (Bonaldo et al., 2010; Jiang et al., 2011; Nogueira et al., 2011; Varnavski and Khromykh, 1999), or from tumor antigens (McAllister et al., 2000) have been developed successfully.

However, the immune mechanisms underlying this virus or live attenuated vaccine remain unclear, although it has been demonstrated that the live attenuated vaccine induces a long-lasting antibody and T cell response (Mudd et al., 2010; van der Most et al., 2002). Reports have suggested that Yellow fever vaccine, in rare instances, can cause illness that resembles infection with wild-type virus, particularly in the elderly (Martin et al., 2001a,b; McMahon et al., 2007; Vasconcelos et al., 2001; Wang and Fan, 2005). This demonstrates the importance of a systematic understanding of YFV pathogenesis and immunology; development of therapeutic intervention is warranted. The role of cell-mediated immunity driven by the YF-17D vaccine virus was first proposed by (Reinhardt et al., 1998), and was confirmed with the identification of YF-specific human effector and memory $CD8^+$ T cells addressed to E, NS1, NS2B, and NS3 proteins of YF 17D (Akondy et al., 2009; Co et al., 2002; Miller et al., 2008). Studies have reported that the E, NS3, and NS5 proteins are major targets of the antiviral T cell response in mice (Maciel et al., 2008; van der Most et al., 2002) and in Indian rhesus macaques (Mudd et al., 2010). There have been relatively few reports concerning the NS4A and NS4B proteins. Both NS4A and NS4B are membrane associated proteins, with molecular masses of 16 and 27 kDa, respectively. Previous studies on YFV suggested that these two proteins play a role in regulating RNA replication (Chambers et al., 1999). However, their function regarding cellular immune response remains largely unknown. Major histocompatibility complex (MHC) I restricted cytotoxic T lymphocytes (CTLs) play a pivotal role in killing virus-infected cells and eliminating potential sources of new virus (Chisari and Ferrari, 1995). Hence, identification of CTL epitopes is crucial for elucidation of the mechanisms underlying cell-mediated immunity; a number of studies have successfully identified pathogen-derived T cell epitopes from respiratory syncytial virus, influenza A virus, foot and mouth disease virus, and other YFV proteins (Barfoed et al., 2006; Co et al., 2002; Rutigliano et al., 2005; Tourdot et al., 2001; van der Most et al., 2002; Yewdell and Haeryfar, 2005).

In this study, the identification of CTL epitopes from the Chinese attenuated YF-17D vaccine virus NS4A and NS4B proteins is described in a mouse model. Peptides derived from the Chinese attenuated YF-17D vaccine virus NS4A and NS4B proteins were screened for their ability to induce interferon (IFN)-γ in splenocytes harvested from BALB/c mice following DNA vaccination and booster vaccination with recombinant vaccinia virus expressing the NS4A or the NS4B protein. Three peptides that elicited IFN-γ production in $CD3^+CD8^+$ splenocytes of vaccinated mice were identified. A multiple amino acid sequence alignment among different NS4A and NSB proteins of YFV indicated that these three peptides are strongly conserved across multiple YFV strains; therefore, further research is warranted.

Table 1
Primers designed for PCR.[a]

Primer	Sequence (5′–3′)	Note
NS4A-F	GC*GTCGAC***ATG**GGAGCTGCTGAAG	*Sal* I
NS4A-R	CG*GGATCC***CTA**CCTTTGTTGCCCTG	*BamH* I
NS4B-F	GC*GTCGAC***ATG**TCCATCCAAGACAAC	*Sal* I
NS4B-R	CG*GGATCC***CTA**CCGGCGTCCAGTTTTC	*BamH* I
pSC11-NS4A-F	ATA*GTCGAC*GCCGCCGCC**ATG**GGAGCTGCTG	*Sal* I
pSC11-NS4A-R	GCC*GTCGAC***CTA**CCTTTGTTGCCCTG	*Sal* I
pSC11-NS4B-F	ATA*GTCGAC*GCCGCCGCC**ATG**TCCATCCAAGAC	*Sal* I
pSC11-NS4B-R	GCC*GTCGAC***CTA**CCGGCGTCCAGTTTTC	*Sal* I
P7.5-F	GCACGGTAAGGAAGTAGAAT	

[a] The bold letter of primers are start or stop codons; the underlined sequence of primers represents the Kozak motif; and the italic sequence of primers represents the restriction enzyme sites of *Sal* I and *BamH* I.

2. Materials and methods

2.1. Viruses and cell lines

The Chinese attenuated yellow fever 17D vaccine strain (YF-17D) was purchased from Beijing Tiantan Biological Company (Beijing, China), and the vaccinia virus WR strain was part of our laboratory collection. The virus was propagated in BHK-21 cells, which were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10%, 56 °C 30 min heat-inactivated, fetal bovine serum (FBS) and antibiotics (0.1 mg/ml of streptomycin and 100 IU/ml of penicillin) (Invitrogen, Carlsbad, CA, USA), in a humidified 37 °C, 5% CO_2 incubator.

2.2. Cloning of YF-17D NS4A and NS4B genes

Total RNA was extracted from the Chinese attenuated YF-17D vaccine strain, and full length cDNA was amplified based on the complete open reading frames (ORFs) of NS4A and NS4B following reverse transcription (GenBank accession number FJ654700) using the NS4A-F and NS4A-R primer pair or the NS4B-F and NS4B-R primer pair (Table 1). The full length cDNA was cloned into the pMD18-T vector (TaKaRa, Dalian, China), referred to as pMD-NS4A and pMD-NS4B, respectively.

2.3. Construction of the eukaryotic expression plasmids pBudCE-NS4A and pBudCE-NS4B

The NS4A and NS4B genes were amplified from pMD-NS4A and pMD-NS4B with the primer pairs NS4A-F and NS4A-R, and NS4B-F and NS4B-R, respectively (Table 1). The PCR products were purified and inserted into the eukaryocyte expression vector pBudCE4.1 (Invitrogen, Carlsbad, CA, USA), named pBudCE-NS4A and pBudCE-NS4B, respectively.

2.4. Expression of pBudCE-NS4A and pBudCE-NS4B in vitro

In vitro expression of NS4A and NS4B was demonstrated by transient transfection of BHK-21 cells using FuGene 6 (Roche, Sandhofer Str, Germany) according to the manufacturer's recommendations. The NS4A and NS4B protein expression levels were detected by indirect immunofluorescence analysis (IFA). Rabbit anti-YFV polyclonal antibody (working dilution 1:200) and FITC-conjugated goat anti-rabbit IgG secondary antibody (Sigma, St. Louis, USA) were utilized for IFA detection of NS4A and NS4B protein levels;