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Characterization of a major neutralizing epitope on the yellow fever virus envelope protein using human recombinant monoclonal antibody fragments generated by phage display

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To my wife, Fanny

To my parents

INDEX

SUMMARY **1**

I-INTRODUCTION **4**

I-1 . Yellow fever (YF). 4
I-1-1. Disease. 4
I-1-2. Epidemiology. 4
I-1-3. Resurgence of yellow fever as a major public health problem. 5
I-1-4. Transmission cycles. 5
I-2. Yellow fever virus (YFV). 6
I-2-1. Taxonomy of flaviviruses. 6
I-2-2. Classification of the yellow fever virus. 6
I-2-3. Structure of the virion. 7
I-2-4. Pathogenesis. 9
I-2-5. The Flavivirus life cycle. 10
I-2-6. Cellular receptor(s) for Flaviviruses. 10
I-2-7. Flavivirus fusion with host cell membranes. 11
I-3. The YFV envelope protein (the E protein). 12
I-3-1. Molecular Structure. 13
I-3-2. Antigenic structure. 13
I-4 The Yellow Fever vaccine. 17
I-4-1. The French Neurotropic Vaccine (FNV). 17
I-4-2. The 17D vaccine. 18
I-5 Immune Response to YFV. 20
I-5-1. Innate immune response. 20
I-5-2. Adaptive immune response. 21
I-6. Adverse effects following YF 17D vaccination. 22
I-6-1. The YELlow fever-Associated Neurotropic Disease (YEL-AND). 22
I-6-2. The YELlow fever-Associated Viscerotropic Disease (YEL-AVD). 23
I-7. Antibodies. 23
I-7-1. Structure of antibodies. 24
I-7-2. Germline organization of the genetic loci of antibodies. 26
I-7-3. Somatic recombination, affinity maturation and isotype switching in antibody diversity. 26
I-7-4. Antiviral antibodies and their mechanism of action. 28
I-7-5. Monoclonal antibodies and biological applications. 28
I-8. Phage Display technology. 29
I-8-1. Principle. 29
I-8-2. Filamentous phages. 30

I-8-3. Phage display system. 30
I-8-4. Phage-displayed scFv libraries. 32
I-8-5. Selection of antibody libraries: “Biopanning”. 34
I-8-6. Human viruses neutralized by recombinant antibody fragments. 36
I-9. Aim of the work 37

II-MATERIAL 39
II-1. Plasticware. 39
II-2. Chemicals. 39
II-3. Enzymes. 40
II-4. Antibodies 40
II-5. Radioactive amino acids. 40
II-6. Commercial Kits. 40
II-7. Vectors. 41
II-8. Viruses. 41
II-9. Bacteria and phages. 42
II-10. Eukaryotic Cells. 42
II-11. PCR primers. 42
II-11-1. Degenerated primers specific for the VH and VL genes. 42
II-11-2. pHEN3-specific primers. 44
II-11-3. YFV E, NS1 and prM proteins-specific primers. 44
II-12. Buffers and solutions. 44
II-12-1. Virus purification. 45
II-12-2. Phage purification. 45
II-12-3. DNA electrophoresis. 45
II-12-4. ELISA. 45
II-12-5. SDS PAGE and Western Blot. 45
II-12-6. Dot Blot buffers. 46
II-12-7. Protein purification 46
II-12-8. pH shift buffers. 47
II-13. Media for bacterial culture. 47
II-14. Media for cell culture. 48

III-METHODS 50
III-1. Schematic representation of the construction of the phage libraries expressing recombinant antibody fragments (scFvs). 50
III-2. Isolation of lymphocytes from two African donors who recovered from yellow fever. 52

III-3. First strand cDNA synthesis. 52

III-4. PCR amplification of the Variable Kappa Light chain (VLκ), Variable Lambda Light chain (VLλ) and Variable Heavy chain (VH) genes of antibodies. 53

III-4-1. PCR conditions and protocol. 53

III-4-2. Combination of degenerated primers used to amplify the Variable Kappa Light chains (VLκ). 54

III-4-3. Combination of degenerated primers used to amplify the Variable Lambda Light chains (VLλ). 55

III-4-4. Combination of degenerated primers used to amplify the Variable Heavy chains (VH). 56

III-5. Cloning of VLκ and VLλ PCR products into the pHEN3 phagemid to generate VLκ and VLλ bacterial sub-libraries. 56

III-5-1. Digestion of the VLκ and VLλ PCR products (reactions 1b to 17b obtained from PCR 1b) and the phagemid pHEN3. 56

III-5-2. Dephosphorylation of the pHEN3 vector. 57

III-5-3. Ligation. 57

III-5-4. Transformation of electrocompetent TG1 *E.coli* cells by electroporation and plating of VLκ and VLλ sub-libraries. 58

III-5-5. Estimation of the library size. 58

III-5-6. DNA preparation of VLκ and VLλ sub-libraries. 59

III-6 Cloning of VH PCR products into VLκ-pHEN3 and VLλ-pHEN3 phagemids to generate the VLκ-VH and VLλ-VH final bacterial libraries. 59

III-7. Colony PCR. 60

III-8. Rescue of recombinant phages displaying scFV fragments from the final bacterial libraries. 61

III-8-1. Preparation of a helper phage working stock. 61

III-8-2. Rescue of recombinant phages from constructed scFv libraries. 62

III-9. Preparation and purification of YFV 17D-204-WHO particles. 63

III-9-1. Preparation of a YFV 17D-204-WHO master stock. 63

III-9-2. Titer determination by plaque assay. 63

III-9-3. Purification of YFV 17D-204-WHO particles. 64

III-9-4. Determination of purified YFV 17D-204-WHO particles antigenicity in ELISA. 64

III-10. Biopanning. 65

III-10-1. Selection step. 65

III-10-2. Rescue of phages isolated from the first round of selection. 65

III-10-3. Polyclonal phage ELISA. 66

III-10-4. Monoclonal phage ELISA. 66

III-10-5. BstNI fingerprintings. 66
III-11. Expression and purification of scFvs in *E.coli* TG1 cells. 67
III-11-1. Cloning in the prokaryotic expression plasmid pAB1. 67
III-11-2. Expression of scFvs in *E.coli* cells. 67
III-11-3. ScFv purification by Immobilized Metal ion Affinity Chromatography (IMAC). 68
III-11-4. Determination of scFvs by Coomassie staining and Western Blot analysis. 68
III-11-5. scFv ELISA. 69
III-12. Dot Blot analysis. 70
III-13. Radioimmunoprecipitation assay (RIPA). 70
III-13-1. Production of radiolabeled soluble viral proteins from radiolabeled YF 17D-204-WHO virions. 70
III-13-2. RIPA. 71
III-14. pH sensitivity experiments. 71
III-15. Competition ELISA using biotinylated antibodies. 71
III-15-1. Biotinylation. 71
III-15-2. Competition ELISA. 72
III-16. Plaque reduction neutralization test (PRNT). 72
III-17. Generation of escape mutants. 72
III-18. Microneutralization assay. 73
III-18-1. Determination of the 50% Tissue Culture Infectious Dose ($TCID_{50}$). 73
III-18-2. Microneutralization assay. 73
III-19. Sequencing analysis. 74
III-19-1. Sequencing analysis of scFvs displayed by YFV-17D-204-WHO specific monoclonal phages 74
III-19-2. Sequencing analysis of prM and E proteins of all YFV strains (17D-204 WHO, wild-type strains and escape mutants). 74
III-20. Molecular Modelling. 75

IV-RESULTS 77
IV-1. Generation of two phage libraries displaying recombinant antibody fragments (scFvs) from two recovered yellow fever patients. 77
IV-1-1. Amplification of the Variable Kappa light chains (VLκ), the Variable Lambda light chains (VLλ) and the Variable Heavy chains (VH) genes by PCR. 77
IV-1-2. Construction of bacterial scFv libraries and rescue of recombinant phages displaying scFvs on their surface. 78

IV-2. Isolation of monoclonal phages with a specific affinity for YFV 17D-204-WHO particles. 79
IV-2-1. Purification of YFV 17D-204-WHO particles. 79
IV-2-2. Enrichment of specific phage binders to the YFV antigen through biopanning. 80
IV-2-3. Screening of monoclonal phages from round 3 and round 4 of the selection step. 81
IV-2-4. Genetic diversity of scFvs displayed by YFV 17D-204-WHO-specific binders. 81
IV-3. Expression and purification of six different scFvs (7A, 5A, R3(27), 1A, 2A and R3(9)) with a specific affinity for YFV 17D-204-WHO virions. 82
IV-3-1. Expression and purification of scFv-7A, 5A, R3(27), 1A, 2A and R3(9) as soluble molecules. 82
IV-3-2. Reactivity of soluble scFv-7A, 5A, R3(27), 1A, 2A and R3(9) with the YFV 17D-204-WHO antigen. 83
IV-3-3. Sequencing analysis of scFvs-7A, 5A, R3(27), 1A, 2A and R3(9). 84
IV-4. Identification of the YFV proteins recognized by the scFvs-7A, 5A, R3(27), 1A, 2A and R3(9) 85
IV-4-1. Western Blot analysis. 85
IV-4-2. Dot Blot analysis. 85
IV-4-3. Radioimmunoprecipitation assay (RIPA). 86
IV-5. Competition ELISA. 87
IV-6. pH sensitivity of the epitopes. 88
IV-7. Neutralization assays. 89
IV-7-1. Plaque Reduction Neutralization Test (PRNT) using the YFV 17D 204-WHO (vaccine strain) and YFV wild-type strain Asibi. 89
IV-7-2. Plaque Reduction Neutralization Test (PRNT) using 5 wild-type YFV strains representing three of the five known African genotypes. 90
IV-8. Generation of YFV 17D-204 WHO variants exhibiting resistance to scFv-7A neutralization. 92
IV-9. Identification of amino acid residues on the E protein associated with resistance to scFv-7A neutralization. 93
IV-10. Location of amino acid substitutions associated with resistance to scFv-7A neutralization in the E protein homodimeric crystal structure of YFV. 93
IV-11. Mutations in the E protein of the YFV Senegal 90 strain. 95
IV-11. Importance of the mutations E-71 and E-155 in terms of neutralization escape using human polyclonal sera from YF patients and 17D vaccinees. 96

V-DISCUSSION **99**
V-1 Quality of antibody libraries and results of selection. 99
V-1-1. Construction of two human immune antibody phage display libraries and their panning against purified YFV 17D-204-WHO virions. 99
V-1-2. Isolation of closely-related scFvs specific for the YFV E protein. 100
V-2.Nature of the epitopes. 101
V-2-1. The epitopes are conformation-dependent and pH-sensitive. 101
V-2-2. The scFv-7A epitope is formed by amino acid residues from domain I (E-153, E-154 and E-155) and from domain II (E-71) in the E protein. 101
V-3. The scFv-7A epitope. 102
V-3-1. E-71 is extremely conserved among all sequenced YF strains and critical for neutralization. 102
V-3-2. Aa residues E-153, E-154 and E-155 contribute to a lesser extent in the scFv-7A epitope. 102
V-4. Three isolated scFvs exhibit a broad and potent neutralizing activity in vitro. 105
V-5. Homology of the scFv-7A with previously described YFV neutralizing epitopes of monoclonal mouse antibodies. 107
V-6. Neutralization of scFv-7A escape mutants generated from a 17D 204-WHO vaccine lot with sera from 17D-immunized travelers. 107
V-7. Neutralization of the YFV Senegal 90 strain. 108
V-8. Potential mechanism of the scFv neutralization. 109
V-9. Potentiel use of these scFvs as a therapeutical tool to treat YF. 111
V-10. Implications for the design of a cDNA-based YF vaccine. 112

VI-LITERATURE. **115**

VII-ANNEXES **132**
VII-1. Figures & Tables. 132
VII-2. Abbreviations. 134
VII-3. Publications, presentations and posters. 136
VII-4. Curriculum Vitae. 138
VII-5. Aknowledgments. 142

SUMMARY

Yellow fever virus (YFV) is a mosquito-transmitted, enveloped, positive stranded RNA virus belonging to the genus flavivirus, which causes hemorrhagic fever in humans in Africa and South America. The YFV is responsible for 200 000 clinical infections per year including 40 000 deaths. Despite the presence of a highly effective YF vaccine called 17D vaccine, this disease is now strongly re-emerging and has to be considered as a public health problem. The present live attenuated 17D vaccine has two major drawbacks: 1) the ancient production method by inoculating viable embryonated eggs which limits the vaccine production capacity and, therefore, impairs attempts to control the disease and may contribute to vaccine supply shortage. 2) this vaccine is a non clonal vaccine which is constituted of heterogenous virion sub-populations. Furthermore, recent reports of several cases of viscerotropic and neurotropic disease associated with 17D vaccination have raised the obvious question of vaccine safety. Taken together, these data show that it appears essential to design a new clonal vaccine which could be based on infectious cDNA clone and produced in animal cell culture. For this purpose, the knowledge of YFV neutralizing epitopes is essential. Because YFV immunity is mainly antibody-mediated, we wanted to isolate human neutralizing antibodies specific for YFV and use them as a tool to characterize the neutralizing epitopes of YFV. The phage display technology provides one of the most convenient systems to isolate such neutralizing recombinant antibody fragments. We generated YF patient-derived antibody phage libraries which were screened against purified virions of the YFV-204-WHO vaccine strain. This step led to the isolation of several single-chain antibody fragments (scFv) which recognized conformational and pH sensitive epitopes in the envelope E protein. Three genetically closely-related and competing scFvs were found to be able to neutralize in vitro the 17D vaccine strain and five wild-type African strains of YFV. To map their epitopes, neutralization escape variants of the YFV-17D-204-WHO were generated using one high-affinity scFv (scFv-7A). Amino acids (aa) E-153, E-154 and E-155 in domain I and aa E-71 in domain II of the E protein were shown to be the critical components of one complex neutralizing epitope. These aa do not form a contiguous epitope on the monomeric E protein, but are in close vicinity in the dimeric form the E protein is predicted to adopt, based on the crystal structures of related flaviviruses. The neutralizing epitope is thus predicted to be formed by contribution of aa from domain I and II of opposing E monomers. The nature of this epitope was supported by the analysis of one wild-type YFV strain (Senegal 90) which is naturally resistant to neutralization by scFv-7A. Microneutralization assays using sera from YFV-

infected patients and 17D-immunized travelers confirm the importance of E-71 in YFV neutralization but also showed that those escape variants, originally present in the vaccine lot, do not carry a risk of neutralization escape in persons who are immunized with the 17D vaccine. The potential neutralization mechanism by which these scFvs act, particularly by preventing the fusion process, and their potential use as a therapeutical tool are discussed. The structural complexity of the epitope identified in this work has implications for understanding the mechanism of antibody-mediated neutralization of YFV and these data may be useful for the design of a new recombinant yellow fever vaccine based on a cDNA-derived infectious clone.

I-INTRODUCTION

I-INTRODUCTION

I-1 . Yellow fever (YF).

I-1-1. Disease.

Yellow fever disease is a viral hemorrhagic fever mainly characterized by severe liver, myocardial and renal injury. The progression of this disease usually follows three stages: a first stage called "period of infection" which occurs three to six days post infection during which infected people suffer from high fever, headache, myalgia and nausea. After three to four days, patients enter into a second phase called "period of remission" at which time all symptoms disappear. At this stage, people either recover without any sequels or they enter into a last stage called "period of intoxication". This stage occurs generally three to six days after onset of symptoms and is characterized by high fever, intense jaundice, multiorgan failure, a generalized hemorrhagic state ("black vomit"), hypotension, coma and, most of the time, death. The case fatality rate of yellow fever disease is around 20 to 40% (Monath and Barrett, 2003) No specific treatment for yellow fever is available so far and only intensive supportive cares may improve the outcome of infected patients. Yellow fever can be prevented using a safe and highly effective vaccine called 17D.

I-1-2. Epidemiology.

Fig 1: Distribution of Yellow fever disease in South America and Africa. In grey are indicated the yellow fever endemic zones. Red stars represent reported YF outbreaks between 1985 and 2003.

Yellow fever disease is endemic in South America and in sub-Saharan Africa (Fig 1). Although an effective and safe live-attenuated 17D strain vaccine exists, YF

remains a serious health problem in African and South American tropical areas with an estimated number of 200 000 clinically apparent infections and 40 000 deaths in the endemic regions annually (Monath, 1999), (Robertson *et al*., 1996).

I-1-3. Resurgence of yellow fever as a major public health problem.

The resurgence of the disease (Fig.2B) is mainly explained by relaxation of immunization programs resulting in a lower YF vaccination coverage in developing countries, particularly in Africa. As shown in Fig.2A, despite the successful eradication in the 70's of mosquitos *Aedes aegypti* which are the urban vector of yellow fever, a reinfestation with this vector is now observed in South America suggesting future changes in yellow fever epidemiology. Changes in human demography (shift from rural to urban residence) and economical development (travelling) are also factors which are responsible for the increase of yellow fever activity (Monath, 1999).

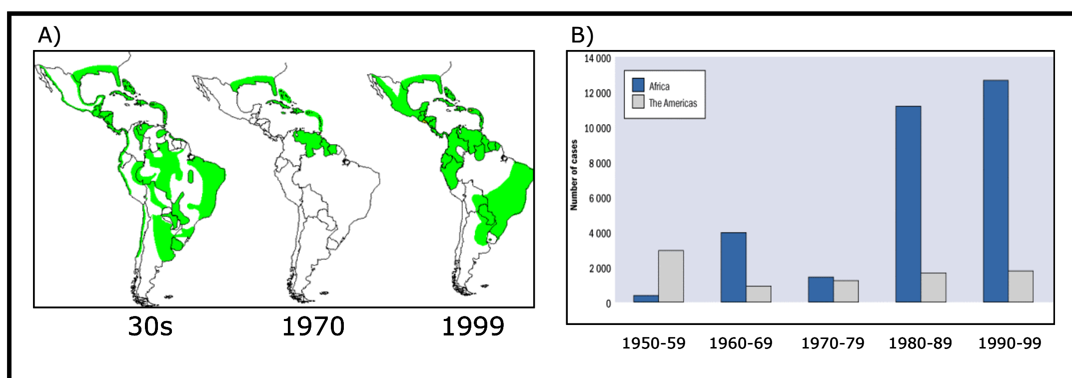

Fig 2. Resurgence of yellow fever as a major public health problem. A) re-invasion of South America by the urban vector of yellow fever, *Aedes aegypti,* during the period 1930-1999 (Monath, 2004). B) number of reported cases of yellow fever per decade from 1950 to 1999.

I-1-4. Transmission cycles.

The yellow fever virus (YFV) which is the causative agent of the yellow fever disease is transmitted to vertebrates (human and non-human primates) by diurnal mosquitos during feeding. Thus, primates are considered as vertebrate host and mosquitos as arthropod host. Since they can be infected for life, mosquitos are considered as the YFV reservoir.

In South America, two cycles are observed: an endemic "sylvatic cycle" in which YFV is transmitted to monkeys by infected mosquitoes (*Heamagogus* species). Non-human primates infected during the sylvatic cycle with YFV either die from infection

or develop immunity and serve as amplifying hosts. Humans are accidentally infected when entering into the jungle, via contacts with infected mosquitos. Infected humans can then spread YFV through urban mosquitos (*Aedes aegypti*) leading to the establishment of the "urban cycle" and a YF urban outbreak. In Africa, YF transmission is identical except that a third cycle called "savannah cycle" has been identified due to the concomitant presence of humans and monkeys in the moist Savannah at the border between the jungle and the city (Barrett and Monath, 2003)

I-2. Yellow fever virus (YFV).

I-2-1. Taxonomy of flaviviruses.

The genus Flavivirus forms together with the two other genera Pestivirus and Hepacivirus the family Flaviviridae It includes 73 viruses divided into non-vector transmitted, tick-borne and mosquito-borne flaviviruses. Flaviviruses are present on all continents. Many members of this genus that includes yellow fever virus (YFV), dengue virus (DV), Japanese encephalitis virus (JEV), Tick-borne encephalitis virus (TBEV) and West Nile virus (WNV) are human pathogens causing a significant morbidity and mortality worldwide (Calisher and Gould, 2003).

I-2-2. Classification of the yellow fever virus.

The YFV is the prototypic member of the genus flavivirus. Recent phylogenetic studies have shown that YFV is closely related to nine other flaviviruses (Banzi, Bouboui, Edge Hill, Jugra, Saboya, Potiskum, Sepik, Uganda S and Wesselsbron), Sepik virus being the most closely related one (Calisher and Gould, 2003).
To date, seven YFV genotypes have been defined based on a phylogenetic analysis from the nucleotide sequences of prM/M and E genes (Fig.3). Two were identified in South America (South America I and South America II) and five in Africa (Angola, East Africa (EA), East and Central Africa (EA/CA), West Africa I (WAI) and West Africa II (WAII)) (Mutebi *et al*., 2004) (Mutebi *et al*., 2001).

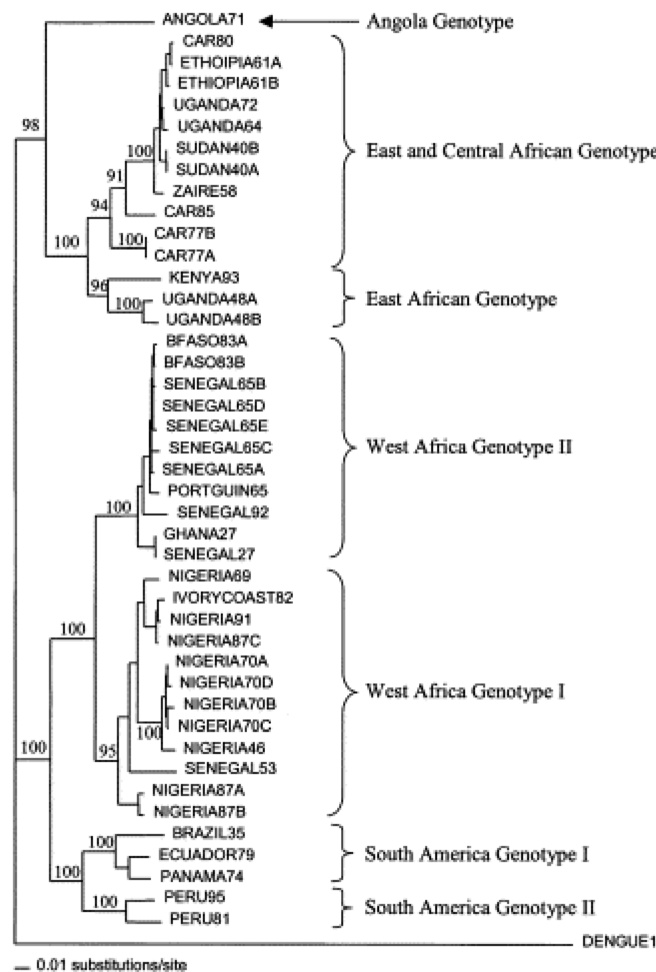

Fig 3: Phylogenetic relationships among genotypes of YFV from Africa and South America, using Dengue virus type 1 as an outgroup. From (Mutebi *et al.*, 2001).

I-2-3. Structure of the virion.

Flaviviruses are spherical enveloped viruses with a diameter of around 50-60 nm. The genome of the YFV consists of a non-segmented 11 kb positive single-stranded RNA (Fig.4A). The genome of the wild-type YFV Asibi strain is composed of 10 862 nucleotides (Rice *et al.*, 1985). It is composed of a single open reading frame (ORF) flanked by two short noncoding regions at the 5´and the 3´termini (5´NCR and 3´NCR). These NCR are not well conserved among flaviviruses but have been shown to form cyclic and secondary structures and may play a major role in virus replication and viral proteins translation (Hahn *et al.*, 1987). In infected cells, the ORF is translated as a single polyprotein which is further processed to yield viral structural and non structural proteins. The N-terminal part of this polypeptide encodes three structural proteins which form the mature virion: the capsid protein

C, the precursor of the membrane protein prM, and the envelope protein E. The rest of the polyprotein encodes for 7 non-structural proteins which are responsible for viral replication and protein processing: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Lindenbach and Rice, 2003).

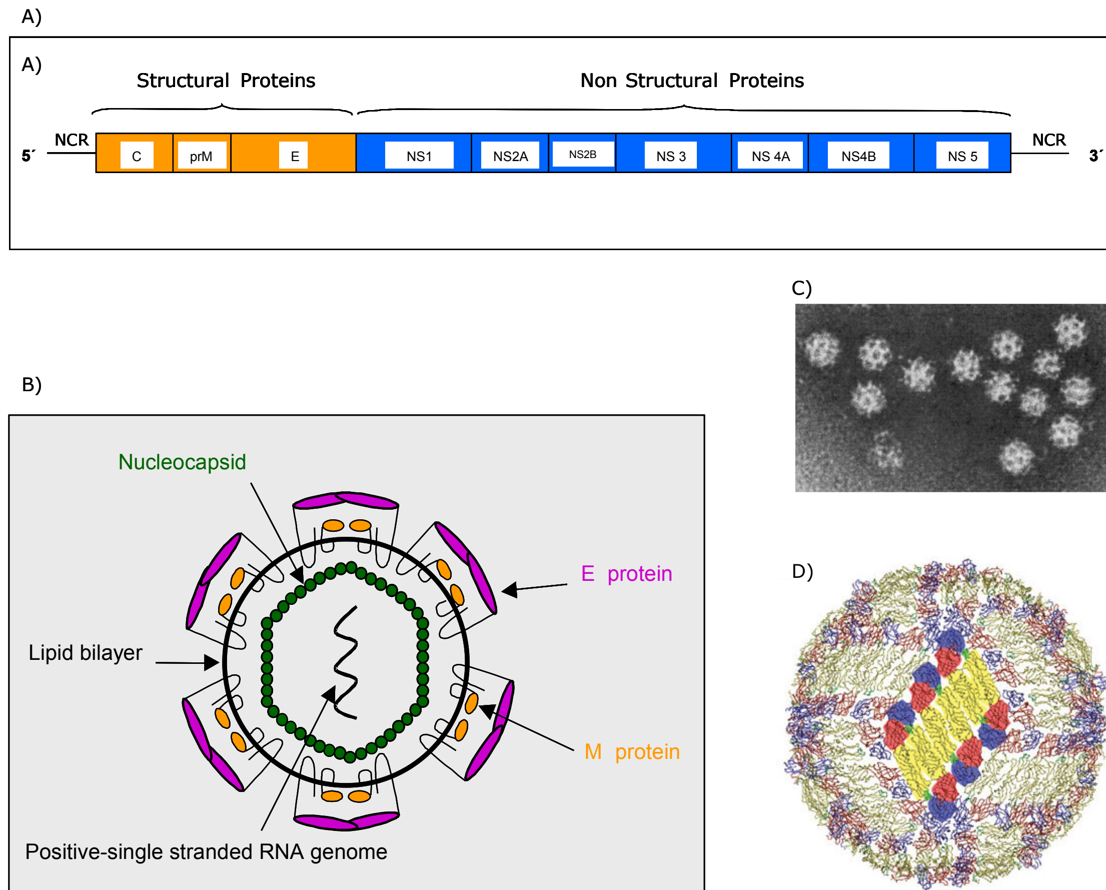

Fig 4: The flavivirus particle. A) Schematic representation of the positive single stranded RNA genome of YFV. B) Schema of infectious YFV particle. C) Electron micrograph of Dengue Virus. D) Model of crystallized mature Dengue virus; E homodimers are depicted. Domain I, II and III of each E monomer are coloured in red, yellow and blue, respectively. Adapted from (Lindenbach and Rice, 2003; Mukhopadhyay *et al*., 2005).

I-2-3-1. The Capsid protein C.

This protein of 11 kDa is a highly basic protein which forms the nucleocapsid of flaviviruses. Charged residues at its N and C termini are probably responsible for the interaction with the viral RNA genome whereas its internal hydrophobic domain mediates membrane association.

I-2-3-2. The Membrane protein M.

The protein M is the minor glycoprotein of flaviviruses which is initially produced in infected cells as its precursor prM (MW approx. 26 kDa). Upon expression, this precursor prM is rapidly folded to form heterodimers with the major envelope

protein E. Studies have shown that this protein may act as a chaperone-like protein ensuring the correct folding of the E protein. The prM protein is further cleaved into its mature form M by a furin-like enzyme in the secretory pathway just before the release of flavivirus particles.

I-2-3-3. The Envelope protein E.

The major envelope protein of flaviviruses, the E protein, is a 55 kDa type I membrane protein. This protein has been crystallized for TBEV (Rey *et al*., 1995) and for DV type 2 (Modis *et al*., 2004).This surface protein is divided into 3 domains and forms homodimers on the viral surface (Fig 4B and 4D). The E protein has been shown to govern virus attachment and to mediate virus entry. The E protein will be extensively described in the section I-3.

I-2-3-4. The Non Structural (NS) proteins.

The NS proteins are less extensively described than the structural proteins. The NS1 protein is found within infected cells, on the cell surface and as a secreted protein. The role of this glycoprotein remains unclear but may play a role in RNA replication. Interestingly, a strong humoral response following infection is developed against this protein and antibodies directed against the cell surface form of NS1 mediate complement-mediated cytotoxicity. The NS2A protein is a small hydrophobic protein which seems to be involved in RNA packaging and RNA replication The NS2B protein is a small membrane-associated protein which forms a complex with the NS3 protein. The NS2B-NS3 complex is involved in polyprotein processing and RNA replication The function of the NS4A and NS4B proteins is still unknown. NS5 is a large protein which plays a major role in RNA replication. This protein has been presumably identified as the polymerase of flaviviruses (for review see Lindenbach and Rice, 2001).

I-2-4. Pathogenesis.

Wild type YFV induces two different patterns of infection and injury in animal models: neurotropism and viscerotropism. In rodents (mice, hamsters and guinea pigs), the YFV is neurotropic (infection of the brain leading to encephalitis). In non-human primates, the virus is viscerotropic (infection of the liver, spleen, heart and kidneys) and leads to a disease which resembles the human disease (Monath and Barrett, 2003).

I-2-5. The Flavivirus life cycle.

Flaviviruses enter into the cell via the endocytosis-mediated pathway through the interaction of the E protein with its cellular receptor (Heinz *et al*., 2004). This cellular receptor has not been with certainty identified so far (see I-2-6). It has been shown that this interaction is driven by the IgG-like domain III of the E protein. Bound particles are then internalised via clathrin-coated vesicles. The release of the nucleocapsid and the genome is mediated through a fusion process with occurs in the endosomal compartment. This fusion process is an acid-dependent process, by which the low pH shift induces irreversible conformational changes of the E protein, leading to the exposure of the fusion peptide (cd loop), its anchoring into the cellular membrane and finally the fusion of both viral and cellular membranes. The capsid is then released into the cellular cytoplasm where viral replication takes place. The decapsidation process allows the release of the positive single-stranded RNA genome which is used as a template for the translation of viral proteins and for the transcription of negative single-stranded RNA molecules. Those molecules are then used as a template for the production of positive single-stranded RNA genomes, which are further encapsidated into new viral particles. Assembly of the virion progeny seems to take place in the ER lumen. The particles have a lipid envelope decorated with E proteins monomers bound to the M protein precursor prM and represent immature virions. These particles are then exported out of the cell via the Golgi network through the exocytosis pathway. Shortly before release, the prM protein is cleaved to M protein, leading to its dissociation with E monomer. The E monomers then dimerize yielding the infectious particle. The association prM-E has been shown to prevent irreversible conformational changes in the E protein induced by pH-shift which may occur during the transport of particles out of the cell. (For review see Heinz et Allison., 2003).

I-2-6. Cellular receptor(s) for Flaviviruses.

The domain III of the E protein is thought to mediate attachment of virions to their target cell through the binding to a cellular receptor. However, this cellular receptor has not been clearly identified yet. Several studies have shown that the dendritic-cell-specific ICAM-grabbing non-integrin protein (DC-SIGN), the glucose-regulating protein 78 (GRP78/BiP), CD14-associated molecules and more recently, the heat shock proteins HSP90 and HSP70 may act as cellular receptors for DV (Navarro-Sanchez *et al*., 2003), (Jindadamrongwech *et al*., 2004) (Chen *et al*., 1999) (Reyes-Del-Valle *et al*., 2005). Heparan sulfates and other glycosaminglycans have

also been proposed to be attachment receptors for flaviviruses (Chen *et al*., 1997; Hung *et al*., 1999; Kroschewski *et al*., 2003; Lin *et al*., 2002). Moreover, it has been shown for DV that the entry process differs between mammalian cells and mosquito cells (Hung *et al*., 2004). As flaviviruses are able to infect a high number of different cell lines, it seems that various receptors would be needed for flavivirus infection and that different flaviviruses could use different receptors. The presence of an additional coreceptor has been postulated to explain the restricted tropism of flaviviruses (Martinez-Barragan and del-Angel, 2001). However, the molecular mechanism by which flaviviruses enter into the cell is still obscure. For YFV and mosquito-borne viruses, a solvent-exposed loop (the Fg loop) in domain III of the E protein and especially the Arg-Gly-Asp (RGD) motif has been proposed to be a recognition motif for integrin-binding (Kuhn and Rossmann, 1995). Nevertheless, mutagenesis of this motif in YFV-17D did not alter the virus-cell binding, suggesting that integrins do not function as a major YFV 17D receptors via the RGD motif (van-der-Most *et al*., 1999). Recently, heparan sulfate has been proposed as a putative cellular receptor for YFV (Germi *et al*., 2002).

I-2-7. Flavivirus fusion with host cell membranes.

I-2-7-1. The fusion peptide.

The fusion peptide responsible for the fusion of viral and cell membranes during the flavivirus life cycle is located at the tip of domain II of the E protein. This fusion peptide is formed by a highly conserved hydrophobic loop previously called cd loop This peptide spans aa 98 to 110 of the E protein. Mutations in this region have been shown to affect fusion and virulence of TBEV (Allison *et al*., 2001).

I-2-7-2. The fusion mechanism.

The fusion mechanism by which flaviviruses enter into the cell has been recently identified as a new fusion process called the class II fusion process (Heinz *et al*., 2004; Modis *et al*., 2004). After endocytosis of flavivirus virions, the release of the genome into the cytoplasm of the infected cell is mediated through low pH shift in the endosomal compartment. Acidification of the endosomal compartment promotes irreversible rearrangements and conformational changes of the E protein on the virion surface, leading to the dissociation of the E homodimers into E monomers and then to E trimerization. These data have been obtained by crystallizing the pre- and post-fusion states of the E protein of TBEV and DV (Allison *et al*., 1995; Stiasny *et al*., 1996; Stiasny *et al*., 2001; Modis *et al*., 2004, Stiasny *et al*., 2004; Stiasny *et al*., 2005). A detailed mechanism for the membrane fusion process of flaviviruses

has been recently proposed (Fig.5) (Modis *et al*., 2004). Following pH shift in the endosomal compartment, E homodimers dissociate into monomers, resulting in the swing of the domain II towards the host cell membrane, the exposure of the buried fusion peptide located at the tip of the domain II, its anchoring into the host cell membrane and finally the formation of E homotrimers. Rearrangement of the domain III brings the viral membrane and the host cell membrane in close contact leading to fusion of both membranes.

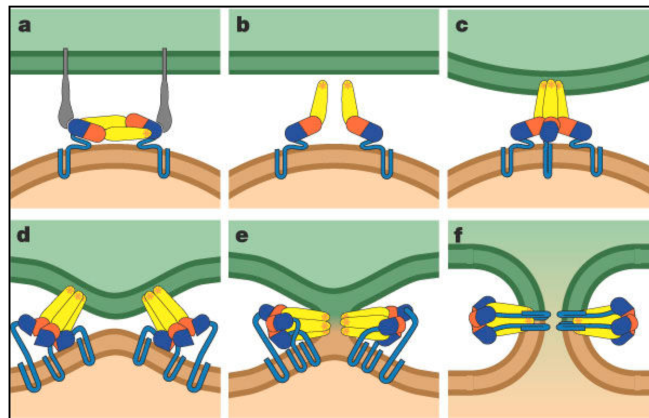

Fig. 5. Proposed fusion mechanism for flaviviruses. From (Modis *et al*., 2004).

I-3. The YFV envelope protein (the E protein).

The E protein is the major envelope glycoprotein of flaviviruses. It has been shown to mediate three crucial functions: attachment of the virus to the host cell through receptor binding, virus-cell membrane fusion upon pH shift and antibody-mediated neutralization.

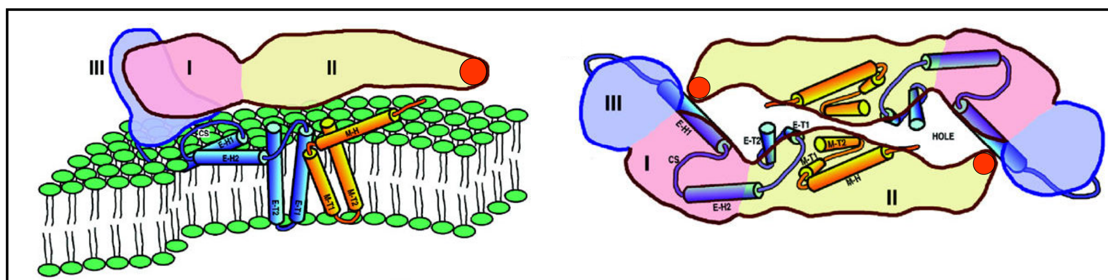

Fig 6: Schematic representation of E protein on the viral surface. The ectodomain, the stem region and the transmembrane domain of the E protein are depicted. Domains I, II and III of the ectodomain are coloured in lilac, yellow and blue, respectively. The red dot at the tip of the domainII represents the putative fusion peptide (cd loop). From (Mukhopadhyay *et al*., 2005).

I-3-1. Molecular Structure.

The molecular weight of the E protein is around 55 kDa and this protein is composed of 495 amino acids. Treatment of TBE or DV virions with trypsin promotes the release of a soluble fragment of the E protein, the ectodomain, which is composed of residues 1-395 of the E protein. The crystal structure of the ectodomain has been resolved at 2 Å resolution for TBEV and for DV type 2 (Rey *et al*., 1995) (Modis *et al*., 2004). This fragment is anchored into the viral membrane via a transmembrane domain (residues 451-495 for DV) which is composed of 2 transmembrane helices (Fig.6, E-T1 and E-T2). A stem region (residues 396-450 for DV) links both domains together. This stem region is presumably formed by two amphiphatic helices (Fig 6, E-H1 and E-H2) (Zhang *et al*., 2003). The E protein forms homodimers with an anti-parallel head-to-tail conformation on the surface of infectious flavivirus particles. The overall dimensions of the E dimer are approx. 150 Å x 55 Å x 30 Å. This dimer is oriented parallelly to the viral membrane and gently curved. Infectious virions contain 90 E dimers (180 E protein monomers) covering the entire viral surface and forming a smooth shell (Kuhn *et al*., 2002) (Fig.4D). The E protein is very conserved among all flaviviruses and shares a common architecture. Conservation of cysteine residues and similar hydrophobicity profiles suggest a common folded structure of the E protein for all flaviviruses. The E ectodomain is divided into 3 domains: a central domain (domain I), a dimerization domain (domain II) and an IgG-like domain (domain III). Domain I contains the N-terminal part of the E protein and is formed by a β-barrel. Domain II forms an elongated structure which promotes dimerization of two E monomers. At the tip of domain II is located a hydrophobic and glycin-rich sequence called cd loop, which has been proposed as the internal fusion peptide for flaviviruses (Allison *et al*., 2001). The domain III contains the C-terminal part of the E protein ectodomain and forms a typical IgG-like domain with an axis which is located perpendicularly to the axis of the virion surface. The domain III has been shown to govern virus attachment through its interaction with a cellular receptor (Bhardwaj *et al*., 2001; Chen *et al*., 1997; Crill and Roehrig, 2001; Hurrelbrink and McMinn, 2001; Mandl *et al*., 2000).

I-3-2. Antigenic structure.

Because of its preponderant role in virus attachment and cell fusion, the E protein is the major inductor of neutralizing antibodies.

I-3-2-1. Flavivirus E-specific neutralizing antibodies and neutralizing epitopes.

The E protein elicits neutralizing antibodies as well as non-neutralizing antibodies suggesting that neutralizing epitopes are restricted to some E protein areas. Moreover, E-specific flavivirus neutralizing antibodies can be either virus-specific of virus cross-reactive; the virus-specific ones having the highest neutralizing activity in vivo (Mandl *et al*., 1989; Roehrig, 2003). Several studies identified neutralizing epitopes for several flaviviruses by generating variants resistant to neutralization for a defined antibody. The Table 1 lists amino acid residues found to form neutralizing epitopes for DV, WNV, YFV, JEV and TBEV. The molecular location of these residues on the crystal structure of the DV E protein shows that flaviviruses neutralizing epitopes are distributed over the entire surface of the E protein, in the three domains. Moreover, most of neutralizing epitopes are located on the upper surface of the E protein and, thus, accessible to antibodies as expected (Fig 7). To date, epitope mapping of neutralizing flavivirus epitopes has been performed using mouse monoclonal antibodies with the exception of a recent paper using monoclonal antibodies generated from chimpanzees infected with DV (Goncalvez *et al*., 2004).

Virus	Antibody	Aa substitution	Domain	Reference
DV-2	mAb(10F2)	69, 71, 112 & 124 402	II III	(Lok *et al*., 2001)
DV-2	mAb(6B2)	311	III	(Lok *et al*., 2001)
DV-1	mAb(M-10)	279	I-II junction	(Beasley and Aaskov, 2001)
DV-1	mAb(M-17)	293	I-III junction	(Beasley and Aaskov, 2001)
DV-2	mAb(4G2)	169 & 275	I-II junction	(Serafin and Aaskov, 2001)
DV-3	mAb(1H9)	386	III	(Serafin and Aaskov, 2001)
DV-2	mAb(G8D11)	307	III	(Lin *et al*., 1994)
DV-2	mAb(6B2)	311	III	(Lok *et al*., 2001)
DV1-2	Fab 1A5 (monkey)	106 & 317	II & III	(Goncalvez *et al*., 2004)
DV-2	mAb(3H5)	383, 384 & 385	III	(Hiramatsu *et al*., 1996)
WNV	mAbs	306, 307, 330 & 332	III	(Sanchez *et al*., 2005)
WNV	mAb(5C5)	307	III	(Beasley and Barrett, 2002)

WNV	mab(5H10)	330	III	(Beasley and Barrett, 2002)
WNV	mAb(5H10)	332	III	(Beasley and Barrett, 2002)
WNV	mAb (E16)	306, 307, 330& 332	III	(Oliphant *et al*., 2005)
WNV	mAb	332	III	(Li *et al*., 2005)
YFV(17D)	mAb(2C9)	71	II	(Lobigs *et al*., 1987)
YFV(17D)	mAb(2E10)	71 & 72	II	(Lobigs *et al*., 1987)
YFV(17D)	mAb(B39)	155 & 158	I	(Ryman *et al*., 1997)
YFV(17D)	mAb(864)	305 & 325	III	(Ryman *et al*., 1997)
JEV	mAb (E3.3)	331 & 332	III	(Lin and Wu, 2003)
	mAb	52	I-II junction	(Morita *et al*., 2001)
	mAb	126	I-II junction	(Morita *et al*., 2001)
	mAb	136	I-II junction	(Morita *et al*., 2001)
	mAb	275	I-II junction	(Morita *et al*., 2001)
	mAb	270	II	(Cecilia and Gould, 1991)
	mAb	333	III	(Cecilia and Gould, 1991)
TBEV	mAb(IC3)	181	I	(Holzmann *et al*., 1989)
	mAb(IE3)	123	II	(Holzmann *et al*., 1989)
	mAb(IO3)	368	III	(Holzmann *et al*., 1989)
	mAb(A3)	71	II	(Mandl *et al*., 1989)
	mAb(A3)	67	II	(Mandl *et al*., 1989)
	mAb(A4)	233	II	(Mandl *et al*., 1989)
	mAb(A5)	207	II	(Mandl *et al*., 1989)
	mAb(i2)	171	I	(Mandl *et al*., 1989)
	mAb(B1)	389	III	(Mandl *et al*., 1989)
	mAb(B4)	384	III	(Mandl *et al*., 1989)

Table 1: Location of neutralizing epitopes identified on the E protein for several flaviviruses.

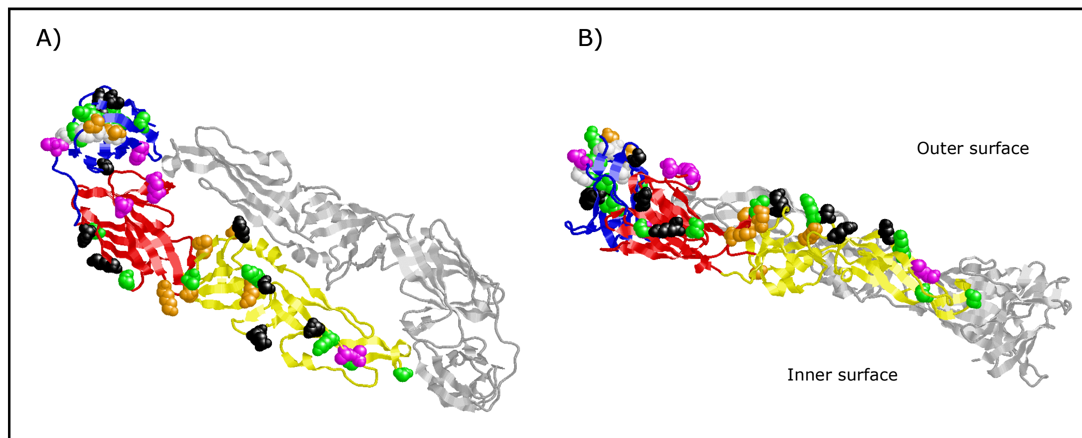

Fig 7: Molecular location of neutralizing epitopes for several flaviviruses on the crystal structure of the homodimer envelope glycoprotein E of DV. A) Top view. B) Side view. Domain I, II and III of one E monomer are coloured in red, yellow and blue, respectively. Neutralizing regions identified for DV, WNV, YFV, JEV and TBEV are colored in green, white, magenta, orange and black, respectively.

I-3-2-2. E protein cross-reactive epitopes for flaviviruses.

Human infection by flaviviruses elicits humoral immune response and the generation of virus-specific antibodies but also flavivirus cross-reactive antibodies. Analysis of these flavivirus cross-reactive antibodies using bioinformatical studies and mutagenesis allowed the identification of some residues (E-104, E106, E-107, E-126, E-226 and E-231) defining three distinct cross-reactive flavivirus epitopes. Three aa residues (E-104, E106, E-107), which define two of these three epitopes, were found to be located within the fusion peptide (Crill and Chang, 2004).

I-3-2-3. Neutralizing determinants on the E protein identified for YFV.

Six aa residues associated with mAb-neutralization escape variants have been identified in the E protein of the YFV 17D-204 vaccine strain. The first study was conducted by Lobigs et al. in 1987 (Lobigs *et al*., 1987). They identified aa E-71 and E-72 as critical residues for the neutralizing activity of the two cross-competing mAbs 2C9 and 2E10 and these aa were identified as components of the first discrete epitope characterized for YFV 17D-204. E-71 and E-72 are both located in domain II of the E protein (Fig.8, in cyan). A second neutralizing epitope was identified by Ryman et al. in the E protein of the YFV 17D-204 strain, formed by E-155 and E-158 and recognized by the mAb B39 (Ryman *et al*., 1997). These aa are located in domain I of the E protein (Fig 8, in green). A third neutralizing epitope has been identified also by Ryman et al. In this study, aa E-305 and E-325 were

found to be part of the neutralizing epitope which is recognized by the mAb 864 (Ryman *et al*., 1997). This epitope is located in domain III of the E protein (Fig 8, in orange).

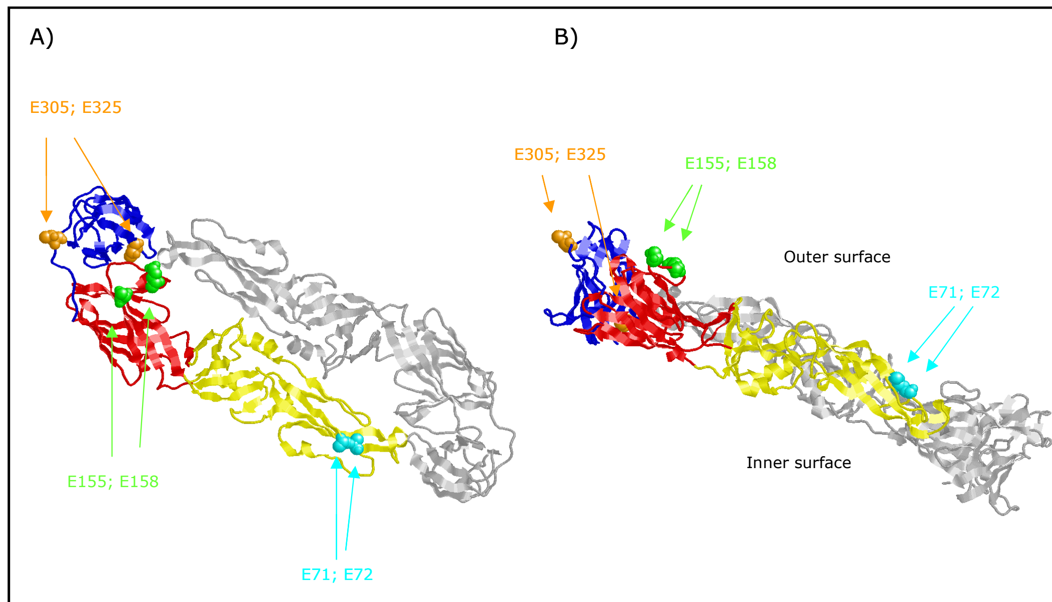

Fig 8: Neutralizing epitopes identified for the YFV and their projection to the E protein crystal structure of DV type 2. The sequence of the YFV was modeled onto the published crystal structure of the Dengue type 2 virus E protein. E-71 and E-72 (in cyan) were identified by Lobigs et al, 1987; E-155 and E-158 (in green) were identified by Ryman et al, 1997; E-305 and E-325 (in orange) were identified by Ryman et al, 1997. A) Top view. B) Side view.

I-4 The Yellow Fever vaccine.

The concomitant isolation of wild-type YF viruses in 1927 by the Pasteur Institute in Dakar, Senegal (the French viscerotropic strain) and by American researchers from the Rockefeller foundation in Ghana (Asibi strain) promoted the development of efforts to generate a live-attenuated YF vaccine by passaging the wild-type strain in a substrate that was restrictive for growth. In the absence of the invention of cell culture, the viruses were initially passaged in Asian non-human primates which are highly susceptible to visceral YFV infection.

I-4-1. The French Neurotropic Vaccine (FNV).

The French Viscerotropic Virus (FVV) was passaged in mouse brains leading to the isolation of an adapted virus without any viscerotropism for monkeys: the French Neurotropic virus (FNV) which could be used as a vaccine. Despite some studies

showing that this FNV vaccine could cause in some instances lethal encephalitis in monkeys, it was widely used for immunizations in French Equatorial Africa starting in 1932. By 1947, around 14 millions, and by 1953, 56 million people were vaccinated with the FNV vaccine. The incidence of FNV-associated encephalitis was estimated around 1:3000 to 1:10 000, which was shown later to be a gross underestimation. During the 60’s, studies on adverse effects associated with FNV underlined that the safety of this strain had not been evaluated carefully and led to a restricted use of this vaccine (to persons over 14 years of age). The production of the FNV vaccine was finally stopped in 1982 (for review see Monath, 2004).

I-4-2. The 17D vaccine.

Empiric passages of the wild-type Asibi strain in 1933 started the history of the 17D vaccine, which is still manufactured and commercialized around the world. Theiler and Smith from the Rockefeller Institute, New York, serially passaged the wild-type Asibi strain in mouse brain and later in embryonic chicken tissue to generate a YFV variant exhibiting loss of viscerotropism and neurotropism for humans. After 18 passages in mice embryos tissues, the new-adapted virus was cultured 74 times in chicken embryos without brain and nervous tissues to avoid a neuroadaptation to take place as observed for the FNV. At passage 176, the obtained YFV variant was tested in monkeys and was found to provide protective immunity without any viscerotropism or neurovirulence. This attenuated strain was called 17D strain. In 1936, the first human trials using this virus were conducted on volunteers. From passage 195, the initial lineage of the 17D vaccine split into 2 different ones. The virus was sent to Brazil, further passaged in chicken embryo tissue and embryonated eggs leading to the generation of the YFV 17DD strain. This strain strain is manufactured and commercialized in Brazil as the 17DD vaccine strain. One 17DD seed was also transferred to Senegal where 17DD vaccines are also still manufactured. The 17D virus was passaged independently at the Rockefeller Institute in parallel subcultures until passage 204. At this time, studies underlined the importance of controlling passage level and virus substrain and led to the establishment of a seed lot system. Primary and secondary seed lots were prepared from the substrain 17D-204. The latter was used to produce multiple vaccine batches by propagating the virus in embryonated eggs. All current 17D-204 vaccines derive from the original 17D–204 strain. With 400 millions doses distributed worldwide, the 17D vaccine is one of the safest and robust live attenuated vaccine (for review see Monath, 2004).

I-4-2-1. Molecular determinants of the YFV-17D vaccine strain attenuation.

To define more precisely mutations which are responsible for the attenuated phenotype of the 17D vaccine, the vaccine strains (17DD and 17D-204-ATCC) and the wild-type Asibi strain were sequenced and compared (Hahn *et al*., 1987). These analysis revealed 20 amino acid changes out of 3411 (0,59%) and 4 nucleotide differences in the 3´NCR. These changes are not distributed homogeneously all over the genome. Many of these substitutions (8/20) are found in the E protein suggesting a preponderant role of these changes in the YFV attenuation process. Nevertheless, it is clear that attenuation and virulence are multigenic involving structural and non structural proteins and, thus, cannot be linked to a specific mutation.

I-4-2-2. Quasispecies nature of the 17D vaccine strain.

Due to uncontrolled repeated passages of the wild-type strain by different manufactures, the nature of the 17D vaccines which are now produced around the world has been shown to be heterogeneous. It is now well established that all 17D vaccine substrains are genetically different and even each 17D-204 batch consists of an heterogeneous mixture of virion sub-populations which defines the quasispecies nature of the 17D vaccine strain. Such variants were found to differ in plaque morphology, mouse virulence, oligonucleotide fingerprinting patterns and antigenicity (Barrett, 1997; Barrett and Gould, 1986; Ledger *et al*., 1992; Liprandi, 1981). The quasispecies nature of the 17D vaccine strain has been illustrated with the ARILVAX vaccine (Pugachev *et al*., 2002). This vaccine was derived from the 17D-204 strain and is produced and commercialized in the United Kingdom by Evans Vaccines. Analysis of one ARILVAX vaccine vial revealed a heterogeneity of 12 nucleotides within viruses from the same vial. Two nucleotides changes leading to amino acid substitutions at position E-240 and NS3-195 were observed. Interestingly, E-240 has already been shown to play a role in YFV neurovirulence in mice (Ryman *et al*., 1998). This aa residue has also been shown to be located within a T-helper cell epitope for Murray Valley encephalitis virus (Mathews *et al*., 1991). The NS3-195 has been described as a critical residue for ATPase activity of the NS3 protein for DV (Li *et al*., 1999). These observations available for the ARILVAX vaccine are probably true for all 17D strain-derived commercialized vaccines. Such a result definitely raises the question whether some variants with higher pathogenicity could, in some instances, be selected and may have an role in vaccine effectiveness and safety.

I-4-2-3. YFV 17D vaccine production.

Currently, the 17D vaccine (17DD and 17D-204) is produced by ten manufacturers in the USA, Brazil, France, United Kingdom, France, India, Colombia, Russia, Senegal and Switzerland (previously Germany). Approx. 55 millions doses are manufactured annually (Monath, 2004). This vaccine is still produced by inoculation into viable embryonated eggs from specific pathogen free (SPF) chickens. This old manufacturing method is a critical factor which strongly impairs the attempts to control the disease by limiting the vaccine production capacity. In 1997, only 4 millions doses were distributed in Africa, whereas 24 millions doses would be required to protect 80% of African children and 240 million doses to start preventive mass campaigns (Monath, 2004). This restricted vaccine production is also a major drawback in case of high demand especially when YF outbreaks occur. Vaccine shortage has been already experienced in Guinea during a YF outbreak in 2000 (Nathan *et al*., 2001). Thus, a novel vaccine production method is strongly needed. Using an infectious clone from a YFV 17D-204 strain-derived cDNA, Marchevsky et al. reported that the progeny virus exhibited comparable features to viruses from the vaccine strain but showed a slightly higher neurovirulence activity in monkeys (Marchevsky *et al*., 1995). Nevertheless, the cell culture-based vaccine production made from a YFV infectious clone may provide an efficient alternative to the present embryonated eggs-based method. Therefore, the knowledge of neutralizing epitopes is important for the standardization of a cDNA-based vaccine.

I-5 Immune Response to YFV.

The human antibody response following YFV-infection has to date not been characterized on the molecular level.

I-5-1. Innate immune response.

Studies on 17D vaccinees have described the activation of natural killer cells (NK cells) and the development of a type I interferon response shortly after virus inoculation during the early phase of viral replication (Bonnevie-Nielsen *et al*., 1995; Fagraeus *et al*., 1982) (Wheelock and Sibley, 1965). In addition, levels of several cytokines and T-cell activation markers were shown to increase after 17D vaccination (Reinhardt *et al*., 1998). Because one in seven wild-type YFV-infected persons develop illness, it is clear that the innate immune response may fail to protect the host. Nevertheless, innate immune responses are believed to be

important for viral haemorragic fevers by delaying the viral replication until the development of the adaptive immune response (Sabin, 1952). On other hand, a study on cytokine/chemokine responses in YF patients has recently shown that a strong activation of these molecules is associated with poor outcome of the patients (ter-Meulen *et al*., 2004).

I-5-2. Adaptive immune response.

YFV wild-type and 17D vaccine infections are followed by a rapid and specific immune response. The humoral immune response has been well described, whereas few studies have performed on the cellular immune response. The E protein has been shown to be the major determinant in terms of neutralizing antibodies induction and cytotoxic T-cell response, and thus, plays a major role in protective immunity.

I-5-2-1. Humoral immune response**.**

Neutralizing antibodies appear 7-8 days after YFV infection or 17D vaccination (Monath and Barrett, 2003; Reinhardt *et al*., 1998). The appearance of IgM neutralizing antibodies following wild-type YFV infection has been shown to occur during the first week of illness and to decline 30 to 60 days after onset (Lhuillier and Sarthou, 1983; Lhuillier *et al*., 1986). IgG antibodies mainly against the E protein appear 10 days post infection The neutralizing antibody peak is observed around 30 to 40 days after infection and slowly declines after 6 months (Monath and Barrett, 2003; Reinhardt *et al*., 1998). Nevertheless, neutralizing antibodies have been shown to persist for many years and have been documented as long as 78 years post illness (Monath, 2001). They provide a complete protection against disease on re-exposure to the virus. Most of the neutralizing antibody specificities which are produced following YFV infection and provide protection have been shown to be directed against the YFV-E protein (Brandriss *et al*., 1986; Gould *et al*., 1986; Pincus *et al*., 1992). However, several studies have shown that non structural proteins, especially NS1, may play a role in YFV humoral immune responses. Passive transfer of anti-NS1 antibodies confered protection after challenge in mice (Gould *et al*., 1986; Schlesinger *et al*., 1986). Mice and monkeys immunized with NS1 were also protected following challenge by developing complement-mediated cytolytic antibodies (Gould *et al*., 1986; Putnak and Schlesinger, 1990; Schlesinger *et al*., 1986). However, the in vivo relevance of those anti-NS1 antibodies in recovery or protection remains uncertain.

I-5-2-2. Cellular immune response.

There are no data on human cellular response following wild-type YFV infection. A few studies have shown, in 17D vaccinees, an increase of $CD8^+$ T cells in the early period of vaccination and lasting up to 13 days post vaccination (Reinhardt *et al*., 1998). For 17D vaccinees, $CD8^+$ T-cell epitopes were found in E, NS1, NS2B and NS3 proteins (Co *et al*., 2002). In mice, studies have shown that $CD4^+$ cells are critical mediators to protect against YF-encephalitis (Liu and Chambers, 2001). It is clear that helper and cytotoxic T lymphocytes play an important role in viral clearance during primary infection but further studies have to be performed to define the cellular immune response following YFV infection in humans.

I-6. Adverse effects following YF 17D vaccination.

Although the YFV-17D vaccine is known to be one of the safest vaccine with more than 400 millions doses given to humans around the world, several published data showed that this vaccine is responsible for a non-negligible number of side effects leading in some instances to the death of the vaccinated patient. The mechanism of these events are not completely understood but could be due to the quasispecies nature of the 17D vaccine lots and to currently unknown host factors. It has been also shown that advanced age of vaccinated travelers (more than 60 years old) is a risk factor to develop such serious adverse effects (Khromava *et al*., 2005). Adverse effects following vaccination with the 17D yellow fever vaccine are nowadays considered as an important problem associated with immunization coverage, especially of travelers, and has been repeatedly described since 1996. The risk of fatal side effects after 17DD-YFV vaccination has been calculated as 0.043 to 2.131 fatalities per million doses administered (Struchiner *et al*., 2004). These side effects are generally categorized as two groups related to the dual tropism of the YFV: the yellow fever-associated neurotropic disease (YEL-AND) and the yellow fever-associated viscerotropic disease (YEL-AVD). The increasing recognition of YF vaccination-related side effects, since the introduction of improved surveillance in the late 90´s, strongly suggests that the number of cases associated with adverse affects has been probably underestimated and thus, an increase of the number of such cases might be observed in the next years.

I-6-1. The YELlow fever-Associated Neurotropic Disease (YEL-AND).

The total number of reported YEL-AND cases has been evaluated to be 25, of which 15 occurred during the 50´s when the vaccine was used without any age restriction

(Monath, 2004). Of those 25 reported cases, 1 was fatal for a 3 year-old boy in 1965. The study of this case in 1994 showed that the variant found in cerebral fluid of the boy differed from the parental 17D-204 virus by two amino acid residues at position 155 and 303 in the E protein (Jennings *et al*., 1994). Nevertheless, it has not been formally evaluated whether such a variant could be the result of a selection process or if mutations occurred *de novo*.

I-6-2. The YELlow fever-Associated Viscerotropic Disease (YEL-AVD).

14 YEL-AVD have been reported worldwide (10 in the last decade), out of which 8 were fatal. The first report described YEL-AVD which occurred in four elderly travelers in USA in 1998, of which three died. Viruses isolated from fluids of two patients did not differ from the parental YFV 17D-204 strain. From one case, sequencing analysis revealed nucleotide differences leading to amino acid substitutions at position M-6 and E-326, when compared to the parental YF 17D-204 virus used for vaccination (Martin *et al*., 2001). The second study reported the death of two persons following 17DD vaccination in Brazil. Both patients died from viscerotropic disease within 1 week. 17DD virus was isolated from fluids and sequencing analysis showed that both viruses, 17DD from patients and 17DD from the vaccine lot, were identical and did not induce disease in monkeys, pointing to a possible role of host factors (Vasconcelos *et al*., 2001). The third study reported the death of a man after YFV 17D-204 vaccination in Australia. Partial sequencing analysis of the virus showed that both viruses (from fluids and from the vaccine lot) were identical. However, the E gene has not been sequenced so far (Chan *et al*., 2001). Thus, it cannot be excluded that a selected variant was responsible for the reported adverse effects. Recently, a fatal YEL-AVD event has been reported in 2004 in Spain following 17D-204 vaccination (Eurosurveillance, 2004). Sequencing analysis does not reveal any mutation in the virus isolated from fluids (M.Niedrig, personal communication). The last reported fatal case occurred in USA but no further informations about the nature of the virus are available yet (Gerasimon and Lowry, 2005).

I-7. Antibodies.

Antibodies or immunoglobulins (Ig) are soluble glycoproteins whose function is the elimination of foreign antigens like infectious microbes. They represent one of the main effectors of the humoral immunity. Their function is based on a specific and physical interaction with the antigen. The diversity of antigenic determinants which can be recognized by the human antibody repertoire is estimated to be 10^7 to 10^9.

I-7-1. Structure of antibodies.

Antibodies are either produced in a membrane-bound form by B-lymphocytes or as secreted molecules by antigen-stimulated B cells (Plasma cells). The membrane-bound form (mainly IgM) mediates the activation of B cells through the interaction with the antigen. The secreted form (IgM, IgG, IgA and IgE) bind the antigen and trigger several effector mechanisms leading to its elimination.
A secreted IgG antibody molecule has a symmetric core structure composed of two identical heavy chains and two identical light chains (Fig.9). The structure is stably maintained by disulfide bridges and non-covalent links. Light chains can be of two different isotypes: the κ isotype or the λ isotype. However, an antibody molecule is always composed of 2 light chains from the same isotype. Each chain is divided into constant domains and variable domains, according to their amino acid sequence variability. The heavy chain is divided into three constant domains (CH1, CH2 and CH3) and one variable domain (VH). The light chain is divided into one constant domain (CL) and one variable domain (VL). The VH and VL domains, named Fragment variable (Fv), form the antigen-binding site and, thus, participate in the antigen recognition function of antibodies. The CH domains (CH2 and CH3) form the Fc receptor of antibodies which mediates effector functions. Due to this symmetric structure, two antigen-binding sites (VH-VL) are present for each IgG molecule and, thus, can bind two antigens. Such a flexibility is conferred by a hinge region located between domains CH1 and CH2. The VH and VL domains can be divided into seven regions: four relatively conserved regions named Framework Regions (FR1, FR2, FR3 and FR4) and three hypervariable regions named Complementary Determining Regions (CDR1, CDR2 and CDR3). Antigen recognition and binding by antibodies is mainly mediated by these hypervariable CDR regions. As shown in Fig.10, CDR loops from both chains (VH ad VL) form a pocket to which the antigen docks. This physical property of the CDR regions explains why they are found to be the most important part of antibodies in terms of antigen specificity. In addition, it has been shown that the VH domains play a more important role in specificity than the VL domains and that the VH-CDR3 is one of the major determinant in terms of antigen specificity (Kabat and Wu, 1991).

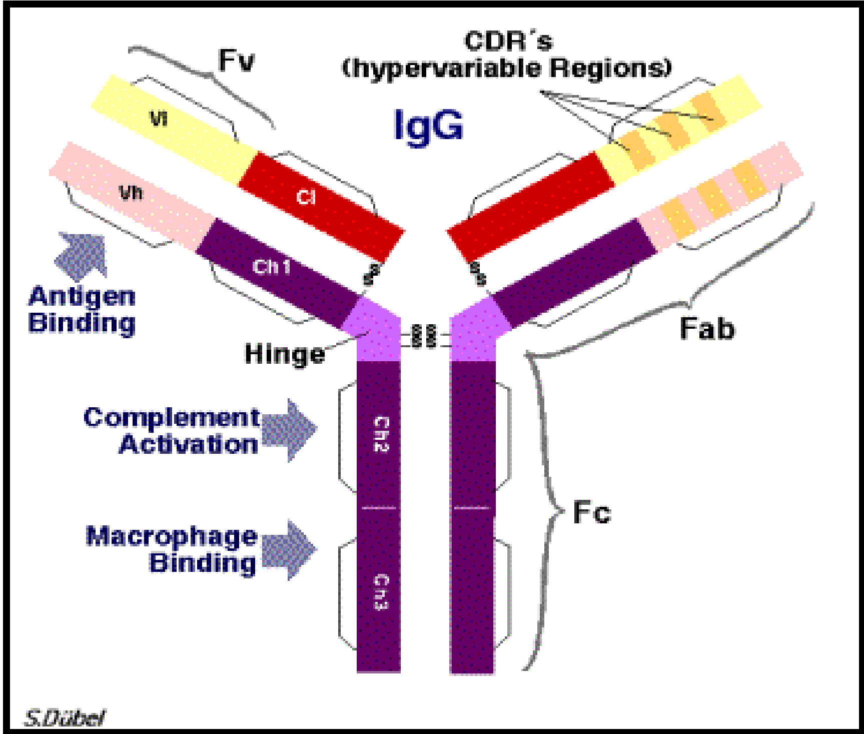

Fig 9: Schematic drawing of an IgG immunoglobulin. From (Kontermann and Duebel, 2001).

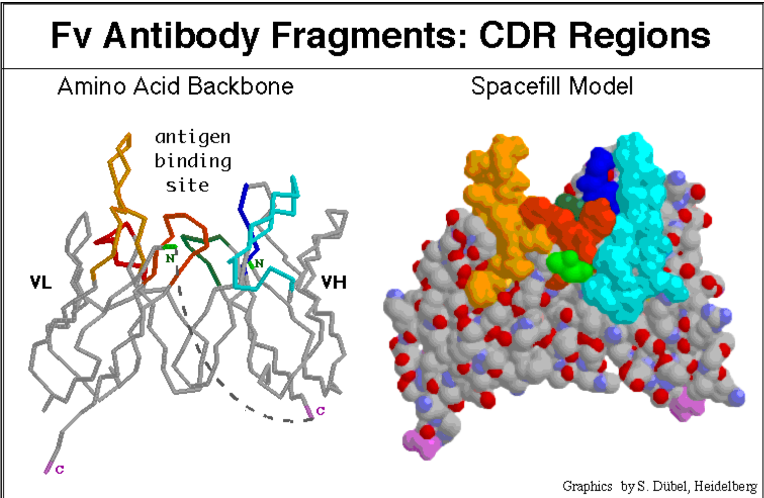

Fig 10. Antigen-binding site and CDR regions. VH, VL and are indicated. CDR1, CDR2 and CDR3 of the VL fragment are indicated in red, light green and orange, respectively. CDR1, CDR2 and CDR3 of the VH fragment are indicated in cyan, dark green and blue, respectively. From (Kontermann and Duebel, 2001).

I-7-2. Germline organization of the genetic loci of antibodies.

The Ig heavy chains, the Ig lambda light chains and the Ig kappa light chains are encoded by three different loci which are located on different chromosomes (in humans, chromosome 14, chromosome 22 and chromosome 2, respectively). The genetic locus encoding for the heavy chain is composed of four genes: V, D and J segments which encode for the VH domain and the C segment which encodes for the CH1, CH2 and CH3 domains of the heavy chain. The genetic locus encoding for the light chain is composed of only two segments V and J, forming the VL domain and the C segment, forming the CL domain. All these segments are not present in one copy but in a cluster of different segments (Vn, Dn, Jn, Cn in Fig.11).

I-7-3. Somatic recombination, affinity maturation and isotype switching in antibody diversity.

The germline organization of antibody loci described previously is present in all cell types. However, membrane-bound or secreted antibodies are produced only in B-cells, through DNA rearrangements which occur in developing B-cells. This process is called somatic recombination. The huge diversity of the antibody repertoire is mainly driven by the somatic recombination process via two distinct mechanisms: the combinatorial diversity and the junctional diversity. The somatic recombination promotes the selection and the physical assembly of one V segment, one D segment (for heavy chain) and one J gene from all DNA segments in each developing lymphocyte to form a VDJ gene (for the heavy chain) or VJ gene (for the light chain). This mechanism is mediated through enzymes called V(D)J recombinases (mainly RAG-1 and RAG-2 complex) which introduce double-stranded breaks in the DNA and rearrangements, resulting in the juxtaposition of V(D)J segments, ("V-J, D-J and V-D-J rearrangements" in Fig.11). The transcriptional processing leads to the production of transcripts containing a V(D)J sequence encoding for the variable domain of the chain and a C domain encoding for the constant domain of the chain. The random combination of germline V, D, J segments among the multiple copies present in Ig loci defines the combinatorial diversity.

The junctional diversity enhances the size of the antibody repertoire by promoting modifications within one germline combination. At the time of V-J, D-J and V-D-J joinings, nucleotides are added (TdT-mediated process) or removed (nuclease-mediated process) at the junction sites. The sites of V(D)J junctions exhibit the

greatest variability and form the CDR3 regions which, as mentioned above, are the critical determinants for antigen specificity.

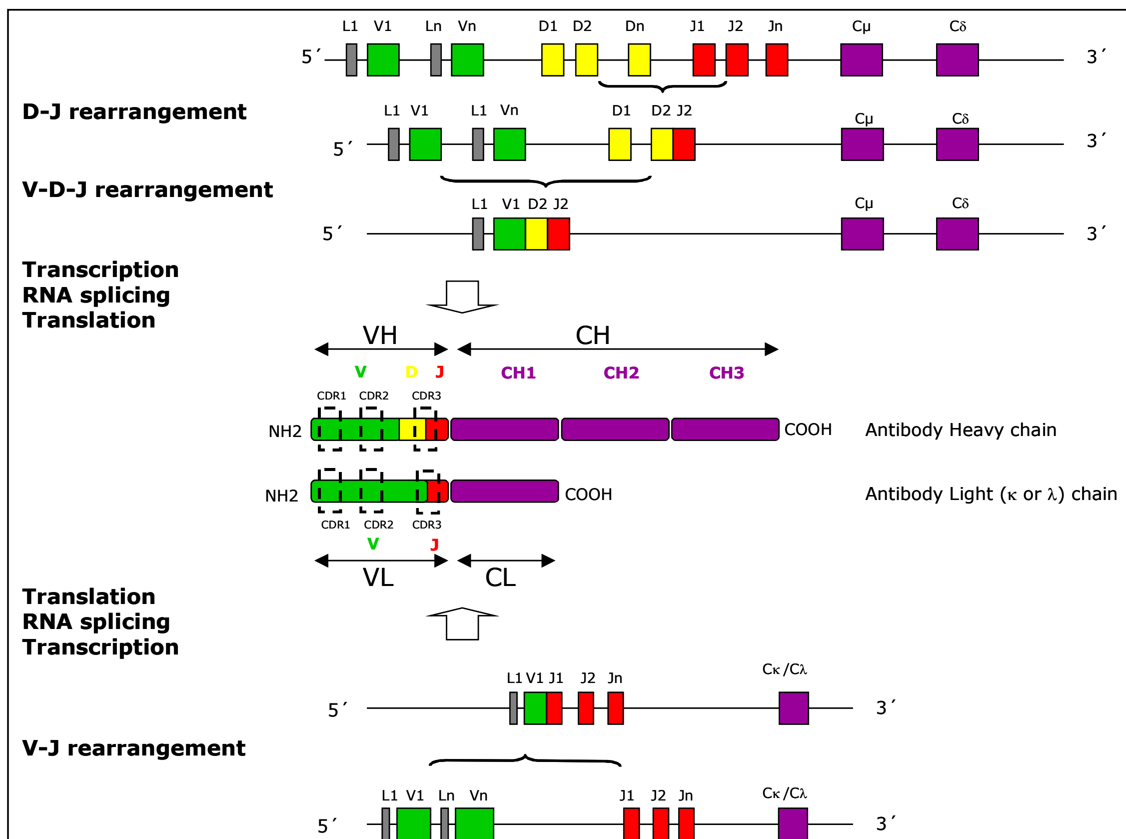

Fig 11: Schematic representation of the somatic DNA recombination process. Adapted from (Abbas AK, 2003)

The third mechanism which governs antibody diversity is called affinity maturation. Whereas combinatorial and junctional diversity mechanism take place during the lymphocyte maturation, the affinity maturation occurs following the development of T-dependent humoral responses. The capacity of antibodies to neutralize foreign elements depends on high affinity and high avidity interactions. The affinity maturation process promotes the production of high-affinity antibodies against one particular antigen. This process is dependent upon the activation of helper T cells and occurs in the germinal center of B cells. The V genes of the activated B-cells undergo point mutations at a high rate, probably after repeated stimulations by helper T cells. The precise molecular mechanism of this process is still unknown. Those V gene mutations will allow the generation of different B cell clones able to produce antibodies with higher affinity for the antigen. Since the mutations occur randomly within the V genes, this mechanism may generate antibodies with a

decreased affinity or even with a loss of affinity for the antigen. Nevertheless, only B-cells which produce high-affinity antibodies will be selected for survival.
Another mechanism, which is dependent upon helper T cells activation and which contributes to antibody diversity, is called heavy chain isotype switching or class switching. In response to signals from helper T cells, activated B-cells undergo a process of heavy chain isotype switching, resulting in the production of antibodies with heavy chains of different classes. Its molecular mechanism is a process called switch recombination, in which CH genes encoding for the constant domain of antibodies are rearranged in a similar way to VDJ rearrangements. In the absence of helper T cells signal, plasma cells produce IgM. The encounter of helper T cells stimuli by activated B-cells promotes switching to other isotypes (IgG, IgA or IgE). The switch to different classes of antibodies which distinct effector functions allows the development of adapted responses against different types of infectious agents.

I-7-4. Antiviral antibodies and their mechanism of action.

The major role of antibodies is to bind foreign elements leading to their elimination from the organism. Antibodies promote their role via several effector functions. The recognition and the binding of infectious microbes like viruses by the variable regions of antibodies promote the physical neutralization of the infectious agent. This process is called neutralization, resulting in the inability for the virus to infect its target cell. The most effective neutralizing antibodies belong to the IgG isotype. Such virus-antibody complexes can be recognized by phagocytes through the interaction between the Fc portion of IgG antibodies and the Fc receptors present on the surface of phagocytes. This process of coating particles for phagocytosis is called Fc-receptor-dependent phagocytosis or opsonization and leads to the phagocytosis and the destruction of the virus. Antibody–coated infected cells are recognized via the Fc part of antibodies by NK cells, which in turn destroy the infected cell. This process is called antibody-dependent cell-mediated cytotoxicity (ADCC). The antigen-antibody complexes are also recognized by soluble molecules of the complement system, promoting phagocytosis.

I-7-5. Monoclonal antibodies and biological applications.

Monoclonal antibodies are molecules which are specific for a particular antigenic determinant. They play a profound role in molecular diagnosis, therapeutical applications and in fundamental research. In the last forty years, efforts have been developed to produce monoclonal antibodies, which could be used for such

purposes. In 1975, Koehler and Milstein revolutionized the field by developing the hybridoma technology, which allows the production of monoclonal antibodies (mAbs) in virtually unlimited quantities. They immortalized antibody-secreting B cells from immunized mice by fusing them with a myeloma cell line, resulting a cell line called hybridoma. Such cell lines retain the immortality of the myeloma cell line and the specific antibody production of the B cell. The major drawback of this method is that such monoclonal antibodies are not human and could generate difficulties when use as therapeutical tools in humans. Attempts to produce human mAbs were performed but were never really successful due to the lack of a suitable human myeloma cell line, to the instability of the hybridoma when fused to a murine myeloma cell line or to low quantity when immortalized following Epstein Barr virus transformation. An alternative is the "chimerization" of those murine mAbs by replacing the murine constant regions by human ones. Those chimeric antibodies display the same specificity for the antigen through the murine V domains but possess human effector functions of the Fc regions which are not recognized as foreign in humans. Another approach is the "humanization" of mouse mAbs by grafting the murine CDRs domains of the mAb to a whole human antibody. All these methods have been successfully used to produce mAbs in large quantity which can be used in therapeutical approaches in human clinical cases. Nevertheless, these methods can be laborious, expensive and the screening of such hybridomas can be limited. Genetic-engineering of antibodies were developed in the last decade and provides an alternative approach to the hybridoma technique. One of the most effective system is the phage display technology.

I-8. Phage Display technology.

Phage display is a very powerful technique to obtain libraries containing millions or even billions of different peptides or proteins. This technology has been successfully used to study protein-ligand interactions, to improve affinity of proteins for their binding partners or to isolate antibodies specific for antigens (Azzazy and Highsmith, 2002).

I-8-1. Principle.

The history of the phage display technology started in 1985 when G. Smith showed for the first time that it was possible to express and display foreign proteins on the surface of filamentous bacteriophages (M13 or fd) (Smith, 1985). This could be done just by fusing, within the phage genome, the nucleotide sequence of the protein of interest to the gene encoding a phage coat protein (typically pIII). Upon

expression, the fusion protein (foreign protein-phage coat protein) is incorporated and displayed on the surface of the recombinant phage particles. Such phage display libraries can then be easily screened against one given antigen to isolate proteins with specific functions. The two major advantages of this rather simple but powerful method based on protein-protein interactions are that specific ligands can be selected from a vast number of different sequences by a simple enrichment method. Moreover, the structural and functional information of the protein of interest displayed on the phage surface is physically linked to its genetic information which is contained in the phage genome.

I-8-2. Filamentous phages.

Bacteriophages (or also called phages) are viruses which infect a variety of gram-negative bacteria using F pili as receptors. Filamentous phages (M13, fd or f1 strains) are a group of bacteriophages with a tube-like shape. Those phages are about 1 µm long and 6 nm in diameter. These particles are mainly composed of the major phage coat proteins pVIII which are helically-arranged. Around 2700 copies of pVIII form a wild-type filamentous phage virion. Three to five copies of another phage coat protein, the pIII protein are found at one tip of the particle. This protein is responsible for the attachment of the phage to the bacterial pili and, thus, mediates the infection of the bacteria. The filamentous phage genome is composed of a 6.4 kb single-stranded DNA consisting of 11 genes. The process of infection is initiated by the attachment of the phage coat protein pIII to the bacterial F pili. This promotes the dissociation of the phage coat proteins on the bacterial surface allowing the ssDNA phage genome to enter into the cytoplasm of the bacteria. A complementary DNA strand is then synthesized by the host DNA replication machinery, resulting a double-stranded replicative form (RF). This RF serves as a template for the generation of new single-stranded DNA genomes as well as for the production of phage proteins. The particles are then assembled in the cell membrane and phages are released into the extracellular compartment through an extrusion process. Filamentous phages are non-lytic bacteriophages; phages are secreted continuously without killing the host (for review see Russel, 1991).

I-8-3. Phage display system.

First attempts to generate recombinant phages displaying foreign proteins on their surface were done by cloning the DNA sequence of the foreign protein in frame with the gene encoding the pIII or pVIII protein directly into the phage genome (mainly

by using the pIII gene, because only very small peptides can be fused to the pVIII protein without altering the phage properties). Nevertheless, such a system was found not to be very effective due to instability during assembly and the low transformation efficiency. To circumvent these problems, a new generation of vectors called phagemids was designed and a system of phage rescue was established (Mead and Kemper, 1988).

I-8-3-1. Phagemids.

Phagemids are double-stranded DNA hybrid molecules from the phage genome and a plasmid vector. The phagemid pHEN3 we used in this work is depicted in Fig.15. It is composed of a plasmid backbone bearing a colE1 ori enabling the phagemid to replicate in the *E.coli* host cell, a M13 ori allowing the phagemid to be packaged into recombinant phage particles, an ampicillin resistance gene for selection of phagemid-containing colonies, a peptide leader pelB promoting the secretion of the fusion protein in the periplasm of the host cells, the LacZ promoter which allows the inducible expression of the fusion protein "protein of interest-pIII coat protein" (its expression is inhibited by glucose and promoted by IPTG) and the gene encoding for the pIII protein of the M13 phage. Restriction sites are also found for cloning of the protein of interest into the phagemid. Two tags (His-tag and Myc-tag) are found on the C-terminal part of the pIII protein for detection of the fusion protein. However, the phagemid lacks all other structural and non structural proteins which are required for a complete phage particle.

I-8-3-2. Helper phages.

Those structural and non structural proteins can be supplied *in trans* by infecting phagemid-transformed bacteria with helper phages. This process is called "phage rescue". Helper phages (such as M13KO7 or VCSM13) are almost identical to wild-type phages except that their genome contains a mutated origin of replication. As a result, this ori is slightly defective for replication, resulting in a lower encapsidation capacity. Thus, phagemids are preferentially packaged into the virion progeny. Since helper phages provide all proteins including wild-type pIII protein, a competition takes place between the phagemid fusion protein pIII and the helper phage wild-type pIII for incorporation into recombinant phages. The resulting phage population consists of recombinant phages displaying on their surface between none and five copies of the fusion protein encoded by the phagemid. Studies have shown that the ratio of pIII-fusion protein vs wild-type pIII in a rescue phage population varies between 1:9 to 1:1000, but typically the majority of recombinant phages bear only a single copy of the fusion protein (Azzazy and

Highsmith, 2002). To circumvent such a limitation, recent studies are in progress to limit the expression of the wild-type pIII. Nevertheless, many articles have shown that this system is extremely powerful and suitable to isolate diverse ligands. One of the most important applications of the phage display technology is the isolation of recombinant antibody fragments against a specific antigen.

I-8-4. Phage-displayed scFv libraries.

The phage display technology provides an easy and efficient tool to access the antibody repertoire by generating phages displaying on their surface recombinant antibodies. (Hoogenboom, 2002), (Azzazy and Highsmith, 2002)

I-8-4-1. scFV antibody fragment.

Due to the structural complexity of an antibody molecule, it is not possible to generate recombinant phages displaying entire antibodies. However, as mentioned above, Fv fragments, which are composed of variable domains of light chains (VL) and heavy chains (VH) of antibodies, are responsible for antigen binding and, thus, determine the antigen specificity of antibodies. For this reason and due to the small size of such fragments, recombinant phages expressing and displaying a recombinant version of Fv fragments called scFv were generated. scFv (single-chain Fragment variable) are synthetic molecules corresponding to the Fv fragments in which VL an VH domains are joined together with a flexible peptide linker. This linker which is composed of a stretch of Glycine and Serine ($(Gly_4Ser)_3$) stabilizes the structure and ensures the formation of the VH and VL antigen-binding site.

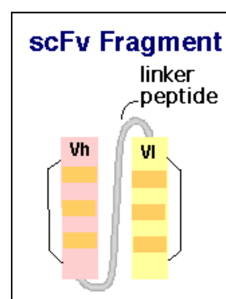

Fig 12: Schematic drawing of a scFv molecule. Vh: variable domain of heavy chains of antibodies, Vl: variable domain of the light chains of antibodies. From (Kontermann and Duebel, 2001).

I-8-4-2. Construction of phage display scFv libraries.

VH and VL genes from the whole antibody repertoire are PCR-amplified by using a set of primers specially designed to amplify all rearranged human V-genes (Marks *et al*., 1991; Marks *et al*., 1991; Orlandi *et al*., 1989). These primers are specific for

regions which are well-conserved among all V-genes families named framework regions (see I-7-1). The first set of primers is specific for the region encoding the N terminal part of VH and VL domains which is found at the beginning of the FR1 region. The second set of primers is specific for the region coding for the junction between the J segment of the V domain and the first C domain. This region is located downstream of the CDR3 in the FR4 region. (see Fig. 11). Furthermore, sets of degenerated primers are used to optimize the amplification of all rearranged V domains. All PCR-amplified regions are then cloned as scFvs molecules in the phagemid, used to transform bacteria resulting in the construction of bacterial scFv libraries. Generation of recombinant phages displaying scFvs on their surface is achieved by infecting these phagemid-transformed bacterial cells with helper phages. Theoretically, those recombinant phages mirror the antibody repertoire initially present in patients whose lymphocytes were taken. The diversity of libraries can even be greater since the cloning method allows the free combination of VH and VL domains which may be not have been originally present in the donor. The construction of libraries is extensively described in Methods and in Fig. 16a; 16b.

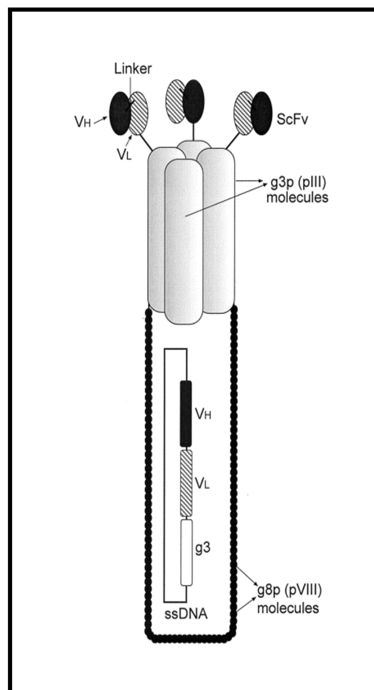

Fig 13: Representation of a recombinant phage expressing and displaying on its surface scFv-pIII fusion proteins. From (Azzazy and Highsmith, 2002).

I-8-4-3. Source of RNAs

Because plasm cells are responsible for the production of antibodies, they represent the biological material which is used to construct phage antibody libraries. Such

cells can be isolated from peripheral blood lymphocytes (PBL), bone marrow or spleen. However, due to technical problems, especially encountered when libraries are constructing from humans, PBL cells harvested from blood are preferentially used.

I-8-4-4. Types of Phage antibody libraries.

Antibody phage librariees are classified according to the nature of the donor and its antibody specificities. Three types of libraries can be constructed.

Naïve libraries.
Such libraries are constructed using B-cells from naïve or non immunized donors. Because, any immune response can biased towards one specific antigen, a single naïve library can be used to isolate antibody fragments against various antigens. This type of libraries can be used to isolate antibodies against self antigens, haptens or small antigens and therefore this potentially limited diversity can reduced the quality content of such libraries.

Immune libraries.
Phage libraries can also be constructed using material from immune or immunized donors. These libraries contain a high content for diverse antibodies against one specific antigen. Furthermore, many of these antibodies have undergone the affinity maturation process during immunization and, therefore, exhibit a high affinity for the antigen.

Synthetic libraries.
A third class of libraries called synthetic libraries can also be designed. In this strategy, residues from the CDRs of the whole antibody repertoire can be randomly PCR-mutated resulting in a large number of specificities, many of which are not present in the natural repertoire..

I-8-5. Selection of antibody libraries: “Biopanning”.

In order to isolate recombinant phages displaying scFvs with a specific affinity for one given antigen from billions different phages from the constructed libraries, a biopanning or selection step is required. This method is based on the interaction between the antibody fragment (scFv) displayed on the phage surface and the antigen of interest. Phages are panned against the antigen of interest which can be

immobilized on a plastic surface, in solution or expressed on cell surfaces (Azzazy and Highsmith, 2002). Multiple and successive selection steps are usually performed to allow an enrichment with antigen-specific phages. Finally, monoclonal phages displaying a specific affinity for the antigen can be isolated and further characterized. (Fig. 14).

Practically, recombinant phages are incubated with the antigen of interest which is usually immobilized onto a plastic surface (Fig.14 [1]). In this case, the antigen is captured onto plastic phase by chemical coupling or non-covalent adsorption to a hydrophobic plastic surface. After the incubation step, unbound phages are eliminated by washes (Fig.14 [2]) and phages displaying scFvs with a specific binding activity for the antigen are retained. These phages are then eluted and used to infect *E.coli* cells resulting in the generation of a new phage population (Fig.14 [3], [4].) This new pool of phages is usually used for another round of selection. Usually, three to four successive rounds of selection are performed to remove all unspecific binders and to ensure the enrichment with phages exhibiting a high specific affinity for the antigen of interest.

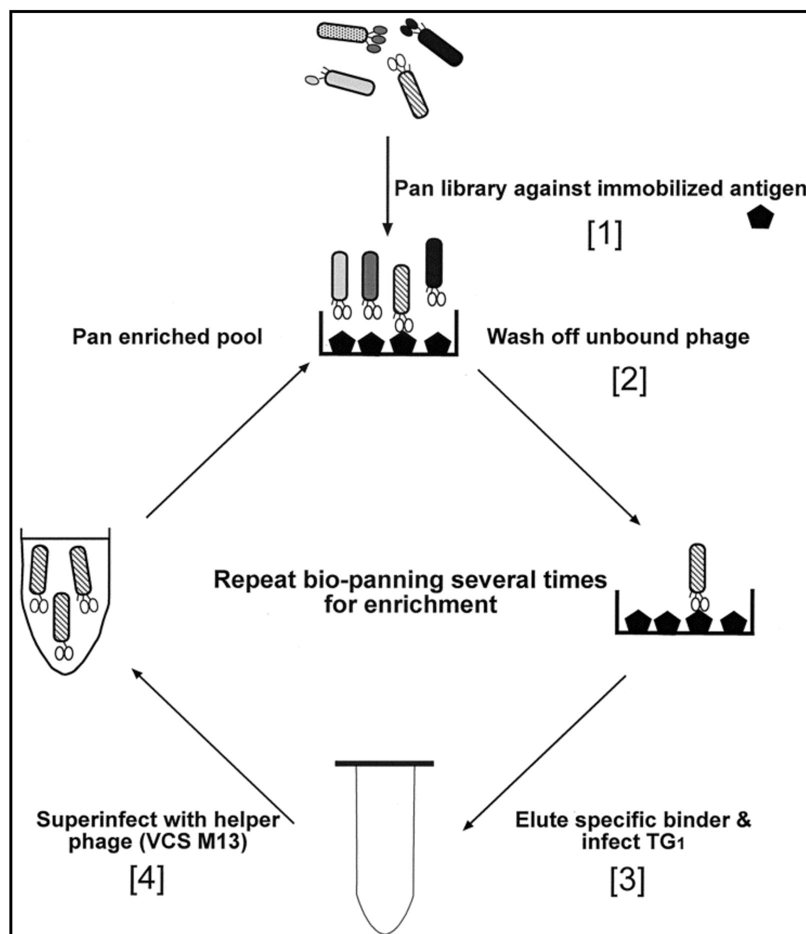

Fig 14: Biopanning or selection step. From (Azzazy and Highsmith, 2002).

I-8-6. Human viruses neutralized by recombinant antibody fragments.

The phage display system has been successfully used to isolate and characterize human neutralizing antibody fragments (scFv or Fab format) against several viruses which are listed in the Table 2.

Virus	Target protein	Format	Reference
Ebola virus	Glycoprotein GP	Fab	(Maruyama *et al*., 1999; Maruyama *et al*., 1999)
Hantaan virus	Glycoprotein G1	Fab	(Liang *et al*., 2003)
Hantaan virus	Glycoprotein G2	Fab	(Koch *et al*., 2003)
Hepatitis A	Capsid protein	Fab	(Schofield *et al*., 2002)
Hepatitis E	Capsid protein ORF2	Fab	(Schofield *et al*., 2000)
HIV-1	Gp140-CD4 complexes	Fab	(Zhang *et al*., 2003)
HIV-1	Gp120-CD4-CCR5 complexes	Fab	(Moulard *et al*., 2002)
HIV-2	Glycoprotein gp125	Fab	(Bjorling *et al*., 1999)
Human cytomegalovirus (HCMV)	B&H glycoproteins	scFv	(Nejatollahi *et al*., 2002)
Human Rotavirus	VP7 protein	Fab	(Higo-Moriguchi *et al*., 2004)
Measles virus	Hemagglutinin H glycoproteins	Fab	(de-Carvalho-Nicacio *et al*., 2002)
Puumala virus	Glycoprotein G2	Fab	(de-Carvalho-Nicacio *et al*., 2000)
Rabies virus	Glycoprotein gp	scFv	(Kramer *et al*., 2005)
Rabies virus	Glycoprotein gp	scFv	(Ray *et al*., 2001)
Resiratory Syncytial Virus	Glycoprotein F	scFV	(Nguyen *et al*., 2000)
Resiratory Syncytial Virus	Glycoprotein F	Fab	(Barbas *et al*., 1992)
SARS-CoV	Glycoprotein spike	Fab	(ter-Meulen *et al*., 2004)
Simian Immunodeficiency virus	Glycoprotein gp120	Fab	(Glamann *et al*., 1998)
Vaccinia virus	unknown	Fab	(Schmaljohn *et al*., 1999)
Varicella—zoster Virus	VZV antigen	scFv	(Kausmally *et al*., 2004)

Table 2. Human neutralizing recombinant antibody fragments isolated against various viruses by phage display.

I-9. Aim of the work

Yellow fever is an “old” disease which can be prevented by vaccination. Despite the presence of a highly effective YF vaccine called 17D vaccine, this disease is currently re-emerging on a large scale. The 17D vaccine is known to be a non clonal vaccine which is constituted of heterogeneous virion sub-populations due to the uncontrolled passage of the virus during the early years. The report of several cases of viscerotropic and neurotropic diseases associated with YFV 17D vaccination raised the obvious question of vaccine safety. Moreover, this vaccine is still produced by inoculation into viable embryonated eggs and this method of production, which limits the vaccine production capacity, strongly impairs attempts to control the disease. Furthermore, vaccine supply shortage has already been experienced during YF outbreaks. Thus, it appears essential to develop new methods for manufacturing the 17D vaccine. One of the easiest way is the use of a clonal vaccine derived from a full length cDNA transfected into a cell culture substrate. This method allows the production of a vaccine composed of a genetically homogeneous virion population at high efficiency and low cost. Because YFV immunity is mainly antibody-mediated, the knowledge of YFV neutralizing epitopes is, therefore, essential. To study this problem, the phage display technology provides a convenient tool to isolate neutralizing recombinant antibody fragments against YFV which could be used to identify and characterize neutralizing epitopes. To this aim, phage antibody libraries composed of phages displaying on their surface recombinant single chain antibody fragments (scFv) from lymphocytes of patients who recovered from YF disease will be generated. Such libraries will be, then, screened against YFV particles to isolate scFv with a specific affinity for the YFV. Those scFvs will be tested for their capacity to block YF infection. The molecular determinants of neutralization, i.e. neutralizing epitopes, will be identified and characterized. These data would be useful for the standardization and the design of novel YF vaccines based on infectious clones.

II-MATERIAL

II-MATERIAL

II-1. Plasticware.

Maxisorp Immunotubes	Nunc, Wiesbaden
Maxisorp Microtiter plates	Nunc, Wiesbaden
Large plates (243 mm x 243 mm)	Nunc, Wiesbaden

II-2. Chemicals.

Acetone	Riedel-de Haen, Seelze
Acrylamid/Bisacrylamid (29 :1)	Roth, Karlsruhe
Agarose	Invitrogen, Karlsruhe
Agarose (Low Melting Point)	Invitrogen, Karlsruhe
Ammoniumpersulfat (APS)	Biorad, Muenchen
Ampicillin	Sigma, Deisenhofen
Bactoagar	Difco, USA
Bromophenolblue	Serva, Heidelberg
Complete (Protease Inhibitor)	Roche, Mannheim
DNA Ladder mix (DNA marker)	MBI Fermentas, St.Leon-Roth
ECL (Enhanced Chemoluminescence)	Pierce, USA
EDTA (Ethylene-Dinitrilo-Tetra-Acetate)	Sigma, Deisenhofen
Ethanol	Roth, Karlsruhe
Ethidiumbromid	Roth, Karlsruhe
Ficoll	Amersham, Braunschweig
Glucose	Sigma, Deisenhofen
Glycerol	Roth, Karlsruhe
Glycin	Merck, Darmstadt
Isopropanol	Roth, Karlsruhe
Iso-Propyl-Thio-Galactoside (IPTG)	MBI Fermentas, St.Leon-Roth
Kanamycin	Sigma, Deisenhofen
Mercaptoethanol	Roth, Karlsruhe
Neutral red	Roth, Karlsruhe
N-hydroxysuccimid (NHS)-L Biotin	Pierce, Rockford, USA
Ni-NTA	Qiagen, Hilden
Nitrocellulose	Schleicher&Schuell, Dassel
n-octyl-β-D-glucopyranoside	Sigma, Deisenhofen
NP40	Sigma, Deisenhofen
Pepton	Merck, Darmstadt
Polyethylenglycol (8000MW)	Sigma, Deisenhofen
Protein G-Agarose beads	Santa Cruz, CA, USA
Rainbow marker (RPN 756)	Amersham, Braunschweig
Rotiphorese Gel 30 (30% Acrylamid)	Roth, Karlsrhue
Sodium-dodecyl-sulfate (SDS)	Roth, Karlsruhe
TEMED (N,N,N,N-Tetramethyl-ethylendiamine)	Biorad, Muenchen
Triethylamine	Sigma, Deisenhofen
Tris (Trishydroxymethylaminomethan)	Roth, Karlsruhe
Triton X-100	Sigma, Deisenhofen
Trypsin-EDTA	Gibco BRL, Eggenstein
Tween-20	Sigma, Deisenhofen

II-3. Enzymes.

ApaLI	New England Biolabs, Schwalbach
MMLV reverse transcriptase (H-)	Promega, USA
NotI	New England Biolabs, Schwalbach
RNase Inhibitor	MBI Fermentas, St.Leon-Roth
SfiI	New England Biolabs, Schwalbach
Shrimp Alcaline Phosphatase (SAP)	MBI Fermentas, St.Leon-Roth
T4 DNA Ligase	MBI Fermentas, St.Leon-Roth
Xho1	New England Biolabs, Schwalbach

II-4. Antibodies

Anti-17D polyclonal sera (immunized travellers)	Provided by Dr M.Niedrig, Robert Koch Institute, Berlin, Germany
Anti-Myc-Tag mouse antibody	Oncogene, CA, USA
Anti-YFV polyclonal serum (monkey)	Provided by Dr M.Niedrig, Robert Koch Institute, Berlin, Germany
Polyclonal serum from a human acute confirmed YF case (S-993)	Provided by Dr L. Koivogui, PFHG, Conakry, Guinea.
HRP-conjugated streptavidin	Pierce, USA
HRP-conjugated mouse anti-M13	Pharmacia, Freiburg
HRP-conjugated rabbit anti mouse IgG	Dako, Denmark
HRP-conjugated rabbit anti human IgM	Dako, Denmark
HRP-conjugated rabbit anti human IgG	Dako, Denmark
Anti YFV-E protein mouse monoclonal antibody (6330)	Provided by Dr M.Niedrig, Robert Koch Institute, Berlin, Germany
Anti-His-tag mouse antibody, (H-3)	Santa Cruz, CA, USA
Anti YFV-E protein mouse monoclonal antibody (6538)	Provided by Dr M.Niedrig, Robert Koch Institute, Berlin, Germany

II-5. Radioactive amino acids.

Redivue Pro-Mix L-[^{35}S] in vitro cell labelling mix:	Amino acid mixture for in vitro labelling containing L-[^{35}S] Methionine and L-[^{35}S] Cysteine, 14.3 mCi/ml, >1000 Ci/mmol. Amersham Biosciences, Freiburg.

II-6. Commercial Kits.

Micro BCA Protein Assay Reagent kit	Pierce, USA
One step RT-PCR kit	Qiagen, Hilden
QIAamp viral RNA Mini kit	Qiagen, Hilden

QIAprep Maxipreps kit	Qiagen, Hilden
QIAprep Minipreps kit	Qiagen, Hilden
QIAquick Gel extraction kit	Qiagen, Hilden
QIAquick PCR purification kit	Qiagen, Hilden
RNeasy Mini kit	Qiagen, Hilden
Sulfo-NHS-LC-Biotinylation kit (EZ-Link)	Pierce, USA
Taq-PCR Core kit	Qiagen, Hilden

II-7. Vectors.

pHEN3	phagemid vector used for the expression of scFv as a fusion protein scFV-pIII. (Kontermann et al, xxx).
pAB1	prokaryotic expression vector allowing the expression of scFvs as soluble molecules. (Kontermann et al, xxx).

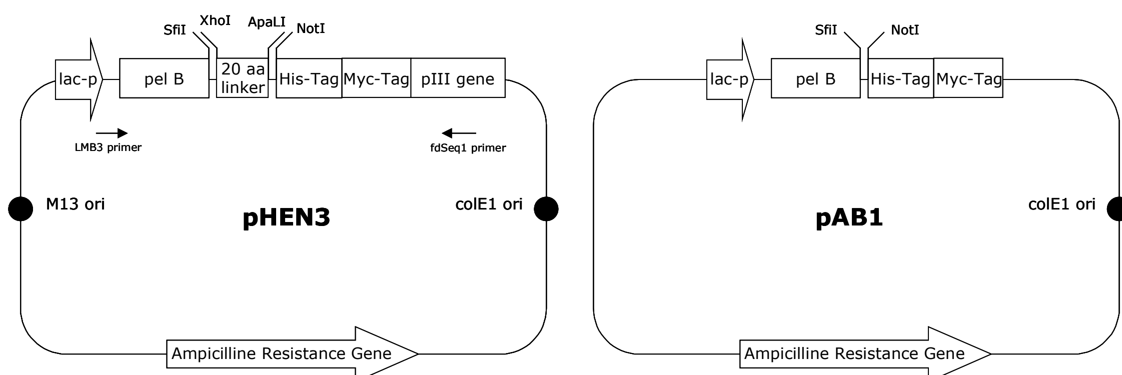

Fig.15: Schematic representation of the pHEN3 phagemid and the pAB1 plasmid. Lac p: promoter LacZ, pelB: pelB leader sequence.

II-8. Viruses.

YF 17D vaccine strain:

17D-204-WHO strain	obtained as a commercial preparation of the 17D-204-WHO vaccine, manufactured by the Robert Koch Institute, Berlin, Germany.

Wild-type YFV strains:

Strain	Year	Origin	Source	Reference
HB1782	1986	CAR	Human	CAR86
ETH2777	1961	Ethiopia	Human	Ethiopia86
BA-55	1986	Nigeria	Human	Nigeria86
ArD76320	1990	Senegal	Mosquito	Senegal90
Asibi strain	1927	Ghana	Human	Ghana27

African wild-type yellow fever viruses obtained as lyophilized stocks made on suckling mouse brain from National Reference Center for Arboviruses and Viral Haemorrhagic Fevers, Lyon, France.

II-9. Bacteria and phages.

E.coli, TG1 strain	Electroporation-competent cells, supE thi-1 Δ(lac-proAB)Δ (mcrB-hsdSM)5 (r_k^- m_k^-) [F´traD36 proAB $lacI^qZ\Delta M15$], Stratagene, Heidelberg.
VCS M13 helper phage	Stratagene, Heidelberg.

II-10. Eukaryotic Cells.

Vero-76 cells	Cell line from African Green Monkey kidney, ATCC CRL-1587
PS cells	Cell line from porcine kidney, obtained from the National Reference Center for Arboviruses and Viral Haemorrhagic Fevers, Lyon, France.

II-11. PCR primers.

The primers used in this study are those described by Marks et al (Marks *et al*., 1991) except that restriction sites (ApaLI, NotI, SfiI and XhoI) were added at 5´ends. These primers were provided by MWG-Biotech AG, Ebersberg, Germany.

II-11-1. Degenerated primers specific for the VH and VL genes.

W=A,T / M=A,C /R=A,G/K=G,T /Y=C,T / S=G,C / V=G,A,C / D=G,A,T / B=G,T,C / H=A,T,C

II-11-1-1. Primers for VLκ genes.

Back primers (for the 1st PCR=PCR 1a and for the 2nd PCR=PCR 1b):

huVκ1Back-Apa	5’ GCCGTTAGTGCACAGATCCAGWTGACCCAGTCTCC 3´
huVκ2Back-Apa	5’ GCCGTTAGTGCACAGGTTGTGATGACTCAGTCTCC 3´
huVκ3Back-Apa	5’ GCCGTTAGTGCACAGATTGTGWTGACRCAGTCTCC 3´
huVκ4Back-Apa	5’ GCCGTTAGTGCACAGATTGTGATGACCCACACTCC 3´
huVκ5Back-Apa	5’ GCCGTTAGTGCACAGACGACACTCACG CAGTCTCC 3´
huVκ6Back-Apa	5’ GCCGTTAGTGCACAGATTGTGCTGACTCAGTCTCC 3

ApaL1 restriction sites are underlined.

Foward Primer (for the 1st PCR=PCR 1a):

huCκFor 5' GAAGACAGATGGTGCAGCCACAGT 3´

Forward Primers(for the 2nd PCR=PCR 1b):

huJκ1For-Not	5' GCCTTCTGCGGCCGCACGTTTGATYTCCASCTTGGTCCC 3´
huJκ2For-Not	5' GCCTTCTGCGGCCGCACGTTTGATATCCACTTTGGTCCC 3´
huJκ3For-Not	5' GCCTTCTGCGGCCGCACGTTTAATCTCCAGTCGTGTCCC 3´

Not I restriction sites are underlined.

II-11-1-2. Primers for VLλ genes.

Back primers (for the 1st PCR=PCR 1a and for the 2nd PCR=PCR 1b):

huVλBack-Apa	5' GGCGGTAGTGCACAGTCTGTGCTGACTCAGCCACC 3´
huVλBack-Apa	5' GGCGGTAGTGCACAGTCTGTGYTGACGCAGCCGCC 3´
huVλCBack-Apa	5' GGCGGTAGTGCACAGTCTGTCGTGACGCAGCCGCC 3´
huVλ2Back-Apa	5' GGCGGTAGTGCACARTCTGCCCTGACTCAGCCT 3´
huVλ3ABack-Apa	5' GGCGGTAGTGCACTTTCCTATGWGCTGACTCAGCCACC 3´
huVλ3BBack-Apa	5' GGCGGTAGTGCACTTTCTTCTGAGCTGACTCAGGACCC 3´
huVλ4Back-Apa	5' GGCGGTAGTGCACACGTTATACTGACTCAACCGCC 3´
huVλ5Back-Apa	5' GGCGGTAGTGCACAGGCTGTGCTGACTCAGCCGTC 3´
huVλ6Back-Apa	5' GGCGGTAGTGCACTTATTTTTATGCTGACTCAGCCCCA 3´
huVλ7/8Back-Apa	5' GGCGGTAGTGCACAGRCTGTGGTG ACYCAGGAGCC 3´
huVλ9Back-Apa	5' GGCGGTAGTGCACWGCCTGTGCTGACTCAGCCMCC 3´

ApaL1 restriction sites are underlined.

Forward Primers (for the 1st PCR=PCR 1a):

HuCλFor1	5' TGAACATTCTGTAGGGGCCACTG 3´
HuCλFor2	5' AGAGCATTCTGCAGGGGCCACTG 3´

Forward Primers (for the 2nd PCR=PCR 1b):

HuCλFor1-Not	5' GAGTCATTCTGCGGCCGCACCTAGGACGGTGACCTTGGTCCC 3´
HuCλFor2-Not	5' GAGTCATTCTGCGGCCGCACCTAGGACGGTCACCTTGGTCCC 3´
HuCλFor3-Not	5' GAGTCATTCTGCGGCCGCACCTAA AACGGTGAGCTGGGTCCC 3´

Not I restriction sites are underlined.

II-11-1-3. Primers for VH genes.

Back primers (for the 1st PCR=PCR 2a *and for the 2nd PCR=PCR 2b*):

huVH1/7Back-Sfi	5’ CTCGCGGCCCAGCCGGCCATGGCCCAGRTGCAGCTGGTGCARTCTGG 3´
huVH2Back-Sfi	5’ CTCGCGGCCCAGCCGGCCATGGCCCAGRTGACCTTGAAGGAGTCTGG 3´
huVH3ABack-Sfi	5’ CTCGCGGCCCAGCCGGCCATGGCCSAGGTGCAGCTGGTGGAGTCTGG 3´
huVH3BBack-Sfi	5’ CTCGCGGCCCAGCCGGCCATGGCCGAGGTGCAGCTGGTGGAGWCYGG 3´
huVH4ABack-Sfi	5’ CTCGCGGCCCAGCCGGCCATGGCCCAGGTGCAGCTACAGCAGTGGGG 3´
huVH4BBack-Sfi	5’ CTCGCGGCCCAGCCGGCCATGGCCCAGSTGCAGCTGCAGGAGTGSGG 3´
huVH5Back-Sfi	5’ CTCGCGGCCCAGCCGGCCATGGCCGARGTGCAGCTGGTGCAGTCTGG 3´
huVH6Back-Sfi	5’ CTCGCGGCCCAGCCGGCCATGGCCCAGGTACAGCTGCAGCAGTCAGG 3´

Sfi I restriction sites are underlined.

Forward Primers (for the 1st PCR=PCR 2a):

huIgGFor1	5’ GACCGATGGGCCCTTGGTGGA 3’
huIgGFor2	5’ GACGGATGGGCCCTTGGTGGA 3’

Forward Primers (for the 2nd PCR=PCR 2b):

huJH1-2,4-5For-Xho	5’ ACTGGTCCGACTCGAGACGGTGACCAGGGTKCC 3’
huJH3,6For-Xho	5’ ACTGGTCCGACTCGAGACGGTGACCRTKGTCCC 3’

Xho1 restriction sites are underlined.

II-11-2. pHEN3-specific primers.

LMB3 primer	5’ CAGGAAACAGCTATGACC 3’
fdseq1 primer	5’ GAATTTTCTGTATGAGG 3’

II-11-3. YFV E, NS1 and prM proteins-specific primers.

EnvF1	5´TGTGAAGATTAATGACAAGTGCC3´
EnvF2	5’ CAATGATAAGTGCCCGAGC 3´
EnvF3	5´CAGGTCATGGCACGGTTG 3´
EnvR1	5´GGATTGACTTCAATTAGGACTTC 3´
EnvR2	5’ CACCTCAATCAGCACTTCATC 3´
NS1R1	5’ CACTATTGATGCAAGCTTCACAG 3´
NS1R2	5’ TCTCCACATTTGAGCTCTCG3´
prM forward	5´GATGTTCTGACTGTGCAATT 3´
M reverse	5´TTCCTCCATGCACCCCCT 3´

II-12. Buffers and solutions.

II-12-1. Virus purification.

30% Glycerol Buffer	Glycerol	30 g
	volume adjusted to 100 ml with PBS and autoclaved	

TNE Buffer (pH 7.5):	Tris	1.2 g
	NaCl	8.8 g
	0.5 M EDTA	10 ml
	volume adjusted to 1 l with dH_2O	
	pH 7.5 adjusted with NaOH	

II-12-2. Phage purification.

PEG $_{6000}$-NaCl solution:	20% PEG $_{6000}$	200 g
	2.5 M NaCl	146.1 g
	volume adjusted to 1 l with dH_2O	

II-12-3. DNA electrophoresis.

50x TAE-Buffer:	Tris	242 g
	Acetic acid	57.1ml
	0.5 M EDTA (pH 8)	100ml
	volume adjusted to 1 l with dH_2O	

10x agarose gel sample buffer:	1 M Tris-HCl pH 7.5	0.5 ml
	Glycerol	6 ml
	Bromophenol blue	25 mg
	Xylene cyanol	25 mg
	volume adjusted to 10 ml with dH_2O	

II-12-4. ELISA.

ABTS Microwell Peroxidase Substrate	KPL, USA
ELISA coating buffer:	one Carbonate/Bicarbonate capsule dissolved in 100 ml dH_2O.

II-12-5. SDS PAGE and Western Blot.

SDS PAGE.

4x SDS PAGE sample buffer:	0.5 M Tris-HCl pH 6.8	4 ml
	SDS	0.4 g
	Glycerol	4 ml
	2% Bromophenolblue	0.1 ml
	dH2O	1.2 ml
	β-mercaptoethanol	0.4 ml
10x SDS PAGE Running buffer:	SDS	10 g
	Tris	30 g

	Glycine	144 g
	volume adjusted to 1 l with dH_2O	

Transfer buffer for Western Blot.

Anodebuffer I:	1 M Tris	300 ml
	Ethanol	200 ml
	adjust volume to 1 l with dH_2O	
Anodebuffer II:	1 M Tris	25 ml
	Ethanol	200 ml
	adjust volume to 1 l with dH_2O	
Cathode buffer:	1 M Tris	25 ml
	Aminocapron acid	5.25 g
	Ethanol	200 ml
	volume adjusted to 1 l with dH_2O	

PBS:	NaCl	8 g
	KCl	0.2 g
	Na_2HPO_4	1.15 g
	$MgCl_2$	0.1 g
	KH_2PO_4	0.2 g
	$CaCl_2$	0.13 g
	volume adjusted to 1 l with dH_2O	

II-12-6. Dot Blot buffers.

TNE + 1%NP-40	
TNE + 1% n-octyl-β-D-glucopyranoside	
TNE + 1%Tween-20	
Rec Blot/Immunoblot substrate	Mikrogen, Freiburg

II-12-7. Protein purification

Periplasmic Preparation Buffer (PPB):	1 M Tris-HCl pH 7.5	30 ml
	0.5 M EDTA	10 ml
	Sucrose	200 g
	volume adjusted to 1 l with dH_2O	

5x Na-Phosphate buffer stock:	Na_2HPO_4	29.8g
	NaCl	147g
	volume adjusted to 1 l with dH_2O	

IMAC loading buffer:	5x Na-Phosphate stock	200 ml
	20 mM imidazole	1.38 g
	volume adjusted to 1 l with dH_2O	

IMAC wash buffer:	5x Na-Phosphate stock	200,ml

	35 mM imidazole volume adjusted to 1 l with dH_2O	2.38 g

IMAC elution buffer:	5x Na-Phosphate stock 100 mM imidazole volume adjusted to 1 l with dH_2O	200 ml 6.81 g

Coomassie Blue staining solution:	Coomassie Brilliant Blue R-250 Methanol Acetic acid volume adjusted to 1 l with dH_2O	2.5 g 450 ml 400 ml

Coomassie Blue destaining solution:	Methanol Acetic acid volume adjusted to 1 l with dH_2O	450 ml 100 ml

RIPA Buffer:	1 M Tris-HCl pH 7.5 5 M NaCl 0.5 M EDTA n-octyl-β-D-glucopyranoside Protease Inhibitor cocktail volume adjusted to 100 ml with dH_2O	1 ml 3 ml 1 ml 0.5 g 1 tablet

II-12-8. pH shift buffers.

Low pH Buffer (pH 5)	0.05 M MES 0.1 M NaCl volume adjusted to 100 ml with dH2O pH 5 adjusted with HCl

Control pH Buffer (pH 8): TNE buffer adjusted to pH 8 with NaOH.

II-13. Media for bacterial culture.

M9 medium:	Na2HPO4 KH2PO4 NaCl NH4Cl Volume adjusted to 1 l with dH_2O and autoclaved	58 g 30 g 5g 10 g

Minimal plates:	H_2O Agar autoclaved 20% glucose 1 M $MgSO_4$ Vitamin B1 (2 mg/ml) M9 medium	880 ml 15 g 10 ml 1 ml 1 ml 100 ml

LB medium:	Bacto-Trypton	10 g
	Yeast extract	5 g
	NaCl	10 g
	Volume adjusted to 1 l with dH_2O and autoclaved	

LB medium-1% glucose	LB medium	1 l
	Glucose	10 g

LB agar plates (+/- 1% glucose)	LB medium (+/- 1% glucose)	250 ml
	Agar	3.75 g
	Volume adjusted to 1 l with dH_2O and autoclaved	

II-14. Media for cell culture.

Dulbecco´s Modified Eagle´s Medium (DMEM)	Gibco BRL, Eggenstein.
Dulbecco´s Modified Eagle´s Medium (DMEM) without Methionine and Cysteine.	Sigma, Deisenhofen.
Fetal calf serum (FCS)	Gibco BRL, Eggenstein.
L-Glutamine	Gibco BRL, Eggenstein.
Penicillin-Streptomycin	Gibco BRL, Eggenstein.
2x DMEM a la Frantz Brandl	Sigma, Deisenhofen.

III-METHODS

III-METHODS

III-1. Schematic representation of the construction of the phage libraries expressing recombinant antibody fragments (scFvs).

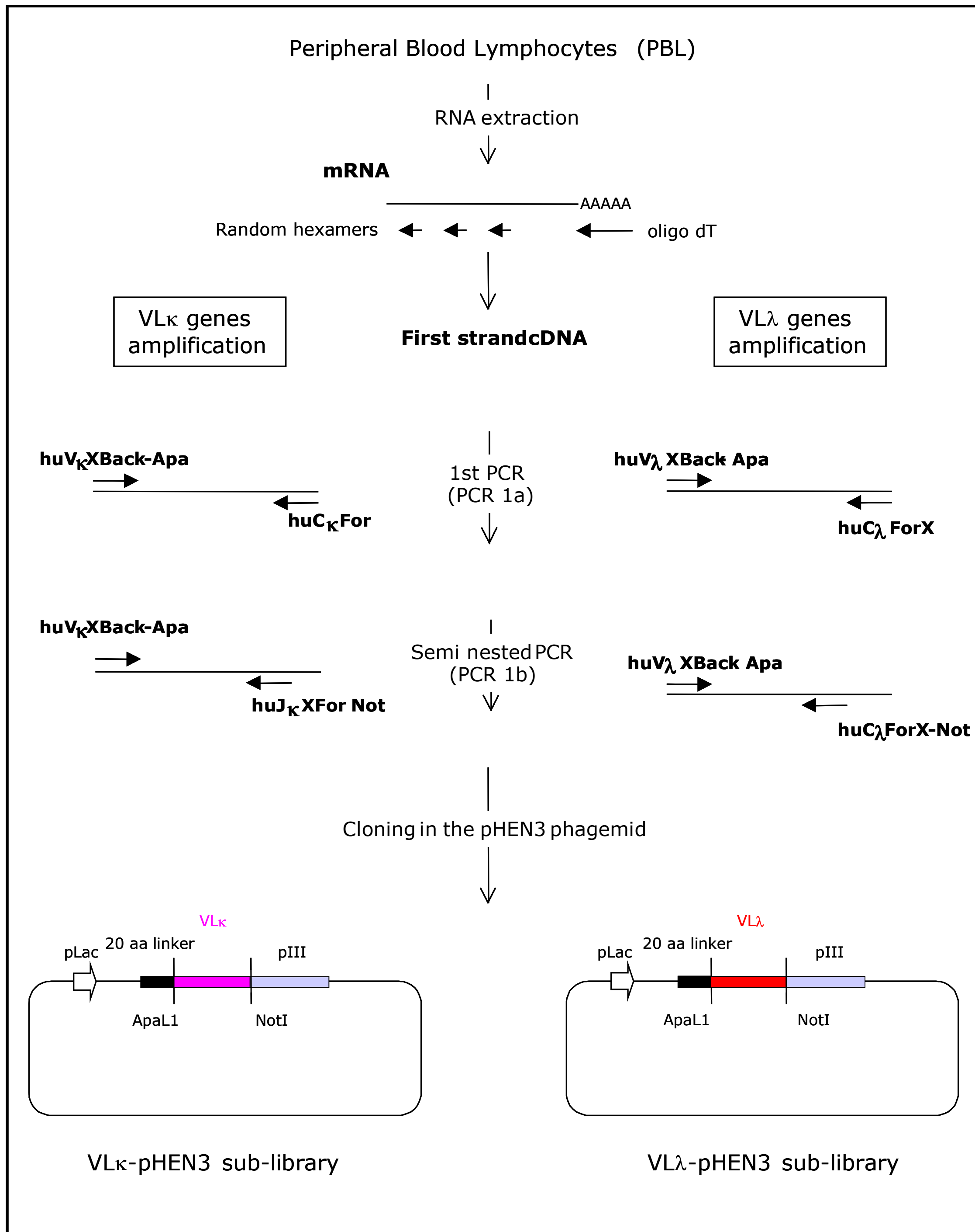

Fig 16a: Overview of the phage libraries construction. Cloning of VL fragments into pHEN3 to obtain VL sub-libraries

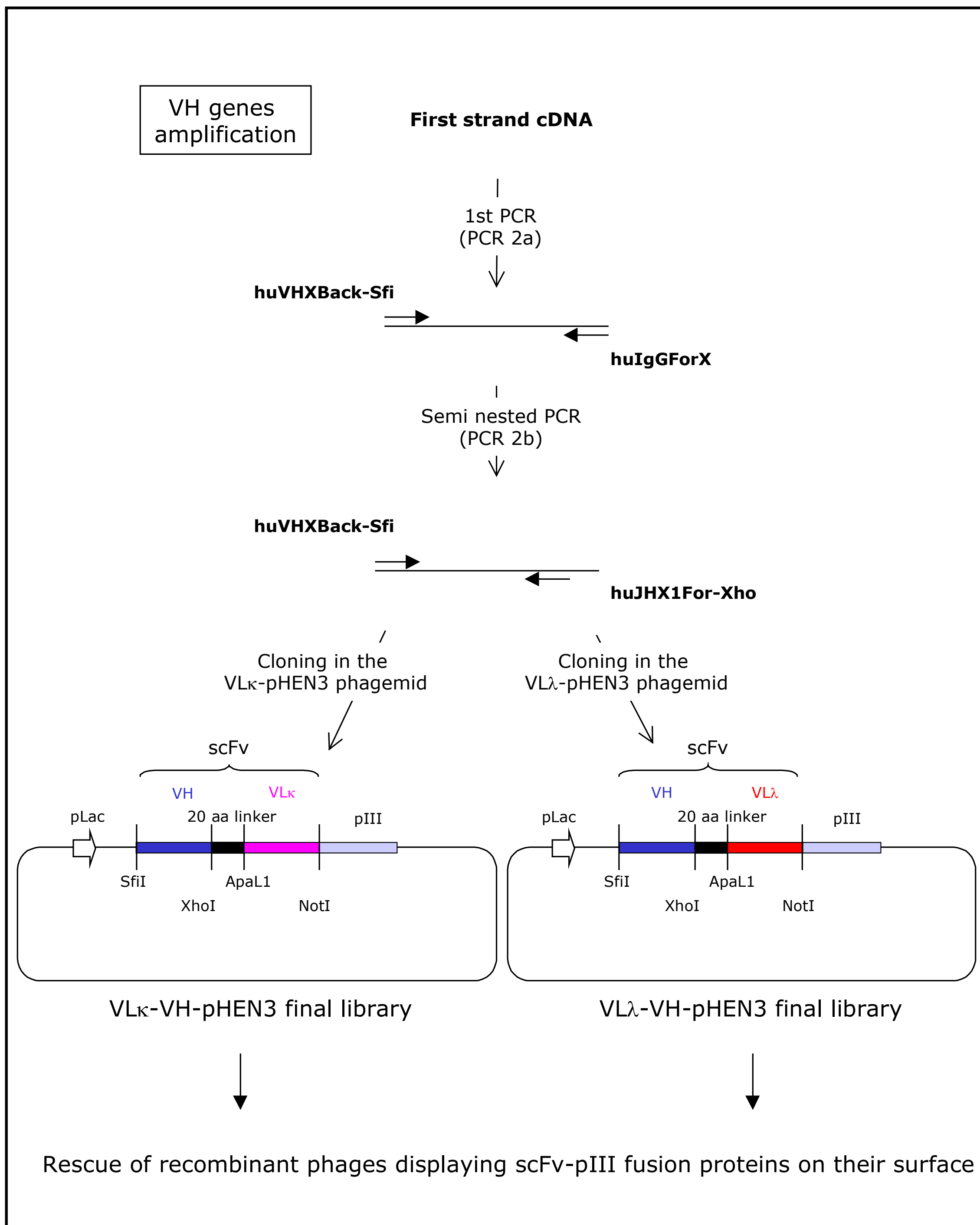

Fig 16b: Overview of the phage libraries construction. Cloning of VH fragments into VL-pHEN3 to obtain scFv libraries.

III-2. Isolation of lymphocytes from two African donors who recovered from yellow fever.

Peripheral blood lymphocytes (PBL) were isolated from blood of two donors from Guinea who were infected with yellow fever virus during an outbreak which occurred in 2000. Blood samples were collected approx. 6 months after acute infection. 20 ml anticoagulated blood were mixed 1:1 with an equal amount of PBS, loaded onto a 10 ml Ficoll layer and centrifuged at 1500 g for 30 min at RT. This step allows the separation of erythrocytes which are found in the ficoll solution at the bottom of the tube from white cells (PBL, leukocytes) which are located at the interface. The top of the tube represents the plasma fraction. PBL cells from the interface were harvested and washed twice with PBS by centrifugation at 1500 g at room temperature (RT). After washes, PBL were directly used for total RNA extraction after trypan blue counting.

III-3. First strand cDNA synthesis.

Total RNA was extracted from 10^7 PBL isolated in III-2 using the RNeasy kit (Qiagen) according to the manufacturer´s instructions. Quantitation of RNA was determined by measuring OD_{260} with a standard spectrophotometer.
The first strand of cDNA was synthesized from total RNA by reverse transcription using two different types of primers: oligo$(dT)_{15}$ primer and random hexamers.

A) Oligo$(dT)_{15}$ primer.

total RNA (isolated in III-2)	4 µg
oligo$(dT)_{15}$ primer (0.5µg)	4 µl
DEPC-treated water to 10 µl	

The reaction was incubated at 65°C for 5 min to melt secondary structures present on mRNA which could interfere with the cDNA synthesis. At this time, the tube was immediately cooled on ice for 5 min to prevent the secondary structures to form. The following components were then added:

+5X RT buffer	5 µl
10mM dNTPs mix	3 µl
+ RNase Inhibitor	2 µl

+ MMLV RT (H-)(200 Units)	1 µl
DEPC-treated water to 25 µl	

The reverse transcription reaction was incubated at 42°C for 1 h. The enzyme was then inactivated by incubating the tube at 70°C for 15 min.

B) Random hexamers.

total RNA (isolated in 2-1)	4 µg
random hexamers (100ng)	2 µl
DEPC-treated water to 10 µl	

The reverse transcription reaction was performed as described above when using the oligo(dT)$_{15}$ primers except for different temperatures conditions. The tube was incubated at 25°C for 10 min and then incubated at 42°C for 50 min. The enzyme was finally inactivated by heating at 70°C for 15 min.
Both cDNA preparations were pooled and stored at –70°C until use as a template for PCR amplification.

III-4. PCR amplification of the Variable Kappa Light chain (VLκ), Variable Lambda Light chain (VLλ) and Variable Heavy chain (VH) genes of antibodies.

VLκ, VLλ and VH genes were amplified using a semi-nested PCR protocol (cf. Fig 16a and 16b) using the cDNA, synthesized in section III-3

III-4-1. PCR conditions and protocol.

The first PCR step [PCR 1a for VL] and [PCR 2a for VH] was performed as follows:

10x PCR buffer	5 µl
5x Q solution	10 µl
10mM dNTPs mix	1 µl
back primer (10 pmol/µl)	3 µl
forward primer (10 pmol/µl)	3 µl
Taq polymerase (5 U/µl)	0.5 µl
H_2O	26.5 µl

First strand cDNA pool (from2-2)	1 µl
Final volume	50 µl

PCR 1a/2a was performed using the following program:

Initial denaturation	30 sec	94°C
Denaturation	30 sec	94°C
Annealing	45 sec	50°C
Elongation	1 min	72°C
Final extension	10 min	72°C
Number of cycles: 20		

The semi nested PCR [PCR 1b for VL] and [PCR 2b for VH] was performed as follows:

10x PCR buffer	5 µl
5x Q solution	10 µl
10mM dNTPs mix	1 µl
back primer (10 pmol/µl)	3 µl
forward primer (10 pmol/µl)	3 µl
Taq polymerase (5 U/µl)	0.5 µl
H_2O	22.5 µl
PCR products from PCR 1a (for PCR 1b) or PCR products from PCR 2a (for PCR 2b)	5 µl
Final volume	50 µl

PCR 2a/2b was performed using the following program:

Initial denaturation	30 sec	94°C
Denaturation	30 sec	94°C
Annealing	45 sec	50°C
Elongation	1 min	72°C
Final extension	10 min	72°C
Number of cycles: 25		

III-4-2. Combination of degenerated primers used to amplify the Variable Kappa Light chains (VLκ).

PCR 1a:

reaction	Back primer	For primer
1a	huVκ1Back-Apa	CκFor
2a	huVκ2Back-Apa	CκFor
3a	huVκ3Back-Apa	CκFor
4a	huVκ4Back-Apa	CκFor
5a	huVκ5Back-Apa	CκFor
6a	huVκ6Back-Apa	CκFor

PCR 1b:

reaction	Back primer	For primer
1b	huVκ1Back-Apa	huJκXFor-Not*
2b	huVκ2Back-Apa	huJκXFor-Not*
3b	huVκ3Back-Apa	huJκXFor-Not*
4b	huVκ4Back-Apa	huJκXFor-Not*
5b	huVκ5Back-Apa	huJκXFor-Not*
6b	huVκ6Back-Apa	huJκXFor-Not*

*huJκXFor-Not: mix of huJκ1For-Not, huJκ2For-Not and huJκ3For-Not (10 pmol/µl for each primer)

III-4-3. Combination of degenerated primers used to amplify the Variable Lambda Light chains (VLλ).

PCR 1a:

reaction	Back primer	For primer
7a	huVλ1aBack-Apa	huCλForX*
8a	huVλ1bBack-Apa	huCλForX*
9a	huVλ1cBack-Apa	huCλForX*
10a	huVλ2Back-Apa	huCλForX*
11a	huVλ3aBack-Apa	huCλForX*
12a	huVλ3bBack-Apa	huCλForX*
13a	huVλ4Back-Apa	huCλForX*
14a	huVλ5Back-Apa	huCλForX*
15a	huVλ6Back-Apa	huCλForX*
16a	huVλ7/8Back-Apa	huCλForX*
17a	huVλ9Back-Apa	huCλForX*

PCR 1b:

reaction	Back primer	For primer
7b	huVλ1aBack-Apa	huVλXFor-Not*
8b	huVλ1bBack-Apa	huVλXFor-Not*
9b	huVλ1cBack-Apa	huVλXFor-Not*
10b	huVλ2Back-Apa	huVλXFor-Not*
11b	huVλ3aBack-Apa	huVλXFor-Not*
12b	huVλ3bBack-Apa	huVλXFor-Not*
13b	huVλ4Back-Apa	huVλXFor-Not*
14b	huVλ5Back-Apa	huVλXFor-Not*
15b	huVλ6Back-Apa	huVλXFor-Not*
16b	huVλ7/8Back-Apa	huVλXFor-Not*
17b	huVλ9Back-Apa	huVλXFor-Not*

*huCλForX: mix of huCλFor1 and huCλFor2 primers(10 pmol/µl for each primer)
*huVλXFor-Not: mix of huVλ1For-Not, huVλ2For-Not and huVλ3For-Not primers (10 pmol/µl for each primer)

III-4-4. Combination of degenerated primers used to amplify the Variable Heavy chains (VH).

PCR 2a:

reaction	Back primer	For primer
18a	huVH1/7Back-Sfi	huIgGForX*
19a	huVH2Back-Sfi	huIgGForX*
20a	huVH3Back-Sfi	huIgGForX*
21a	huVH3bBack-Sfi	huIgGForX*
22a	huVH4Back-Sfi	huIgGForX*
23a	huVH4bBack-Sfi	huIgGForX*
24a	huVH5Back-Sfi	huIgGForX*
25a	huVH6Back-Sfi	huIgGForX*

PCR 2b:

reaction	Back primer	For primer
18b	huVH1/7Back-Sfi	huJHXFor-Xho*
19b	huVH2Back-Sfi	huJHXFor-Xho*
20b	huVH3Back-Sfi	huJHXFor-Xho*
21b	huVH3bBack-Sfi	huJHXFor-Xho*
22b	huVH4Back-Sfi	huJHXFor-Xho*
23b	huVH4bBack-Sfi	huJHXFor-Xho*
24b	huVH5Back-Sfi	huJHXFor-Xho*
25b	huVH6Back-Sfi	huJHXFor-Xho*

*huIgGForX: mix of huIgGFor1 and huIgGFor2 primers (10 pmol/µl for each primer).
*huJHXFor-Xho: mix of huJH1-2, 4-5For-Xho and huJH3, 6For-Xho primers(10 pmol/µl for each primer).

PCR fragments obtained after the semi-nested PCR (1b to 25b) were individually separated on a 1% agarose gel and extracted using the Gel Extraction kit (Qiagen) in a 43.5 µl H_2O eluate and stored at -20°C until cloning.

III-5. Cloning of VLκ and VLλ PCR products into the pHEN3 phagemid to generate VLκ and VLλ bacterial sub-libraries.

III-5-1. Digestion of the VLκ and VLλ PCR products (reactions 1b to 17b obtained from PCR 1b) and the phagemid pHEN3.

VL (κ and λ) PCR products (reactions 1b to 17b)	43.5 µl	pHEN3 phagemid (5 µg)	2 µl
10x Buffer 4 (ApaL1).	5 µl	10x Buffer 4 (ApaL1).	3 µl
BSA (10 mg/ml).	0.5 µl	BSA (10 mg/ml).	0.5 µl
ApaLI (10 U/µl)	1 µl	ApaLI (10 U/µl)	1.5 µl
		H_2O	23 µl
Final volume	50 µl	Final volume	30 µl

The reaction was incubated overnight at 37°C. Digested DNA was purified using the PCR Purification kit (Qiagen) and eluted in 43.5 µl H_2O. To add a second restriction site at 5´end, DNA was subjected to the second digestion reaction as following:

ApaL1-digested VL (κ and λ) PCR products	43.5 µl	ApaL1-digested pHEN3	43.5 µl
10x Buffer 3 (Not1)	5 µl	10x Buffer 3 (Not1)	5 µl
BSA (10 mg/ml).	0.5 µl	BSA (10 mg/ml).	0.5 µl
NotI (10 U/µl)	1 µl	NotI (10 units)	1 µl
Final volume	50 µl	Final volume	50 µl

The reaction tube was incubated for 6 h at 37°C and the double-digested DNA was purified using the PCR Purification kit (Qiagen) and eluted in 43.5 µl H_2O

III-5-2. Dephosphorylation of the pHEN3 vector.

The SAP (Shrimp Alkaline phosphatase) exonuclease enzyme removes the 5´phosphate groups of DNA. The treatment is performed to avoid the recircularization of the vector during the ligation reaction and, thus, to optimize the number of fragment-inserted vectors clones.

ApaL1-Not1-digested pHEN3	50 µl
10x SAP buffer	6 µl
SAP	2 µl
H_2O	2 µl
Final volume	60 µl

The tube was incubated for 1 h at 37°C and the dephosphorylated double digested pHEN3 vector was purified using the PCR purification kit.
ApaLI/NotI-digested inserts (VLκ and VLλ PCR products) and vector (pHEN3) were run on a 1% agarose gel for quantitation.

III-5-3. Ligation.

Ligation was performed as follows:

ApaLI-NotI-digested and dephosphorylated pHEN3 (10 µg)	X µl
ApaLI-NotI-digested inserts (2-4 µg)	X µl

10x T4 Ligase buffer	6 µl
T4 ligase (2 U/ µl)	2 µl
H_2O	
Final volume	60 µl

The reaction was incubated overnight at 16°C. Ligated products were concentrated through ethanol-precipitation: 1/10 vol., 3 M NaOAc pH 5 and 2.5 vol. 100% ethanol were added to the ligation reaction and the mixture was stored at –20°C for 4 h. At this time, the DNA was precipitated by centrifugation at 20 000 g for 30 min. The pellet was washed two times with 70% ethanol, air-dried and resuspended in 10 µl dH_2O.

III-5-4. Transformation of electrocompetent TG1 *E.coli* cells by electroporation and plating of VLκ and VLλ sub-libraries.

To generate the VLκ and VLλ sub-libraries, TG1 *E.coli* cells were transformed with the ligation products obtained above. Since the diversity of the sub-libraries is dependent on the efficient introduction of DNA into bacteria, the electroporation method, which is one of the most efficient transformation method, was used. This technique is based on subjecting a mixture of bacteria and DNA to an intense electric field which creates pores in the cell membrane promoting the entry of the DNA into the bacteria. 2 µl of the concentrated ligation reaction were mixed with 40 µl electro-competent TG1 cells (Stratagene) into a chilled electroporation cuvette. The mixture was electroporated using a Bio-Rad electroporator using the following settings: 1700 V, 200 Ohms and 25 µF. The mixture was then transferred into 1 ml LB medium and incubated for 1 h at 37°C while shaking. Next, 990 µl of this culture was plated onto large plates (243 mm x 243 mm) containing LB agar/1% glucose/100 µg/ml ampicillin and incubated o.n at 30°C. Large plates were used to maximize the number of recovered clones, thus, increasing the diversity of the library. Transformed bacteria were incubated at 30°C to ensure an homogeneous growth of all clones and to avoid the overgrowth of some clones compared to others. The presence of glucose ensures the repression of the Lac promoter which drives the expression of the fusion protein from the phagemid, thus, preventing growth dysregulation due to the expression of the fusion protein.

III-5-5. Estimation of the library size.

The size of each library was determined by serial dilutions. 10 µl of the electroporated cells were mixed with 990 µl LB medium and serially diluted (10^{-1} to

10^{-6}). 100 μl of each dilution was plated onto LB agar plates/1% glucose/100 μg/ml ampicillin and incubated o.n. at 30°C. The day after, clones were counted and the size of each sub-library was estimated.

III-5-6. DNA preparation of VLκ and VLλ sub-libraries.

Colonies obtained on large plates were scraped in 5 ml LB medium and used to inoculate 100ml LB medium/1% glucose/100 μg/ml ampicillin. Large scale DNA (VLκ and VLλ phagemids) of each sub-library was prepared by using the Maxipreps kit (Qiagen) according to the manufacture´s instructions and used for further steps.

III-6 Cloning of VH PCR products into VLκ-pHEN3 and VLλ-pHEN3 phagemids to generate the VLκ-VH and VLλ-VH final bacterial libraries.

VH-PCR products (reactions 18b to 25b) and VL-phagemids (from III-5-6) were digested with SfiI restriction enzyme, as described:

VLκ-pHEN3 phagemid (from Maxipreps III-5-6)	5 μg	VLλ-pHEN3 phagemid (from Maxipreps III-5-6)	5 μg
10x Buffer 2 (SfiI)	5 μl	10x Buffer 2 (SfiI)	5 μl
BSA (10 mg/ml).	0.5 μl	BSA (10 mg/ml).	0.5 μl
SfiI (20 U/μl)	1 μl	SfiI (20 U/μl)	1 μl
Final volume	50 μl	Final volume	50 μl

VH-PCR products (reactions 18b to 25b)	43.5 μl
10x Buffer 2 (Sfi1)	5 μl
BSA (10 mg/ml).	0.5 μl
SfiI (20 U/μl)	1 μl
Final volume	50 μl

All reactions were incubated overnight at 50°C and digested DNA was purified using the PCR Purification kit (Qiagen) and eluted in 43.5 μl H_2O. The second digestion reaction using the restriction enzyme XhoI was performed as below:

SfiI-digested VH-PCR products	43.5 µl	SfiI-digested VLκ-pHEN3 and VLλ-pHEN3 phagemids	43.5 µl
10x Buffer 4 (XhoI)	5 µl	10x Buffer 4 (XhoI)	5 µl
BSA (10 mg/ml).	0.5 µl	BSA (10 mg/ml).	0.5 µl
XhoI (10 U/µl)	1 µl	XhoI (10 U/µl)	1 µl
Final volume	50 µl	Final volume	50 µl

The reaction was incubated for 6 h at 37°C and double-digested DNA was purified using the PCR Purification kit (Qiagen) and eluted in 43.5 µl H_2O. The SfiI/XhoI-digested VLκ-pHEN3 and VLλ-pHEN3 phagemids were dephosphorylated as described in III-5-2. Both digested inserts and phagemids were run on a 1% agarose gel electrophoresis for quantitation. Ligation was performed as follows:

SfiI/XhoI-digested VLκ-pHEN3 and VLλ-pHEN3 phagemids (10 µg)	X µl
SfiI/XhoI-digested VH inserts (2-4 µg)	X µl
10 x T4 Ligase buffer	6 µl
T4 ligase (2U/µl)	2 µl
H_2O	
Final volume	60 µl

The reaction was incubated overnight at 16°C. Ligation products were precipitated and resuspended in 10 µl dH_2O. The VLκ-VH and VLλ-VH final libraries were constructed by transforming TG1 cells as described for the VLκ and VLλ sub-libraries in section III-5-4.

III-7. Colony PCR.

The proportion of colonies (from the sub-libraries and the final libraries) having an insert of the correct size was evaluated by PCR of single colonies using primers specific (LMB3 and fdseq1) for the pHEN3 backbone (see Fig 15). Single clones were picked up from agar plates and transferred directly into a the PCR reaction mix. An initial denaturation step of 5 min at 94°C allows the burst of bacterial cells and the release of the phagemid which can be then amplified by PCR.

colony PCR reaction mix:

10x PCR buffer	2 µl
5x Q-solution	4 µl
10mM dNTPs mix	0.5 µl
LMB3 primer (10 pmol/µl)	2 µl
fdseq1 primer (10 pmol/µl)	2 µl
Taq polymerase (5 U/µl)	0.3 µl
H_2O	9.2 µl
Final volume	20 µl

colony PCR program:

Initial denaturation	5 min	94°C
Denaturation	30 sec	94°C
Annealing	45 sec	50°C
Elongation	1 min	72°C
Final extension	10 min	72°C
Number of cycles: 35		

The presence of the insert was checked by running the PCR products on a 1% agarose electrophoresis gel.

III-8. Rescue of recombinant phages displaying scFV fragments from the final bacterial libraries.

III-8-1. Preparation of a helper phage working stock.

Helper phage stock, used to promote the rescue of recombinant phages from bacterial libraries, was prepared by propagation of an aliquot of the commercial VCSM13 helper phage (Pharmacia). VCSM13 is a helper phage which contains a slightly defective origin of replication. When the VCSM13 helper phage infects cells carrying the phagemid pHEN3, its genome is normally processed to provide *in trans* all proteins required to generate recombinant phages. However, due the mutation in the helper phage genome ori, this genome is less efficiently encapsidated than the phagemid into virion progeny, resulting in the production of recombinant phages which contain preferentially the phagemid. Nevertheless, this helper phage can be amplified to generate a working stock. In the absence of the phagemid, the helper phage genome ori is well enough encapsidated to produce sufficient new helper phages.

VCSM13 helper phages (Pharmacia) were serially diluted in LB medium and each dilution (100 µl) was mixed with 100 µl log-phase TG1 and 3 ml of top agar. The mixture was plated onto LB-agar plates and incubated overnight at 37°C. A log-phase culture is used to ensure the presence of F-pili on the surface of the bacteria and thus, to ensure the ability of those bacteria to be infected by helper phages. The semi-solid top agar overlay prevents the diffusion of bacteriophages to the whole bacteria lawn, resulting in the generation of plaques. As the M13 phage is a non lytic phage, real lysis plaques cannot be observed. Nevertheless, phage-infected cells exhibit a reduced growth rate and therefore, single "plaques" can be visualized. A single "plaque" from the lawn corresponding to an identical helper phage population was picked from the agar plate and transferred into 3 ml LB medium containing 100 µl log-phase *E. coli* TG1 cells. The mixture was shaken for 3 h allowing the infection process to take place. The grown plaque was then large-scale amplified by inoculating 500 ml LB medium containing 25 µg/ml kanamycin. The addition of antibiotics ensures the selection of bacteria carrying the VCSM13 genome which contains a kanamycin resistance gene. After overnight incubation, the culture was centrifuged and the supernatant which contains helper phages was filtered through a 0.45 µm filter, aliquoted and stored at -20°C. Those working stocks aliquots were titered as follows: 10 µl of working stock was 10 fold-serially diluted in 990 µl LB medium and 100 µl of each dilution was mixed with 100 µl log-phase TG1 and 3 ml of top agar. The mixture was plated onto LB-agar plates and incubated overnight at 37°C. Plaques were counted on each plate and used to determine the helper phage working stock titer. Stocks were stored at –70°C until subsequent use.

III-8-2. Rescue of recombinant phages from constructed scFv libraries.

100 ml LB medium/1% Glucose/ 100 µg/ml ampicillin was inoculated with TG1 cells from each library to get an initial OD_{600} = 0.1. The culture was incubated at 37°C while shaking until OD_{600}=0.5 was reached (log-phase of TG1). At this time, around 10^{12} VCSM13 helper phages (from stock produced in section III-8-1) were added to the culture and incubated for 1 h standing at 37°C and for 1 h shaking at 37°C to allow the infection process to take place. Bacteria were then pelleted by centrifugation for 10 min at 4000 g and the pellet was resuspended with 400 ml LB medium with 100 µg/ml ampicillin (to ensure the presence of the phagemid pHEN3 which encodes for the scFv-g3p fusion protein), 25 µg/ml kanamycin (to ensure the presence of the VCSM13 helper phage genome) and without glucose (to avoid the

repression of the Lac promoter and, thus, to promote the expression of the scFv-g3p fusion proteins). The culture was then incubated overnight at 30°C to allow the production of recombinant phages and their release into the supernatant. After overnight incubation, the culture was spun at 4000 g for 10 min and the supernatant was transferred into a new vessel. 1/8 volume (50ml) of a PEG_{6000}-2.5 M NaCl solution was added to precipitate phages from the supernatant. The mixture was incubated 2 h at 4°C and centrifuged at 4000 g to pellet phages. The phage pellet was resuspended with 1 ml PBS and centrifuged to pellet down all remaining bacterial debris. The supernatant was harvested, dispatched into 100µl aliquots and stored at -20°C. Phages obtained from each library were titered as following: 10 µl of the phage preparation was 10 fold serially-diluted into 990 µl LB medium and 100 µl of each dilution was incubated with 100 µl log-phase TG1 cells for 1 h standing and 1 h shaking at 37°C (to promote the infection process as described previously). 100 µl of each dilution was then spread onto LB/1% glucose/100 µg/ml ampicillin plates and incubated overnight at 37°C. The number of colonies from each plate was counted and the phage titre was calculated for each library.

III-9. Preparation and purification of YFV 17D-204-WHO particles.

III-9-1. Preparation of a YFV 17D-204-WHO master stock.

A YFV 17D-204-WHO master stock which was used for all subsequent experiments was obtained by infecting subconfluent Vero cells in a T75 flask with a commercial seed lot of the YFV 17D-204-WHO vaccine from the Robert Koch Institut, Berlin, Germany. The vaccine preparation was diluted in 5 ml DMEM without FCS and spread on Vero cells for 1 h at 37°C to allow virus adsorption. At this time, the inoculum was removed and replaced with 20 ml DMEM supplemented with 4% FCS, L-Glutamine and Penicillin-Streptomycin. 4 days post infection, the supernatant was harvested and centrifuged at 5000 g for 20 min at 4°C. This master stock was titered by plaque assay and 500 µl aliquots of the YFV 17D-204-WHO were stored at –70°C until subsequent use.

III-9-2. Titer determination by plaque assay.

The titer of the YFV 17D-204-WHO stock was determined by plaque assay. 100 µl of the stock was mixed with 900 µl DMEM without FCS. The mixture was then 10 fold serially-diluted in DMEM without FCS (10^{-1} to 10^{-7}) and each dilution was used to infect Vero cells as described above in a 6 well plate format. After 1 h at 37°C,

the mixture was removed, cells were washed once with PBS and overlaid with a mix[1:1] of 2% [v/v] low melting point (LMP) agarose and 2X DMEM supplemented with 4% FCS, 2 mM glutamine and antibiotics. Plates were kept at RT for 10 min until the agarose contained in the overlay had solidified. Plates were then incubated at 37°C for 4 days. This method using a semi-solid overlay prevents virus diffusion from one infected cell to the whole culture. As a result, small round plaques form in the cell monolayer as the virus replicates and spreads from cell to cell. For the detection of these plaques, cells were overlaid 4 days post infection with 0.005% of neutral red which is vital dye diluted in 2% LMP agarose. One day after staining, plaques were counted and the titer of the virus stock determined.

III-9-3. Purification of YFV 17D-204-WHO particles.

A T75 flask of 90% confluent Vero cells was infected with YF 17D-204 WHO viruses from the working stock at a multiplicity of infection (moi) =0.1 for 5 days at 37°C. The moi corresponds to the ratio [virus PFU / number of cells]. The supernatant was then harvested, purified by centrifugation for 10 min at 500 g and re-used to infect six T225 flasks of 90% confluent Vero cells (dilution 1:1000) for 5 days. YFV 17D-204-WHO particles contained in supernatants were purified by centrifugation at 70 000 g in a Beckmann SW28 rotor for 2 h at 4°C through a 30% glycerol cushion diluted in PBS (v/v). Pelleted glycerol-purified virions were resuspended in TNE buffer, aliquoted and stored at-70°C. Proteins were quantified using the BCA assay kit (Pierce) according to the manufacture’s instructions.

III-9-4. Determination of purified YFV 17D-204-WHO particles antigenicity in ELISA.

The antigenicity of purified YFV 17D-204-WHO virions (from III-9-3) was tested in ELISA using various sera specific for the YFV. Microtiter plates were coated o.n. at 4°C with 2.5 µg per well (in a final volume of 100 µl) either with YFV 17D-204 WHO virions or with BSA. After washing with PBS and blocking with 200 µl 2% MPBS per well, polyclonal sera, from YFV 17D-204-WHO-infected monkeys diluted 1:300 or from a human acute confirmed YF case (S-993) diluted 1:100, were added to each well for 2 h at RT. Anti YFV-E protein monoclonal antibodies 6538 and 6330 diluted 1:500 were also used. ELISA plates were then washed 3 times with PBS/ 0.1% Tween-20 and 3 times with PBS. Detection of antibody binding to YFV 17D-204-WHO particles was performed by adding for 1 h at RT either HRP-conjugated rabbit anti human IgG, HRP-conjugated rabbit anti human IgM or HRP-conjugated rabbit

anti mouse IgG, all diluted 1:1000. After 3 washes with PBS/ 0.1% Tween-20 and 3 washes with PBS, the assay was developed by adding 100µl of ABTS substrate in each well. The colorimetric reaction was stopped by adding 100µl of 0.1% SDS and the absorbance was read at 405 nm on a standard ELISA microtitre plate reader.

III-10. Biopanning.

The biopanning procedure was mainly performed as described in the laboratory manual "Antibody engineering" (Kontermann and Duebel, 2001)

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III-10-1. Selection step.

The two phage libraries were panned against glycerol-purified YFV 17D-204-WHO particles. Maxisorp immunotubes were coated o.n. at 4°C with purified YFV 17D-204-WHO virions at a concentration of 25 µg/ml in 5 ml 0.05 M carbonate buffer pH 9.6. The use of this buffer promotes the binding of virions on the plastic surface through adsorption. After washing with PBS and blocking with 2% skimmed milk in PBS (2% MPBS) for 2 h at RT, 10^{15} phage transforming units (t.u) from each library (from III-8-2) were diluted in 5 ml (2% MPBS) and incubated for 1 h at RT. The phage solution was then removed and the immunotube was washed 5 times with PBS/ 0.1% Tween-20 and 5 times with PBS (by pouring in and out). Finally, bound phages were eluted by incubating with 1 ml of a 100 mM triethylamine solution for 8 min (the acidic pH 2.2 of this buffer promotes the release of phage particles from the antigen without affecting their infectivity) and immediately neutralized with 500 µl of 1 M Tris-HCl pH 7.5. One ml of those eluted phages was incubated with 10 ml of log-phase TG1 for 1 h standing at 37°C and 1 h shaking at 37°C. Bacteria were pelleted and resuspended with 1 ml LB medium. To determine the titer of eluted phages, 10 µl of this preparation was 10 fold serially-diluted in LB medium and 100 µl of each dilution was spread onto LB agar/ 1% glucose/100 µg/ml ampicillin plates and incubated overnight at 30°C. The rest of the bacteria infected with eluted phages (990 µl) was spread on LB-agar 1% glucose/ 100 µg/ml ampicillin large plates. Those infected bacteria represented the phage population which have been "positively selected" against the YF 17D-204-WHO antigen.

III-10-2. Rescue of phages isolated from the first round of selection.

To amplify the phage population obtained after the first round of selection, bacteria which have grown on large plates were scraped in 10 ml LB medium and added to 50 ml LB medium/ 1% glucose/ 100 µg/ml ampicillin to get a starting OD_{600} = 0.1.

The culture was then incubated at 37°C until OD_{600} = 0.4-0.5. 10^{12} t.u of helper phages were added and recombinant phages were rescued, purified and titered as described in section III-8-2. The new phage population was used to perform the next round of selection against the YF 17D-204-WHO antigen as described in III-10-1. In total, four successive rounds of selection were performed using different stringency of washes (5x for the first round and 10x for the second, the third and the fourth round).

III-10-3. Polyclonal phage ELISA.

Microtiter plates were coated o.n. at 4°C with 2.5 µg per well (in a final volume of 100 µl) either with YFV 17D-204 WHO virions or with BSA. After washing with PBS and blocking with 200 µl 2% MPBS per well, 10^{13} t.u. phages of the polyclonal phage solution rescued from each round of selection were added for 2 h at RT. ELISA plates were then washed 3 times with PBS/0.1% Tween-20 and 3 times with PBS. Polyclonal phages which bound to YFV 17D-204-WHO were detected by adding 100 µl per well of horseradish peroxidase-conjugated mouse anti-M13 antibody diluted 1:2000. The assay was developed by adding 100µl of ABTS substrate. The colorimetric reaction was stopped by adding 100µl of 0.1% SDS and the absorbance was read at 405 nm on a standard ELISA microtitre plate reader.

III-10-4. Monoclonal phage ELISA.

Single clones from the third and the fourth round were picked up from the agar plates, transferred into 500 µl LB/ 1% glucose/ 100µg/ml ampicillin amd grown overnight. 5 µl of the overnight culture was used to inoculate 300µl LB/ 1% glucose /100 µg/ml ampicillin and incubated for 2 h at 37°C until OD_{600} was approx. = 0.5. At this time, 20 µl helper phages (10^{13} tu) were added in each culture and the infection process was done as described above in section III-10-1. Bacteria were pelleted, resuspended into 500 µl LB/ 100 µg/ml ampicillin/ 25µg/ml kanamycin/ without glucose and incubated overnight at 30°C. The protocol used to perform monoclonal phage ELISA was identical to the one used for polyclonal phage ELISA except that 100 µl of cleared bacterial supernatant-containing monoclonal phages were used instead of 100 µl of polyclonal phages.

III-10-5. BstNI fingerprintings.

To check scFv diversity, BstNI fingerprintings were performed. scFV fragments from monoclonal phages which were found to be positive in ELISA were PCR-amplified as described in section III-7. 10 µl of the PCR reaction was digested by BstN1 endonuclease as follows:

PCR-amplified scFv	10 µl
10X Buffer 3	3 µl
BSA (10 mg/ml).	0.3 µl
BstNI (20 U/µl)	1 µl
H_2O	15.7 µl
Final volume	30 µl

The reaction was incubated at 60°C for 4 h. BstN1 patterns from each clone were visualized on a 2 % agarose gel.

III-11. Expression and purification of scFvs in *E.coli* TG1 cells.

III-11-1. Cloning in the prokaryotic expression plasmid pAB1.

For soluble expression, scFv fragments were sub-cloned into the pAB1 plasmid. This vector introduces a His-tag at the C-terminus of the scFv and allows inducible scFv expression in the periplasmic space of *E.coli* cells. Phagemids of interest (representing each BstNI fingerprintings pattern observed above) were extracted from *E.coli* cells by the alkaline lysis procedure using the Minipreps kit (Qiagen). Each purified phagemid was digested by SfiI and NotI endonucleases as previously described to release the whole scFv fragment (VH chain-linker-VL chain). The digestion reaction was run on a 1% agarose gel and the band corresponding to scFv nucleotide sequence (approx. 700bp) was excised. DNA was extracted from agarose using the Gel Extraction kit (Qiagen) and ligated into SfiI/NotI-digested pAB1 plasmid. Ligation products were used to transform bacteria and positive clones (i.e. containing a pAB1-scFv recombinant plasmid) were identified by Miniprep screening.

III-11-2. Expression of scFvs in *E.coli* cells.

Two liters of LB medium containing 0.1% glucose and 100 µg/ml ampicillin were inoculated with 15 ml of an o.n. culture of the positive clones previously identified and grown at 37°C until OD=0.9. At this time, 0.27 g IPTG was added to the culture to a final concentration of 1mM IPTG. The culture was grown at 25°C for 4

hours to allow the induction of the Lac Z promoter by IPTG, the expression of scFvs and their accumulation in the periplasmic space of the bacteria. The culture was then centrifuged at 5000 g for 10 min at 4°C. The supernatant was removed and the bacterial pellet was resuspended in 120 ml of hypertonic solution (20% sucrose) to allow the disruption of the outer bacterial membrane, resulting in the release of scFvs from the periplasmic space. To ensure the complete cell membrane disruption, 5 mg/ml of lyzozyme was also added and the mixture was left on ice for 30 min. At this time, 1 M MgSO4 was added to stabilize the spheroplasts (bacteria without their outer membrane) and the mixture was centrifuged at 15 000 g for 10 min at 4°C. The supernatant containing soluble scFvs was harvested and dialyzed overnight against 2 liters of PBS/ 20 mM imidazole. This step allows the reduction of sucrose concentration and the enrichment with imidazole, which is used for further purification of scFv.

III-11-3. ScFv purification by Immobilized Metal ion Affinity Chromatography (IMAC).

The dialyzed scFv solution was centrifuged at 20 000 g for 10 min at 4°C and the supernatant was incubated with 3 ml Ni-NTA solution for 1 h rotating at 4°C. During this step, scFvs with a fused Histidine tag at the C terminus will bind to the Ni^{2+}-ions. The mixture was then loaded onto a filter column. Contaminants and other proteins pass through the column, whereas Ni-NTA-scFvs complexes are retained. The column was then washed with 60 ml washing buffer (containing 35 mM imidazole) to further remove all contaminants which did not interact specifically with the Ni-NTA matrix. Finally, scFvs were eluted by adding 7 ml of elution buffer (containing 150 mM imidazole). Fractions of 1 ml each were collected in different tubes.

III-11-4. Determination of scFvs by Coomassie staining and Western Blot analysis.

To determine the fractions containing the highest amounts of scFvs and to check the purity, 15 µl of each fraction were run on a 12 % SDS PAGE. The scFvs were detected by Western Blot and their purity was evaluated by Coomassie staining.

III-11-4-1. SDS-Polyacrylamid gel electrophoresis (SDS-PAGE).

Protein samples were separated on a 12% denaturing polyacrylamid gel. 10µl of protein sample were mixed with 2.5µl of 4x SDS PAGE sample buffer and denatured

for 5 min at 95°C. The polyacrylamid gel was cast using a 6% stacking gel and a 12% resolving gel. The sample was loaded on this polyacrylamid gel and run until separation was sufficient. 5µl of a protein marker was run in parallel.

III-11-4-2. Coomassie staining.

After running, the SDS-PAGE gel was soaked in a G250 Coomassie solution, incubated for 30 min and washed extensively with the Coomassie destaining solution.

III-11-4-3. Western blot (WB) analysis.

ScFv proteins were selectively detected by Western Blot using an antibody specific for the his-tag located a the C-terminus. To this aim, proteins were run on a 12% SDS-PAGE and transferred onto a nitrocellulose membrane by electroblotting as follows:

Anode

	3 Whatman paper soaked in Anode buffer I 3 Whatman paper soaked in Anode buffer II
	Nitrocellulose membrane soaked in dH_2O
	Gel
	6 Whatman paper soaked in Cathode buffer

Cathode

Transfer conditions: 43 mA per membrane (9 cm x 6 cm) for 1h

After the transfer, the membrane was blocked with 5% MPBS for 1 h at RT. The first antibody (anti His-tag mouse antibody) diluted 1:100 in 5% MPBS was added to the membrane for 1h at RT. Unbound antibodies were removed by washing 3 times (10 min each wash) the membrane with PBS/ 0.1% Tween. HRP-conjugated goat anti mouse antibody was diluted 1:2000 in 5% MPBS and incubated with the membrane for 1h at RT. At this time, the membrane was washed again 3 times (10 min each wash) with PBS/ 0.1% Tween. The Super Signal Kit (Pierce) was used as a substrate for the peroxidase. After a 5 min incubation time with the substrate, the membrane was exposed to a film (Amersham Pharmacia) allowing the detection of the scFvs.

III-11-5. scFv ELISA.

The protocol used to perform scFv ELISA was identical to that used for polyclonal phage ELISA except that 2 µg of scFv were used instead of 100 µl of polyclonal phage solution. Detection of scFv binding to YFV 17D-204-WHO particles was performed using anti His-tag mouse antibody diluted 1:100 for 1 h at RT. After 3 washes with PBS/0.1% Tween-20 and and 3 washes with PBS, HRP-conjugated goat anti mouse antibody diluted 1:2000 was added. 100 µl/well of ABTS was added as a substrate for the peroxidase and the absorbance was measured at 405 nm using a ELISA spectrophotometer.

III-12. Dot Blot analysis.

10 µl of 17D-204-WHO antigen (from section III-9-3) were mixed with 10 µl of each detergent-containing solution and incubated for 20 min at RT. At this time, a drop of this preparation was allowed to adsorb on a nitrocellulose membrane by air-drying. The membrane was blocked by adding a 5% MPBS for 1 h at RT. 100 µl of scFv were then incubated with the blotted membrane for 2 h at RT. After washes with PBS/0.1 % Tween, the anti-His-tag mouse antibody diluted 1:100 was added and incubated at RT for 1h, followed by HRP-conjugated anti-mouse goat antibody diluted 1:2000. Binding of scFv to YFV was visualized by incubation with RecBlot/Immunoblot substrate.

III-13. Radioimmunoprecipitation assay (RIPA).

III-13-1. Production of radiolabeled soluble viral proteins from radiolabeled YF 17D-204-WHO virions.

A 90% confluent T75 flask with Vero cells was infected with YFV 17D-204-WHO at moi=1. 24 h post infection, the culture medium was removed and replaced with cysteine and methionine-free DMEM supplemented with 2% FCS, 2 mM glutamine and antibiotics for 1 h at 37°C. At that time, 1 mCi of [^{35}S] methionine and [^{35}S] cysteine was added. After 48 h, the culture supernatant was harvested and radiolabeled virions were purified through a 30 % glycerol cushion as described above (III-9-3). Pelleted virions were resuspended in TNE buffer containing 0,5 % n-octyl-β-D-glucopyranoside with a protease inhibitor cocktail and sonicated 3 times for 10 sec at 4°C. The lysed radiolabeled virion preparation was centrifuged at 20 000 g for 1 h at 4°C and the supernatant was used for immunoprecipitation.

III-13-2. RIPA.

150 µl of supernatant containing soluble radiolabeled YF viral proteins (from III-13-1) were incubated with 1.5 µg of scFv for 4 h at 4°C. Antigen-scFv complexes were immunoprecipitated by addition of 1 µg anti His-tag mouse antibody and 20 µl protein G-coupled agarose beads overnight, rotating at 4°C. As positive control, the radiolabeled viral protein mixture was incubated with 1 µg of YFV-E specific mAbs 6538 or 6330 and 20 µl protein G-coupled agarose beads. As negative controls, soluble radiolabeled YF viral proteins were incubated either with 1.5 µg of irrelevant scFv, 1 µg of anti His-tag mouse antibody and 20 µl protein G-coupled agarose beads, or with 1 µg anti His-tag mouse antibody and 20 µl protein G-coupled agarose beads only. The agarose beads were pelleted and washed 3 times with TNE buffer containing 0.5 % n-octyl-β-D-glucopyranoside with the protease inhibitor cocktail and once with Tris-HCl buffer pH 7.5. Samples were resuspended in 15 µl of Laemmli buffer, boiled 5 min, centrifuged 5 min and separated on a 12% SDS-PAGE gel as described above. The gel was fixed in 20 % methanol-10% acetic acid solution, dried and exposed to X-ray film for the detection of YFV radiolabeled proteins which were immunoprecipitated by scFvs.

III-14. pH sensitivity experiments.

The ability of scFvs to bind their epitopes upon low pH treatment was performed as follows. 2.5 µg (10µl) of purified YFV 17D-204-WHO virions were coated o.n. at 4°C in an ELISA plate as described previously. pH treatment of the YFV particles was performed by adding either 100µl of a pH 5 buffer (0.05 M MES, 0.1 M NaCl) or 100 µl of a pH 8 buffer (TNE buffer) for 1 h at 37°C. At this time, the preparation was back-neutralized by adding 10 µl of pH 8 buffer (1 M Tris-HCl, pH 8). Ability of mAbs or scFvs to bind YFV antigen was performed by ELISA as described in section III-11-5.

III-15. Competition ELISA using biotinylated antibodies.

III-15-1. Biotinylation.

ScFv-7A were biotinylated using the EZ-Link Sulfo-NHS-LC-Biotinylation Kit (Pierce). 10 µg of purified scFv-7A were mixed with Sulfo-NHS-LC-Biotin and

incubated for 2 h on ice to allow the coupling of N-terminal group of scFvs with NHS-biotin to obtain biotinylated scFvs. Removal of excess of biotin and the purification of biotinylated scFvs were performed by dialyzing the samples o.n. against PBS.

III-15-2. Competition ELISA.

2 µg of biotinylated scFv-7A were mixed with increasing amounts of non-biotinylated competitor, either scFvs-5A, R3(27), 1A, 2A, R3(9) or mAbs 6538, 6330. The mixture was then added to a microtiter plate coated with YFV-17D-204 WHO virions as described in section III-9-4 and incubated for 1 h at RT. The plate was washed 3 times with PBS/ 0,1 %Tween and 3 times with PBS. Bound biotinylated scFv-7A were detected by using HRP-conjugated streptavidin diluted 1:10 000. Competition was expressed as the percentage of binding after incubation with competitors compared to biotinylated scFv-7A alone.

III-16. Plaque reduction neutralization test (PRNT).

The ability of each scFv to block YFV infection was tested by a plaque reduction neutralization assay (PRNT) using PS cells (porcine kidney) or Vero cells (African Green Monkey kidney). 100 PFU of YFV 17D-204-WHO from the virus stock diluted in DMEM without FCS, supplemented with 2 mM glutamine and antibiotics were incubated for 1 h at 37°C with various amounts of each scFv to obtain final concentrations ranging from 0.07 µg/ml to 10 µg/ml scFv. As a control, 100 PFU were diluted and incubated for 1h at 37°C without scFv. The virus-scFv mixture was spread on 90 % confluent PS cells or Vero cells in a 6 well plate format to allow adsorption. After 1 h at 37°C, the mixture was removed, cells were washed once with PBS and overlaid with 2% LMP Agarose-2X DMEM [1:1] supplemented with 4% FCS, 2 mM glutamine and antibiotics. Plates were incubated at 37°C for 4 days. For plaque detection, cells were overlaid with 0.005% neutral red dye. Plaques were counted and the percentage of neutralization was expressed as: 100 – [(number of plaques virus + scFv / number of plaques virus alone) x 100].

III-17. Generation of escape mutants.

To map the neutralizing epitopes on the envelope E protein, scFv-7A neutralization-resistant virus variants were generated. For this purpose, the YFV-17D-204-WHO strain was passaged three times in Vero cells in the presence of sub-neutralizing

concentrations of scFv-7A. After the third passage, escape mutants were plaque-purified and tested in PRNT. Practically, 100 PFU of YFV 17D-204-WHO were incubated for 1 h at 37°C with 95% of the scFv-7A concentration which had been shown to completely neutralize the inoculum (7µg/ml, see results). The mixture was inoculated on Vero cells for 1 h at 37°C in a 6 well plate format. The inoculum was removed and replaced with fresh medium supplemented with scFv-7A (7µg/ml). Three days post-infection, 50 µl of the supernatant were incubated with 1ml DMEM /scFv-7A (7 µg/ml) for 1 h at 37°C and inoculated on cells for 1 h at 37°C. After removing the inoculum, fresh medium containing scFv-7A (7 µg/ml) was added. This selection procedure was repeated twice. After the third round of selection, twenty single clones were picked up from the plate, propagated, plaque-titered and tested in PRNT with various concentrations of scFv-7A as described in section III-16.

III-18. Microneutralization assay.

To test the ability of sera from wild-type YFV-infected patients or from YFV 17D-204-WHO-vaccinated donors to neutralize escape variants, microneutralization assays were performed.

III-18-1. Determination of the 50% Tissue Culture Infectious Dose ($TCID_{50}$).

Each virus was 10-fold serially diluted in DMEM without FCS and 100 µl of each dilution was added to 2.5 x 10^4 Vero cells in a 96 well plate format and incubated at 37°C for 1 h. Each dilution was tested in five replicates. The inoculum was then removed and cells were washed 3 times with PBS. 100 µl of DMEM supplemented with 4% FCS was added to each well and the plates were incubated at 37°C. Six days post infection, the cytopathic effect (CPE) was observed in each well and the $TCID_{50}$ for each virus was calculated according to the Reed-Muench formula:

$TCID_{50} = 10^{\text{log total dilution above 50\%} - (I \times \log h)}$.
I (interpolated value of the 50% endpoint) = (% of wells infected at dilution above 50% - 50%)/(% of wells infected at dilution above 50% - % of wells infected at dilution below 50%).
h = dilution factor.

III-18-2. Microneutralization assay.

Sera were inactivated for 30 min at 56°C and serially diluted in DMEM without FCS on a log2 scale. 25 µl of each dilution were mixed with 25 µl of virus corresponding to 100 $TCID_{50}$ and the mixture was incubated at 37° C for 1h. The mixture was then spread on $2.5x10^4$ Vero cells and incubated for 1h at 37°C. Cells were then washed 3 times with PBS, 100 µl of fresh medium was added and the CPE was observed in each well six days post infection. Each dilution was tested in five replicates. The 50% end-point dilution of each serum, corresponding to the dilution at which 50% of wells were completely protected from infection, was determined according to the Reed-Muench formula as described above.

III-19. Sequencing analysis.

100 ng of the DNA (PCR products) to be sequenced were mixed together with 10 pmol of the primer and 6 µl of dH_2O. The sequencing analysis was performed through technical assistance using a MegaBACE sequencer (Amersham Pharmacia).

III-19-1. Sequencing analysis of scFvs displayed by YFV-17D-204-WHO specific monoclonal phages

Phagemids from bacterial clones, from which YF-17D-204-WHO specific monoclonal phages were rescued, were PCR amplified using LMB3 and fdseq1 primers. Those primers are specific for the pHEN3 backbone and are located upstream and downstream of the cloned scFv as shown in Fig 15. PCR products were directly sequenced in both directions using either the LMB3 or fdseq1 primers.
The closest germline gene for VL and VH chains were identified by a Blast search of the antibody-database IgBlast (http://www.ncbi.nlm.nih.gov/igblast/).

III-19-2. Sequencing analysis of prM and E proteins of all YFV strains (17D-204 WHO, wild-type strains and escape mutants).

Viral RNA of the different viruses was isolated from cell culture supernatants using the QIAamp viral RNA mini kit (QIAGEN). The E protein of the neutralization escape mutants was amplified by RT/PCR and sequenced as described above. RT-PCR and sequencing of the M protein were performed using a prM forward primer and an M reverse primer. Wildtype YFV strains were first genotyped by amplifying a 670 nucleotide fragment within the prM-E region. The rest of the E protein was sequenced using two sets of primers that generate two overlapping fragments (see section II-11-3 in Material). Primers were designed by aligning wild-type YFV

sequences accessible in the GenBank and designated EnvF1, EnvF2, EnvF3, EnvR1, EnvR2, NS1R1 and NS1R2. Due to the sequence variability of the envelope protein gene, the East/Central African strains were amplified using primer combinations EnvF2/EnvR1 and EnvF3/NS1R2, and the West African strains using EnvF1/EnvR2 and EnvF3/NS1R1.

III-20. Molecular Modelling.

The sequence of the YFV-E protein and the molecular location of substitutions were modeled on the 3D structure of the Dengue virus type 2 envelope glycoprotein (SWIS-prot PDB code:1K4R) using the RasMol software (www.umass.edu/microbio/rasmol).

IV-RESULTS

IV-RESULTS

IV-1. Generation of two phage libraries displaying recombinant antibody fragments (scFvs) from two recovered yellow fever patients.

IV-1-1. Amplification of the Variable Kappa light chains (VLκ), the Variable Lambda light chains (VLλ) and the Variable Heavy chains (VH) genes by PCR.

The light chain variable domains (kappa locus and lambda locus) of the whole antibody repertoire of each patient (YF2 and YF3) were PCR-amplified from total RNA extracted from circulating lymphocytes using combinations of degenerated primers (see Materiel and Methods) by a semi-nested PCR. As seen in Fig. 17, all primer combinations were able to amplify VLλ and VLκ domains (350 bp). The amplification of heavy chain variable domains (VH) was performed with the same method using VH-specific primers. All combinations amplified a 400 bp fragment corresponding to VH-segments. Some differences in amplification efficiency are observed due to the varying of some degenerated primers to anneal to the template. This underlines the great variability especially of the VH genes.(Fig 17, PCR reactions 11b, 13b, 18b, 19b, 24b).

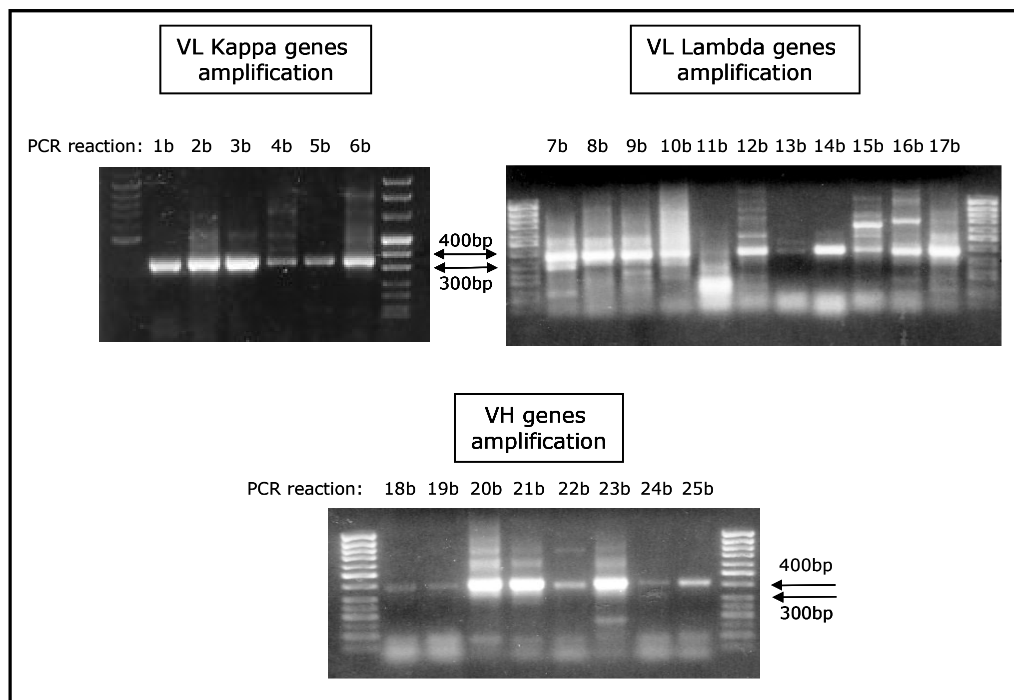

Fig 17. PCR amplification of genes encoding for variable domains of light chains (Kappa and Lambda) and heavy chains.

IV-1-2. Construction of bacterial scFv libraries and rescue of recombinant phages displaying scFvs on their surface.

IV-1-2-1. Construction of bacterial VLκ-pHEN3 and VLλ-pHEN3 sub-libraries.

The size of each sub-library was determined by serial dilutions and found to range between 4.10^6 and 3.10^7 individual clones. The presence of the insert was found in more than 85% of tested clones.

	VLκ sublibrary-YF2	VLλ sublibrary-YF2	VLκ sublibrary-YF3	VLλ sublibrary-YF3
Size	3.10^7	4.10^6	4.10^6	4.10^6
% insert	90%	90%	85%	92%

IV-1-2-2. Construction of bacterial VLκ-VH-pHEN3 and VLλ-VH-pHEN3 final libraries.

The size of each final library was found to range between 2.10^6 and 10^7 individual clones. PCR screening identified the presence of an entire scFv (VH-VL) in more than 80 % of total clones.

	VLκ-VH-pHEN3 library-YF2	VLλ-VH-pHEN3 library-YF2	VLκ-VH-pHEN3 library-YF3	VLλ-VH-pHEN3 library-YF3
Size	5.10^6	6.10^6	2.10^6	10^7
% insert	80%	85%	90%	80%

IV-1-2-3. Rescue of recombinant phages from YF2 and YF3 libraries.

Each final bacterial library (VLκ-VH-pHEN3 and VLλ-VH-pHEN3 libraries from YF2 and YF3 donors) was infected with helper phages to produce recombinant phages displaying scFvs on their surface. The phage titer was determined for each final library:

	VLκ-VH-pHEN3 library-YF2	VLλ-VH-pHEN3 library-YF2	VLκ-VH-pHEN3 library-YF3	VLλ-VH-pHEN3 library-YF3
Phage Titer	10^{16} t.u/ml	10^{15} t.u./ml	10^{13} t.u./ml	10^{13} t.u./ml

All four libraries were then used for biopanning against purified YFV 17D-204-WHO virions.

IV-2. Isolation of monoclonal phages with a specific affinity for YFV 17D-204-WHO particles.

IV-2-1. Purification of YFV 17D-204-WHO particles.

To isolate recombinant phages displaying scFvs specific for YFV, large scale-produced YFV-17D-204-WHO virions were purified through a 30% glycerol cushion. To test the quality of the antigen to be used in further selection steps, purified YFV virions were coated on a 96 well ELISA plate at different concentrations. For detection, a polyclonal serum from YFV 17D-infected monkeys, two mouse monoclonal antibodies specific of the YFV-E protein and one human serum isolated from an acute confirmed YF case were employed. As a negative control, a polyclonal from a normal donor (i.e. whithout YFV antibodies) was used. As seen in Fig. 18, all sera specific for YFV react with the purified YFV-17D-204-WHO virions, whereas the irrelevant serum does not. Thus, this result showed that the purified YFV 17D-204-WHO particle preparation was a suitable antigen which could be further used for the biopanning.

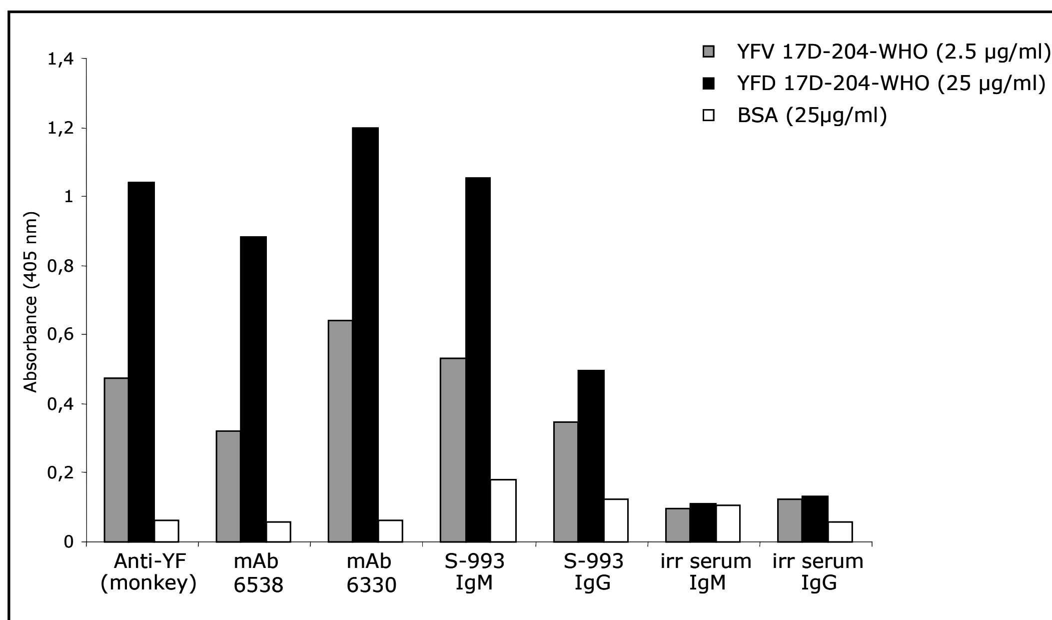

Fig 18. Antigenicity of purified YFV-17D-204-WHO particles in ELISA. Anti YF (monkey): polyclonal serum obtained from YFV 17D-204-WHO-infected monkeys. mAb 6358 & 6330: anti YFV E protein mouse monoclonal antibodies. S-993: serum isolated from a human acute YF case. irr serum: naïve human serum. IgG:immunoglobulines G, IgM: immunoglobulines G.

IV-2-2. Enrichment of specific phage binders to the YFV antigen through biopanning.

In order to isolate recombinant phages displaying scFv with a specific affinity for YFV, selection steps were performed as follows. Recombinant phages rescued from all constructed libraries were pooled and panned against purified YFV 17D-204-WHO particles. This antigen was used because it can be handled under biosafety level 2 conditions without any chemical or physical inactivation. Thus, sensitive conformational epitopes are protected from denaturation. Four rounds of selection were performed successively, in order to enrich phages specific for the YFV antigen. Expanded phages from each round (polyclonal phages) were then tested in ELISA for their ability to bind the YFV antigen.

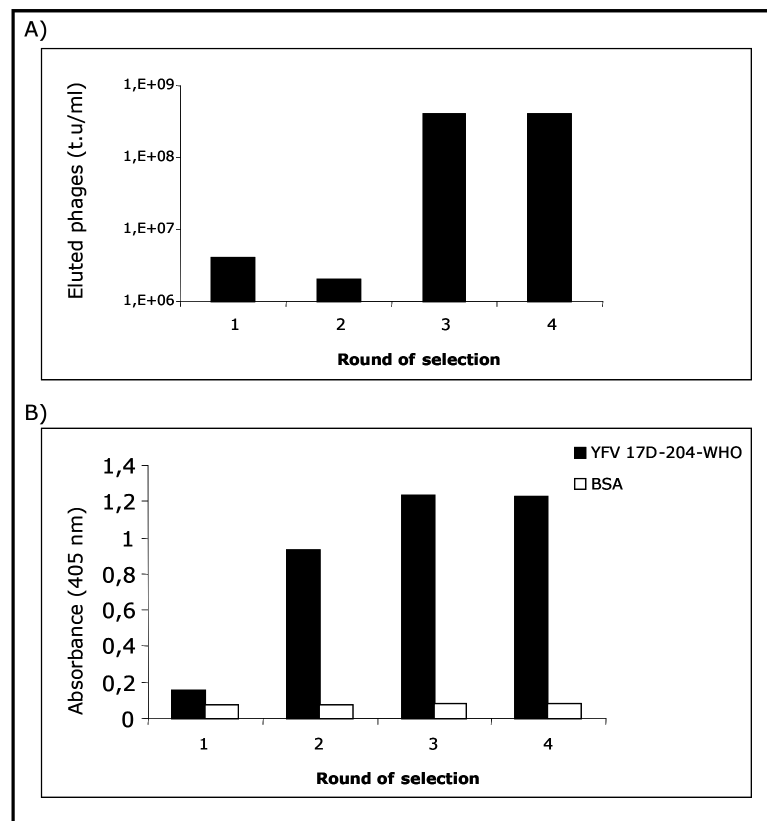

Fig 19. Biopanning of YF-patient-derived antibody phage libraries against the YFV-17D-204-WHO antigen. A)Titer of eluted phages isolated from each round of selection after panning against the antigen. B)Polyclonal phage ELISA using rescued phages isolated after each round of selection. BSA was used as a negative control antigen.

As shown in Fig 19A, the number of bound phages strongly decreased between the first and the second round and strongly increased during the second and the third round which shows a characteristic enrichment of specific binders for the antigen.

This enrichment process was confirmed by the polyclonal phage ELISA (see Fig 19B), which showed an increase of the signal representing a increase of the binding activity of the polyclonal phages for the YFV antigen. The plateau after the third round suggests that the maximal binding activity was obtained after three rounds of selection. Taken together these data show that the selection step succeeded and that three successive rounds of selection were sufficient enough to allow an optimal enrichment of phages with a specific affinity for the YFV 17D-204-WHO antigen.

IV-2-3. Screening of monoclonal phages from round 3 and round 4 of the selection step.

Monoclonal phages were rescued from 58 single bacterial clones which were obtained during rounds 3 (30 clones) and 4 (28 clones) of the selection step. Their binding activity to YFV 17D-204-WHO particles was tested in ELISA. As shown in Figure 20, 47 monoclonal phages (81%) were found to bind specifically the YFV antigen in ELISA (21/30 (70%) from round 3 and 26/28 (93%) from round 4). These results show that several monoclonal phages specific for the YF 17D-204 antigen were isolated.

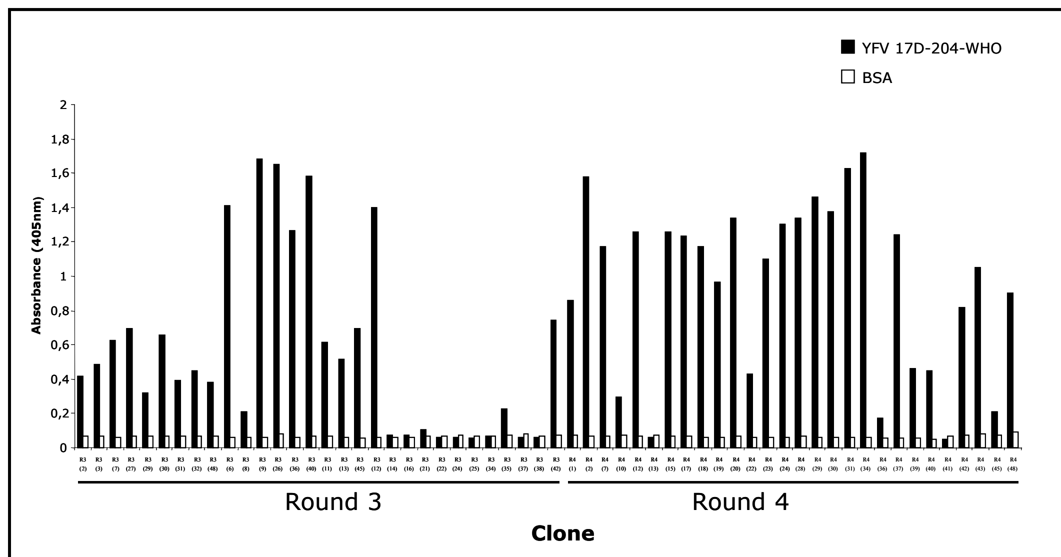

Fig 20. Reactivity of 58 monoclonal phages isolated from round 3 and round 4 during the biopanning against purified 17D-204-WHO virions. As negative control antigen, BSA was used.

IV-2-4. Genetic diversity of scFvs displayed by YFV 17D-204-WHO-specific binders.

To check the diversity of scFvs displayed by these 47 YFV-specific monoclonal phages, BstN1 fingerprinting was performed. BstNI is an endonuclease which recognizes and cleaves a frequent site on DNA. Thus, this enzyme is useful to evaluate simultaneously the diversity of several DNA sequences, by comparing their restriction pattern after digestion. Phagemids of all YFV-specific monoclonal phages were isolated, digested with BstNI, and restriction fragments were run on agarose gel for comparison. Results are summarized in Table 3. From 47 clones, six different BstNI patterns were observed. All of them except pattern 4 were present in round 3 and in round 4.

BstN1 pattern	round	frequency	clone
1	3 & 4	12/47 (25.5%)	7A
2	3 & 4	8/47 (17%)	R3(27)
3	3 & 4	7/47 (14.9%)	5A
4	3	1/47 (2.1%)	1A
5	3 & 4	6/47 (12.8)	2A
6	3 & 4	13/47 (27.7%)	R3(9)

Table 3: Diversity of monoclonal phages specific for YFV tested by BstN1 fingerprintings.

One clone representing each BstN1 pattern (7A, R3(27), 5A, 1A, 2A and R3(9)) was chosen and used for further characterization.

IV-3. Expression and purification of six different scFvs (7A, 5A, R3(27), 1A, 2A and R3(9)) with a specific affinity for YFV 17D-204-WHO virions.

IV-3-1. Expression and purification of scFv-7A, 5A, R3(27), 1A, 2A and R3(9) as soluble molecules.

scFvs sequences from each of these six YFV-specific monoclonal phages were isolated and cloned into the prokaryotic expression plasmid pAB1. This plasmid contains a lac Z promoter to allow IPTG induction, a pelB leader sequence to allow the release of expressed scFv into the periplasmic space of the bacteria and a Myc-tag and a His-tag for detection. ScFvs were expressed in E.coli TG1 cells upon IPTG induction and purified from periplasmic fractions by IMAC chromatography as described in Methods. Purity of each elution fraction was tested by Coomassie staining and Western Blot using an anti-His tag monoclonal antibody. As seen in Fig

21, each eluted fraction contains only 32 kDa molecules which correspond to pure scFvs. Fractions 2, 3, 4 and 5 were pooled for each scFv, dialyzed and used for further experiments. scFvs were quantified using the micro BCA kit (Pierce) and the quantity of purified scFvs were found to range between 50 µg to 270 µg of proteins per liter of bacterial culture, the efficiency varying between each clone.

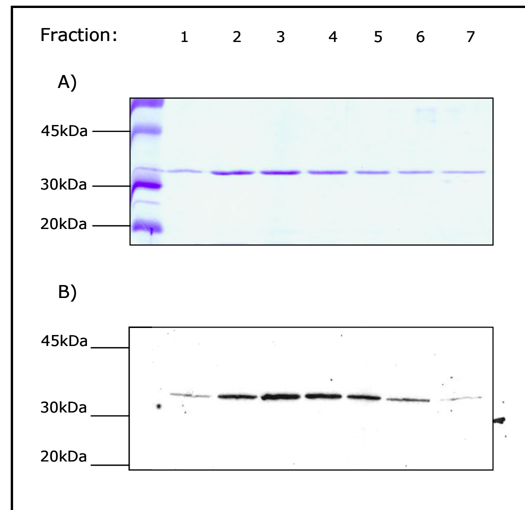

Fig 21. Expression and purification of scFvs in *E. coli* cells. 10 µl of each eluted fractions (1 to 7) were separated by SDS-PAGE (12% polyacrylamide) and scFvs were detected by A) Coomassie staining and by B) Western Blot using an anti His-tag mouse antibody.

IV-3-2. Reactivity of soluble scFv-7A, 5A, R3(27), 1A, 2A and R3(9) with the YFV 17D-204-WHO antigen.

To test whether these scFvs, when expressed as soluble molecules, were able to bind the YFV antigen, an ELISA was performed. As shown in Figure 22, all scFvs have an intrinsic capacity to bind YF 17D-204 WHO virions and this binding was specific, no binding being observed with BSA.

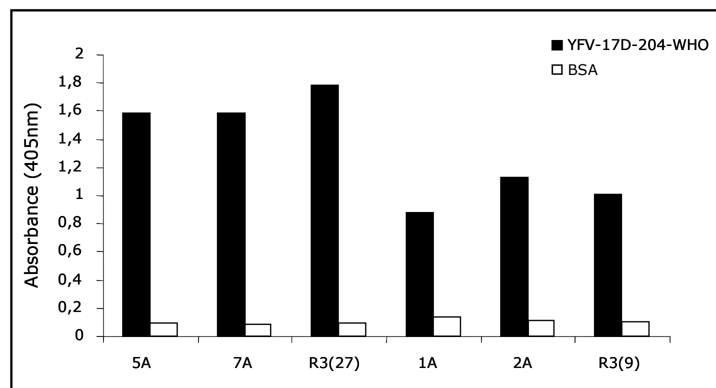

Fig 22: scFv ELISA. Purified soluble scFvs from YFV-specific monoclonal phages were tested for their reactivity against the YFV 17D-204-WHO antigen in ELISA. As negative control antigen, BSA was used.

IV-3-3. Sequencing analysis of scFvs-7A, 5A, R3(27), 1A, 2A and R3(9).

To identify the nucleotide sequences of each scFv, a sequencing analysis was performed. As mentioned previously, the scFv molecule is composed of a variable domain of the heavy chain (VH) and a variable domain of the light chain (VL) of antibodies. Sequencing analysis of YFV-specific scFvs revealed usage of only two different VH and three different VL germline genes published in the database. According to their nucleotide sequence relationships, the scFvs could be divided into 2 groups (Fig. 23). The first group includes scFvs-7A, 5A and R3 (27). Their CDR regions of the VH domains were identical and closely related to the germline gene VH4-59. Some differences were observed in the FR1 and FR4 regions, probably due to the usage of degenerated primers for PCR amplification. The VL domains of these three scFvs differ from each other by up to 12 substitutions and are closely related to germline gene VKL-12. The substitutions are mainly located in CDR2 and CDR3. The second group is composed of scFv-1A, 2A and R3(9). The VH chains of these three scFvs were almost identical and closest to the germline gene VH3-11. The VL chains of scFv-1A and scFv-2A are related to the same germline gene VL1-19. Substitutions were located in CDR2, CDR3 and FR4 regions. The VL chain of R3(9) was different from those of scFv-1A and 2A and closely related to the germline gene VL2-14.

VH

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	Closest germline V-gene
7A	EVQLVESGPGLVKPSETLSLTCTVSGGSTY	NHHWS	WIRQPPGRGLEWIG	YISYSGKSNYNPSLKS	RVTISLEPSTTQFSLKLNSLTAADTAVYYCAR	EYRDDTNYYYYSLDV	WGPGTLVTVSS	VH4-59
R3(27)	----------------A-------------	-----	--------------	----------------	--------------------------------	---------------	-----T-----	VH4-59
5A	---------R--------------------	-----	--------------	----------------	--------------------------------	---------------	-----M-----	VH4-59

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	
1A	QVQLVESGGGLVKPGGSLRLSCAASGFTFS	DYYMS	WIRQAPGKGLEWVS	YVTSSGRTKYYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR	PQEASEAFDI	WGQGTLVTVSS	VH3-11
2A	------------------------------	-----	--------------	-----------------	--------------------------------	----------	-----------	VH3-11
R3(9)	E-----------------------------	-----	--------------	-----------------	--------------------------------	----------	-----M-----	VH3-11

VL

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	
7A	QIQMTQSPSTLSASVGDRVTITC	RASQSISSWLA	WYQQKPGKAPNLLIY	KASNLET	GVPSRFSGSGSGTEFTLTITSLQPDDFATYYC	QQYNSFPIT	FGQGTRLEIKRAAA	VκL-12
R3(27)	-----------------------	-----------	---------------	---S--S	------------------------D-------	----SYPFT	--P--K--------	VκL-12
5A	--V--------------------	------G----	---------------	---S--S	------------------------E-------	----NYSYT	--P--K--------	VκL-12

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	
1A	QSVLTQPPSVSAAPGQKVTISC	SGSSSNIGNNYVS	WYQQLPGTAPKLLIY	DNDKRPS	GIPDRFSGSKSGTSATLGITGLQTGDEADYYC	GTWDTSLSAPYV	FGTGTKVTVLGAAA	VL1-19
2A	----------------------	-------------	---------------	-------	--------------------------------	----SSLSAGSYV	------L-------	VL1-19

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	
R3(9)	QSVLTQPPSVSVAPGQTARITC	GGNNIGSKSVH	YQQKPGQAPVLVV	YDDSDRPS	GIPERFSGSNSGNTATLTISRVEAGDEADYYC	QVWDSSSDHSYV	FGTGTQLTVLGAAA	VL2-14

Fig 23: Alignment of the variable heavy (VH) and the variable light chains (VL) of the YFV-specific scFv-7A, 5A, R3(27), 1A, 2A and R3(9). Framework regions (FR) and complementarity-determining regions (CDR) are indicated in bold. The closest germline V-gene for each chain was identified using IgBLAST (http://www.ncbi.nlm.nih.gov/igblast/).

IV-4. Identification of the YFV proteins recognized by the scFvs-7A, 5A, R3(27), 1A, 2A and R3(9)

IV-4-1. Western Blot analysis.

To identify the viral proteins which are recognized by these scFvs, Western blot analysis using YFV 17D-204-WHO particles was performed. Proteins from purified YFV 17D-204-WHO particles were separated on SDS-PAGE and transferred onto a nitrocellulose membrane. YFV proteins were probed in Western Blot using each of the soluble scFv. As a positive control, two monoclonal antibodies specific for the YFV E protein (MAb 6538 and MAb 6330) were used. As seen in Fig. 24, none of the six scFvs was able to recognize any YFV protein. Only one of the two mAbs (mAb 6330) was able to detect a 55 kDa protein which corresponds in site to the YFV envelope glycoprotein (E protein). This result strongly suggests that all six scFvs recognize YFV proteins in their native conformation and not under denaturing conditions which are obtained after SDS and beta-mercaptoethanol treatment.

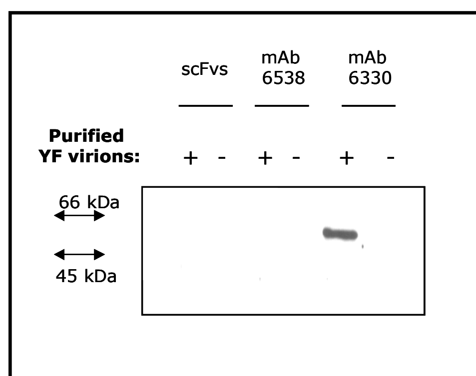

Fig 24: Reactivity of scFvs with YFV proteins in Western Blot. YFV proteins from purified virions were separated on a 12% SDS-PAGE, transferred onto a nitrocellulose membrane and blotted using scFvs or two E-specific mAbs (mAb 6538 and mAb 6330).

IV-4-2. Dot Blot analysis.

To circumvent the problem of protein/epitope denaturation following SDS-PAGE analysis, an immunoprecipitation assay using scFvs and soluble viral proteins was performed. The critical step of this experiment was to find a balance between solubilizing the proteins in their native conformation and not disrupting the antigen-antibody interaction. Therefore, the choice of the detergent used to solubilize virions was important. Preliminary Dot Blot experiments were performed in order to identify a detergent which preserves the integrity of viral proteins after

solubilization. For this purpose, YFV 17D-204-WHO virions were solubilized in the presence of several ionic and non-ionic detergents and directly blotted onto a nitrocellulose membrane. scFvs were then added to check their ability to recognize their epitopes after treatment. As shown in Table 4, a treatment with non-ionic detergents like Tween-20 or β-octyl-n-D-glucopyranoside did not interfere with the scFv-YFV binding and could be used to perform immunoprecipitation. Thus, β-octyl-n-D-glucopyranoside was used for further experiments.

	scFv 7A	scFv 5A	scFv R3(27)	scFv 1A	scFv 2A	scFv R3(9)	mAb 6538	mAb 6330
Western Blot:	-	-	-	-	-	-	-	+
Dot Blot:								
TNE	+	+	+	+	+	+	+	+
"RIPA buffer": TNE +10% Triton-X100 +10% DOC +10% SDS	-	-	-	-	-	-	-	+
TNE + 1% NP40	-	-	-	-	-	-	+	+
TNE + 1% Triton X-100	+/-	+/-	+/-	+/-	+/-	+/-	+	+
TNE + 1% Tween-20	+	+	+	+	+	+	+	+
TNE + 1% β-octyl-n-D-glucopyrannoside	+	+	+	+	+	+	+	+

Table 4: Reactivity of scFvs with YFV proteins solubilized under different reaction conditions. Purified 17D-204-WHO virions were treated with buffers containing various detergents (ionic and non-ionic) and blotted onto a nitrocellulose membrane. Each scFv was incubated with the blot and bound scFv were detected with an anti His-tag antibody.

IV-4-3. Radioimmunoprecipitation assay (RIPA).

Since the epitopes recognized by the scFvs are dependent on the native conformation of the viral proteins, we performed a radioimmunoprecipitation assay. For this purpose, [^{35}S] radiolabeled YFV 17D-204-WHO virions were produced and radiolabeled viral proteins were solubilized using the detergent β-octyl-n-D-glucopyranoside, which has been shown to preserve the integrity of epitopes recognized by scFvs. Solubilized radiolabeled YFV proteins were then mixed with each scFv and the scFv/viral protein complexes were immunoprecipitated with an anti His-tag mouse antibody/agarose beads mixture. As positive control, two mAbs (6538 and 6330) specific for the YFV E protein were used. Both precipitated a 55 kDa protein corresponding to the YFV E protein. As shown in Fig. 25, scFvs were able to immunoprecipitate a protein of the same size. As negative control, a scFv from the same library without any YFV binding activity was used as well as the anti His-tag antibody alone. Both negative controls were not able to immunoprecipitate this 55 kDa protein corresponding to the YFV E protein. Taken together these data

show that scFvs are able to bind specifically the YFV-E protein and that scFvs recognized epitopes which are conformation-dependent.

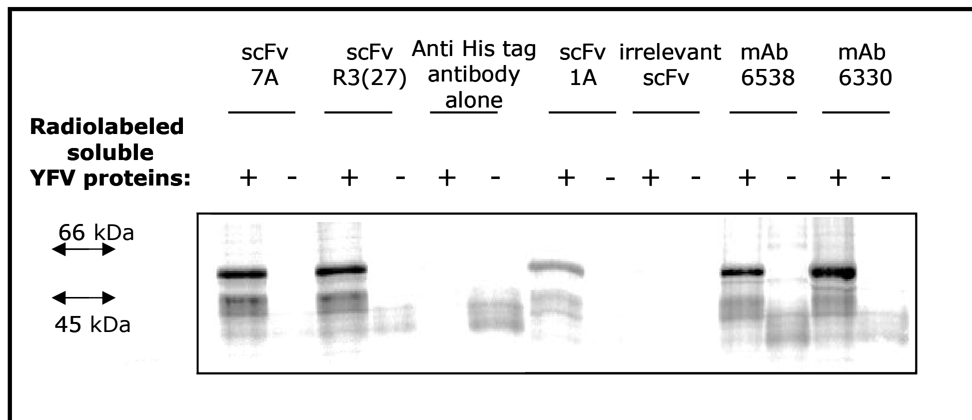

Fig 25: Immunoprecipitation of radiolabeled soluble YFV proteins. Radiolabeled soluble YFV proteins were immunoprecipitated with each scFv. YFV-E specific mAbs 6538 and 6330 were used as positive controls. As negative controls, an irrelevant scFV from the same library and an anti His-tag antibody were used.

IV-5. Competition ELISA.

To determine whether the six scFvs were directed against the same or overlapping epitopes, a competition ELISA assay using biotinylated scFv was performed. Biotinylated scFv-7A was mixed with increasing amounts of non-biotinylated scFvs as well as mAbs and then incubated with YFV 17D-204-WHO virions coated onto an ELISA plate to test whether the scFvs could compete with each other for the binding to YFV virions. The amount of bound biotinylated scFv was detected using HRP-conjugated streptavidin. As expected, a competition between unlabeled and biotinylated scFv-7A was observed, the amount of biotinylated scFv-7A dropping from 100% in the absence of unlabeled scFv-7A to 30% binding in the presence of 5µg of the same scFv as competitor. As a negative control, an irrelevant scFv not competing with biotinylated scFv-7A was used. The two closely-related scFvs-5A and R3(27) competed with biotinylated scFv-7A, reducing the binding from 100% to approx. 40% by using 5 µg each. ScFv-1A, 2A and mAbs (6330 and 6538) did not displace biotinylated scFv-7A suggesting that they recognize different epitopes on the E protein. Interestingly, scFv-R3(9), which differs from scFv-7A, 5A or R3(27), can partially compete with scFv-7A (40% reduction of binding). Taken together

these data show that scFv-7A, 5A and R3(27) are specific for the same or an overlapping epitope on the E protein.

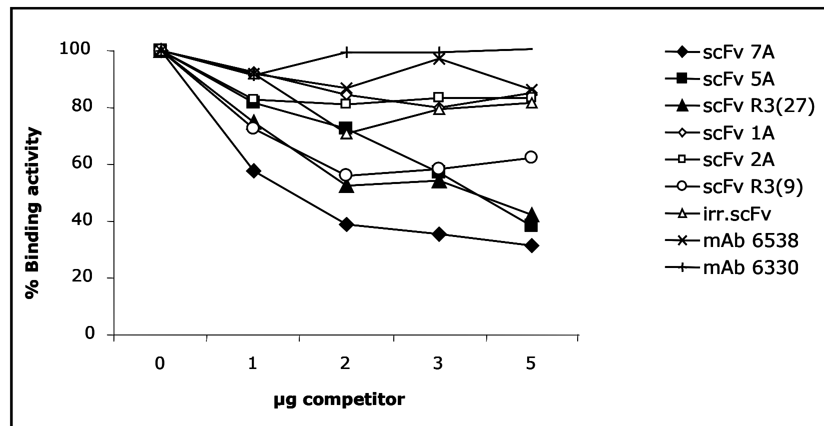

Fig 26: Competition of scFv or E-specific mAbs with biotinylated scFv-7A for binding to YFV 17D-204-WHO virions. Biotinylated scFv-7A and various amounts of unlabeled scFvs or mAbs were incubated with coated YFV virions. The detection of bound biotinylated scFv-7A was performed using HRP-conjugated streptavidin.

IV-6. pH sensitivity of the epitopes.

Since the scFvs are specific for the E protein and since the E protein undergoes irreversible conformational changes upon pH shift during the fusion process, we investigated whether those epitopes and their recognition by scFvs are affected by a low pH treatment. It has been previously shown that the treatment of TBE or DV particles with a mildly acidic pH (pH 6 or below) induces identical irreversible conformational changes on the E protein which are known to take place during the fusion process, whereas a pH 8 treatment does not affect the E protein conformation (Allison *et al.*, 1995; Modis *et al.*, 2004; Stiasny *et al.*, 1996). To this aim, purified YFV 17D-204-WHO virions were coated in an ELISA plate and treated either with low pH (pH 5) or with slightly alkaline pH (pH 8) and back-neutralized to pH 8. An ELISA test was then performed using the scFvs as well as the mAbs in order to check whether those epitopes are still recognized after pH shift. As shown in Fig 27, all six scFvs as well as the two E-specific monoclonal antibodies are able to bind their epitope when YFV particles are pretreated at pH 8. In contrast, when YFV virions are pre-treated at an acidic pH (pH 5) which leads to the reorganization of the E proteins on the viral surface, the six scFvs and the monoclonal antibody mAb 6538 cannot bind the YFV virions anymore, suggesting that their epitopes were lost during the pH-induced conformational changes. In contrast, low pH

treatment of YFV particles does not alter or only slightly reduces the capacity of the second monoclonal antibody mAb 6330 to bind its epitope. These results correlate with those obtained in IV-4-1 (Western Blot analysis) in which we have shown that only the mAb 6330 was able to recognize the YFV under reduced conditions. All these data show that the scFvs recognize conformational epitopes which are sensitive to low pH on the E protein of YFV, located in regions which undergo pH-induced rearrangements.

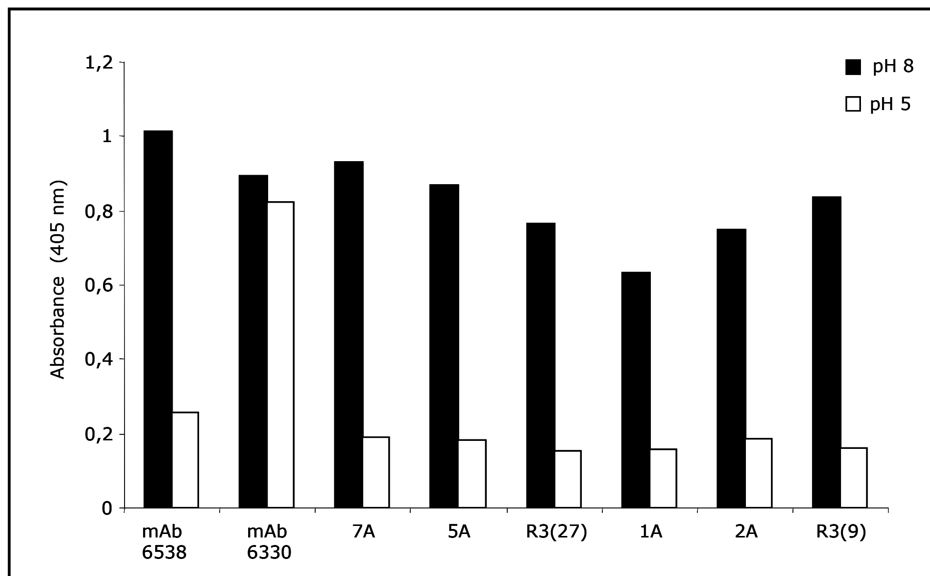

Fig 27: pH sensitivity of the epitopes recognized by the scFvs. Coated-YFV virions were treated either at pH 5 or at pH 8. The ELISA procedure was then performed using scFvs and mAbs as described in methods.

IV-7. Neutralization assays.

IV-7-1. Plaque Reduction Neutralization Test (PRNT) using the YFV 17D 204-WHO (vaccine strain) and YFV wild-type strain Asibi.

To investigate the ability of scFvs to block infection of cells by YFV in vitro, PRNT was performed. YFV 17D-204-WHO were incubated with various concentrations of each scFv, spread on Vero cells and plaque formation was observed. As shown in Fig. 22, the three closely related scFvs-5A, 7A and R3(27) showed 50% and 100% neutralizing activity against the YFV vaccine strain at concentrations of approx. 1 µg/ml and 10 µg/ml, respectively, whereas the three other scFv did not neutralize at any tested concentrations. Two YFV type-specific mAbs directed against the E protein were used as controls (mAb 6538 and 6330). MAb 6538 showed 100%

neutralizing activity at a concentration of 1 µg/ml, whereas the potency of mAb 6330 was lower (50% neutralization at 10 µg/ml). An irrelevant scFv included as negative control did not neutralize YFV 17D-204-WHO. To test whether scFv have a neutralizing activity against a wild-type YFV strain, PRNT was also performed using the Asibi strain.

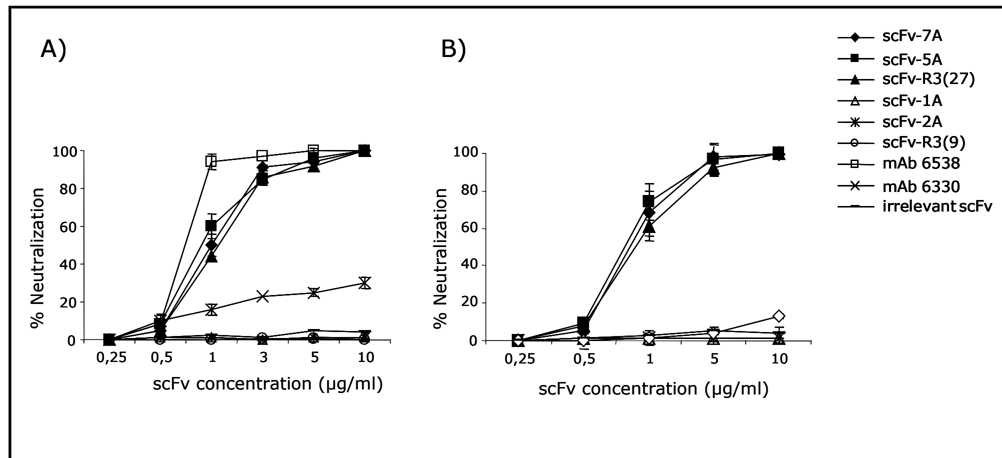

Fig 28: Neutralization of the 17D-204-WHO (A) and the Asibi (B) strains of YFV by scFvs in plaque reduction neutralization test (PRNT) on Vero cells. YFV-specific mAbs 6538 and 6330 were used as positive controls. As negative control, an irrelevant scFv from the same library was used. Each assay was run in triplicate.

IV-7-2. Plaque Reduction Neutralization Test (PRNT) using 5 wild-type YFV strains representing three of the five known African genotypes.

To test whether these scFvs could neutralize other YFV wild-type strains, PRNT experiments were performed using five strains representing three of the five known African genotypes: CAR86 (East Africa/Central Africa), Ethiopia61 (East Africa/Central Africa), Senegal90 (West Africa II), Nigeria87 (West Africa I) and Ghana 1927–Asibi-(West Africa II). As shown in Fig. 23, the three closely related scFv-5A, 7A and R3(27) are able to neutralize the CAR86, Nigeria87, Ethiopia61 and Asibi strains with a comparable efficiency; 90% neutralization being observed at concentrations ranging from 0.3 to 1 µg/ml of scFv, scFv 5A showing the most potent neutralizing activity. Interestingly, the Senegal 90 strain was neutralized by those three scFvs four to ten fold less efficiently than the four other wild-type strains. Of the second group of closely related scFvs, only scFv R3 (9) showed some activity, neutralizing the Nigeria87 strain to maximally 60%. Cross reactivity of those scFvs was also tested by doing PRNT using another flavivirus, a West Nile virus strain isolated in 2000 but none of them exhibit any neutralizing activity against this virus, showing that these neutralizing scFvs are YFV-type specific.

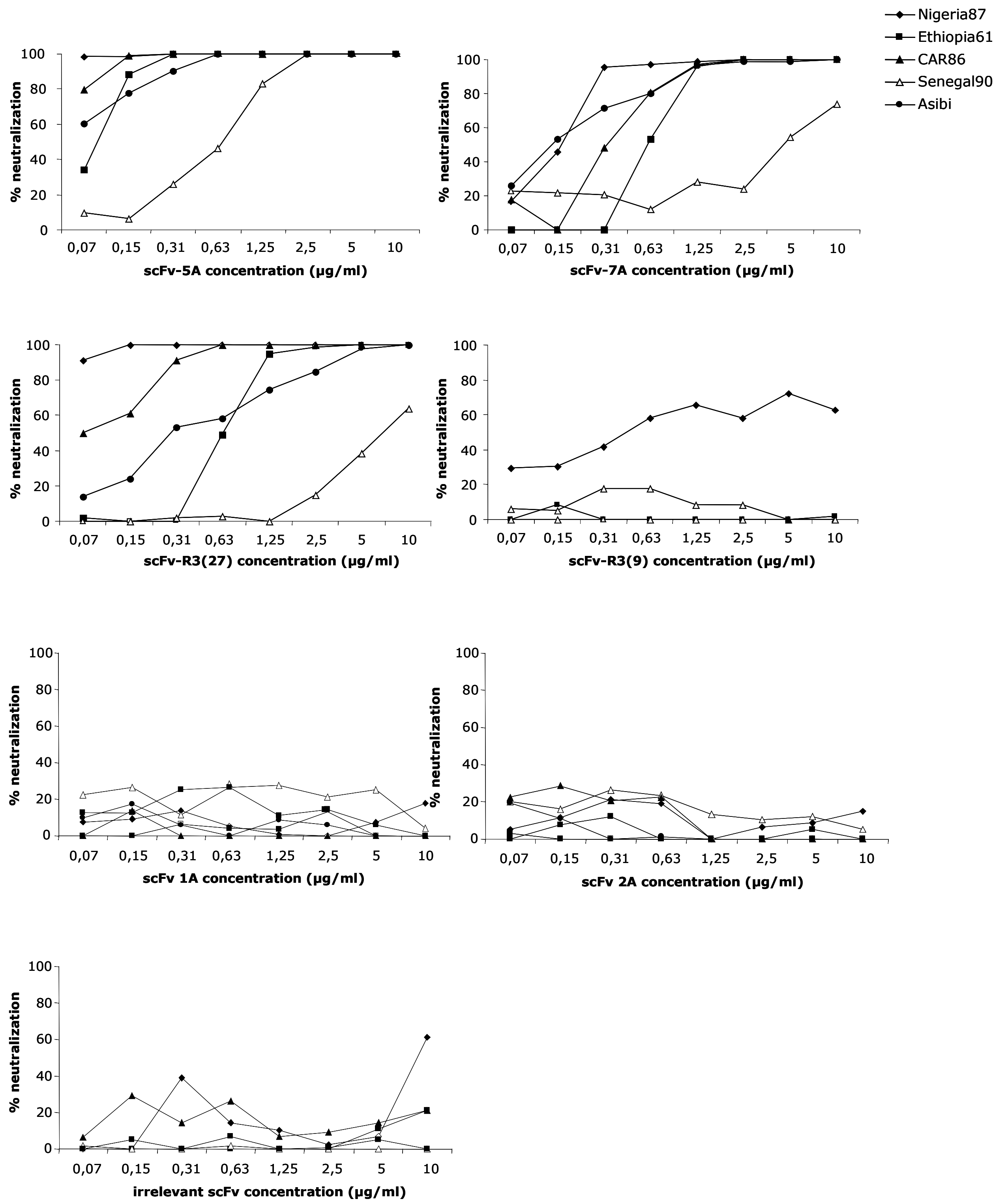

Fig 29: Neutralization of several wild-type strains of YFV by scFvs in PRNT in PS cells. As negative control, an irrelevant scFv (not reactive with YFV) from the same library was used.

IV-8. Generation of YFV 17D-204 WHO variants exhibiting resistance to scFv-7A neutralization.

To identify viral determinants associated with scFv neutralization, YFV mutants which were able to escape neutralization were generated. To this aim, the YFV 17D-204-WHO vaccine strain was passaged three times in the presence of a sub-neutralizing concentration of scFv-7A (representing a 95% neutralization concentration) to promote the growth of YFV 17D-204-WHO variants which could escape the neutralization by scFv-7A. After the third passage, 20 YFV variants were plaque purified, propagated, titered and tested in PRNT. As shown in Fig. 30, four mutants found to be resistant to scFv-7A neutralization were isolated.

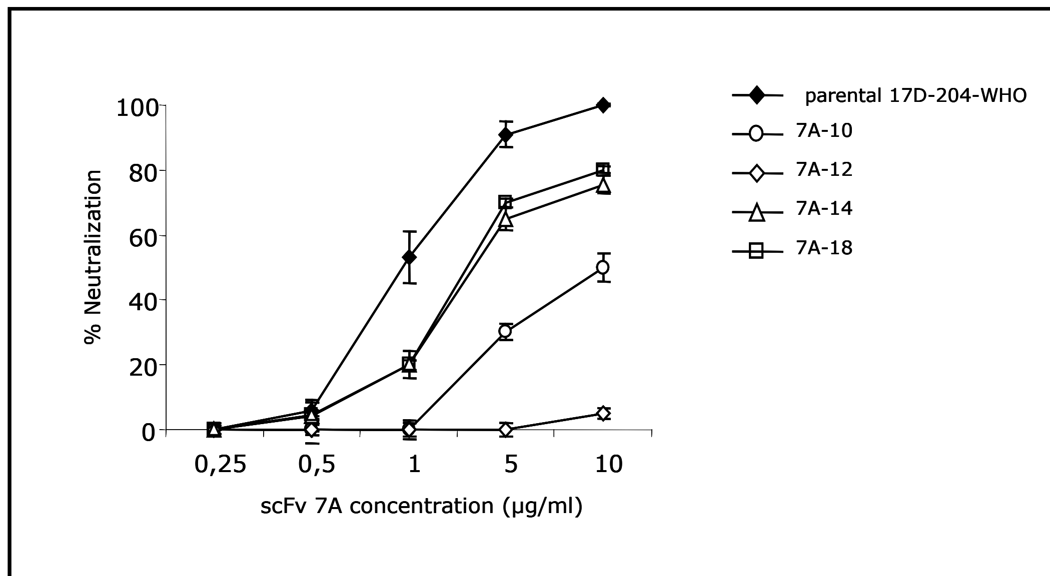

Fig 30. Neutralization sensitivity of YFV-17D escape mutants to scFv-7A. The scFv-7A neutralization resistance of four escape mutants (7A-10, 7A-12, 7A-14 and 7A-18) which were selected in the presence of subneutralizing concentrations of scFv-7A was tested in PRNT using various concentrations of scFv-7A. As a positive control, the parental YFV-17D-204-WHO was used. Each assay was performed in triplicate.

Mutants 7A-14 and 7A-18 exhibited a low neutralization resistance (75% neutralization at 10 µg/ml scFv-7A). 50% neutralization was seen with the 7A-10 variant at the highest antibody concentration. The fourth mutant, 7A-12, was almost completely resistant to neutralization by scFv-7A (5% neutralization at 10 µg/ml scFv-7A).

IV-9. Identification of amino acid residues on the E protein associated with resistance to scFv-7A neutralization.

To determine mutations in the E protein associated with neutralization resistance, its gene was sequenced for all four escape variants as well as for the parental YFV 17D-204-WHO strain. Because changes could also have occurred in the other YFV envelope protein, the gene encoding the M protein was also sequenced for all viruses (escapes and parental viruses). Mutations were detected only in the E protein gene. As shown in Table 5, a total of four amino acid changes associated with resistance to neutralization by scFv-7A were identified, one each at positions E-71 (domain II), E-153, E-154, and E-155 (all in domain I). The mutation at E-71 conferred the highest neutralization resistance.

	Virus	Nucleotide changes	Amino acid changes	Deduced amino acid sequence (E-71, E-153, E-155)	Neutralization by scFv-7A (10 µg/ml)
Parental virus	**17D-204-WHO**	-	-	...CYNAVLTHVKINDK........KQENWTTDIKT...	100%
Escape mutants	**7A-10**	**nt 1437** A → G	**E-155** Asp→Gly	...--------------........-------G---...	50%
	7A-12	**nt 1186** T → G	**E-71** Asn→Lys	...-----------K--........-----------...	5%
	7A-14	**nt 1430** A → G **nt 1433** A → G	**E-153** Thr→Ala **E-154** Thr→Ala	...--------------........-----AA----...	75%
	7A-18	**nt 1430** A → G	**E-153** Thr→Ala	...--------------........-----A-----...	75%

Table 5: Nucleotide and amino acid changes of the E protein for 17D-204-WHO variants which are associated with scFv-7A neutralization resistance.

IV-10. Location of amino acid substitutions associated with resistance to scFv-7A neutralization in the E protein homodimeric crystal structure of YFV.

The YFV E protein has not been crystallized yet. However, the crystal structure of two others closely-related flaviviruses (Dengue virus and TBE virus) has been resolved and published. Therefore, we used the crystal structure of the Dengue virus E protein to locate the four substitutions that we found to be associated with scFv-7A neutralization resistance.

As shown in Fig. 31, aa E-153, E-154 and E-155 are located in domain I and aa E-71 in domain II of the E protein. As expected, all substitutions were found to be located on the outer surface of the E dimer (Fig 31, right side) and, thus, accessible to antibodies. Another interesting point is that aa E-71 and aa E-153, E-154 and E-155 are spatially separated by 65-70 Å in the monomeric protein. This distance is far beyond the surface area which the VH and VL chain of an antibody fragment can cover, ranging typically from 15 Å x 20 Å to 20 Å x 30 Å. (Davies and Cohen, 1996; Davies *et al*., 1990). Therefore, aa residues E-71, E-153, E-154 and E-155 are unlikely to participate in forming an epitope on the monomeric E protein. In contrast, in the homodimeric, anti-parallel orientation which the molecules are predicted to adopt on the surface of the virion, domains I and II are in close vicinity, with a separating distance of 15-20 Å between aa E-71 and E-155 (Fig. 32). It is thus likely that the scFv-7A neutralizing epitope is formed by two E proteins, which each contribute a different domain. We therefore postulate that the epitope of the antibody fragment 7A is not located on the protein E monomer, but spans adjacent domains I and II of the homodimeric form of the molecule.

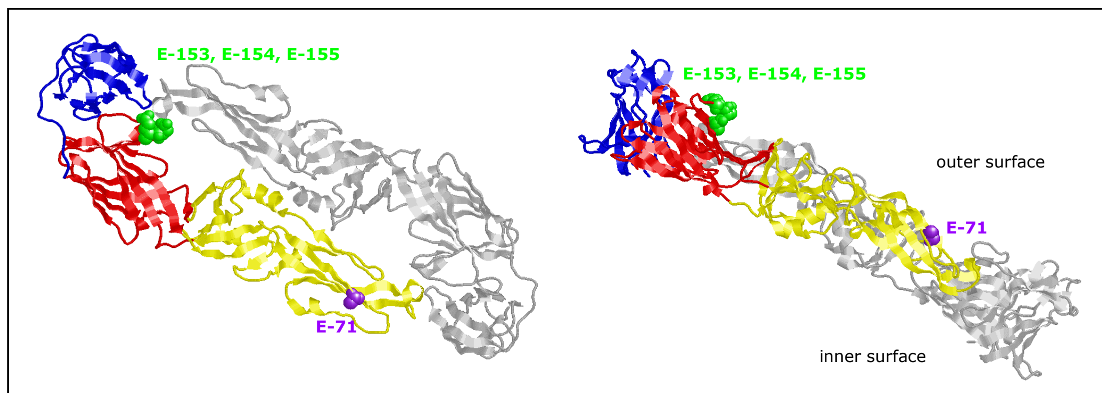

Fig 31. Location of amino acid residues E-71, E-153, E-154 and E-155 associated with scFv-7A neutralization escape in the YFV E protein modeled on the 3D structure of the dengue virus type 2 E protein. (left side): top view of the E protein. Domains I, II and III indicated in red, yellow and blue, respectively. (right side): side view.

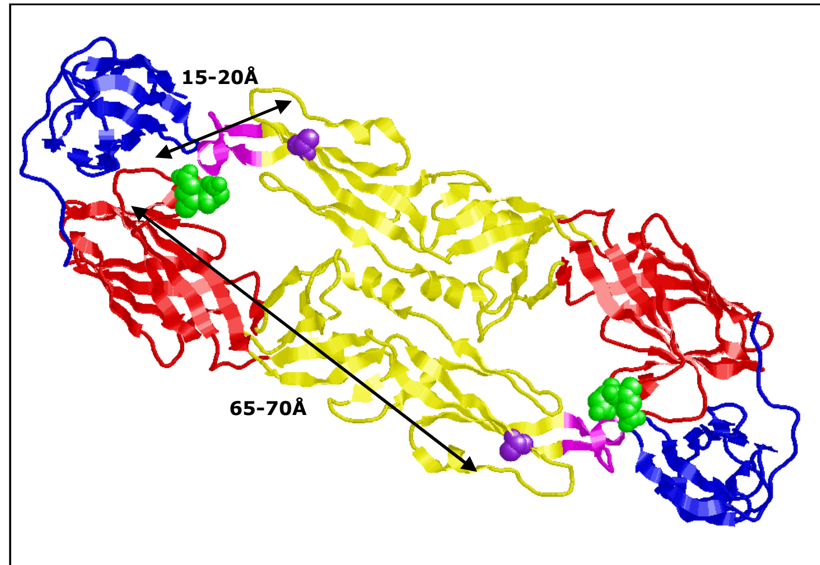

Fig 32: The scFv-7A epitope. E-71 is indicated in purple and E-153, E-154 and E-155 are colored in green. The fusion peptide is depicted in pink.

IV-11. Mutations in the E protein of the YFV Senegal 90 strain.

As shown in Fig. 29, the YFV Senegal 90 strain is four to ten fold less efficiently neutralized by scFv-7A, 5A and R3 (27) when compared to all other YFV wild-type strains tested (Asibi, CAR86, Ethiopia61 and Nigeria87). The gene encoding for the E protein of those five wild-type YFV strains were sequenced. Comparison of their E protein sequences showed that four aa substitutions were observed between the Senegal 90 strain and all other wild-type strains at position E-7, E-54, E-153 and E-249. These substitutions were modeled on the crystal structure of the Dengue virus E protein (Fig. 33). Interestingly, three of these four substitutions (E-7, E-153 and E-249) were found to be located in close vicinity of the scFv-7A epitope or even directly within (E-153). These modelling data, taken together with the neutralization data, strongly suggest that the Senegal 90 strain could represent a naturally-occurring scFv-7A escape variant, equivalent to the escape variants (7A-14, 7A-18, 7A-10 and 7A-12) that we found to be present in the YFV 17D-204-WHO vaccine preparation.

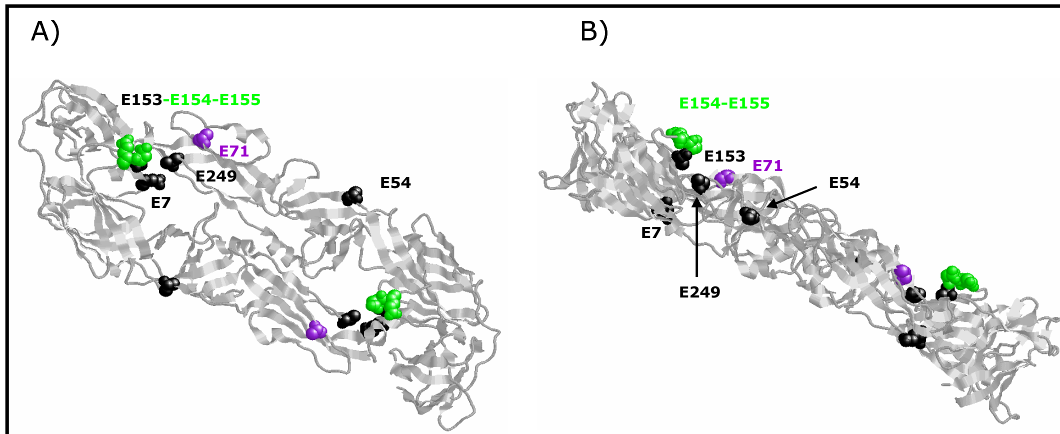

Fig 33: Location of residues of the YFV Senegal 90 strain which differ from other YFV wild-type strains. Residues E-7, E-54, E-153, E-249 are colored in black. Residues of the scFv-7A epitope are also indicated (green and purple). A) Top view. B) Side view.

IV-11. Importance of the mutations E-71 and E-155 in terms of neutralization escape using human polyclonal sera from YF patients and 17D vaccinees.

To evaluate the impact of the escape mutants in a human context and to determine the importance of the neutralizing epitope E-71,-E-153,-E-154,-E-155, we tested their ability to escape neutralization of sera from YF-patients and from travelers who were routinely immunized with the YFV 17D-204-WHO vaccine strain. To this aim, microneutralization assays were performed with the two most interesting mutants 7A-10 and 7A-12 using polyclonal sera obtained from recovered YF-patients and from travelers immunized with the YFV 17D-204-WHO vaccine. Fig. 34 represents the inverse of the 50% end point dilution of each serum for each virus (escape mutants vs parental 17D-204-WHO) expressed in a $\log_{10}$ scale. Both sera from YF-patients (YF2 and YF3) neutralized the variant 7A-12 up to ten fold less effectively when compared to the parental YFV 17D-204-WHO strain. In contrast, the sera of the immunized travelers (sera 3, 5, 7, 14, 20, 37, 85 and 86) showed full or only slightly reduced neutralizing activity against this variant. Paradoxically, the neutralizing titers of most sera against the variant 7A-10 were significantly higher (up to 10 fold) compared with titers against the parental YFV 17D-204-WHO strain.

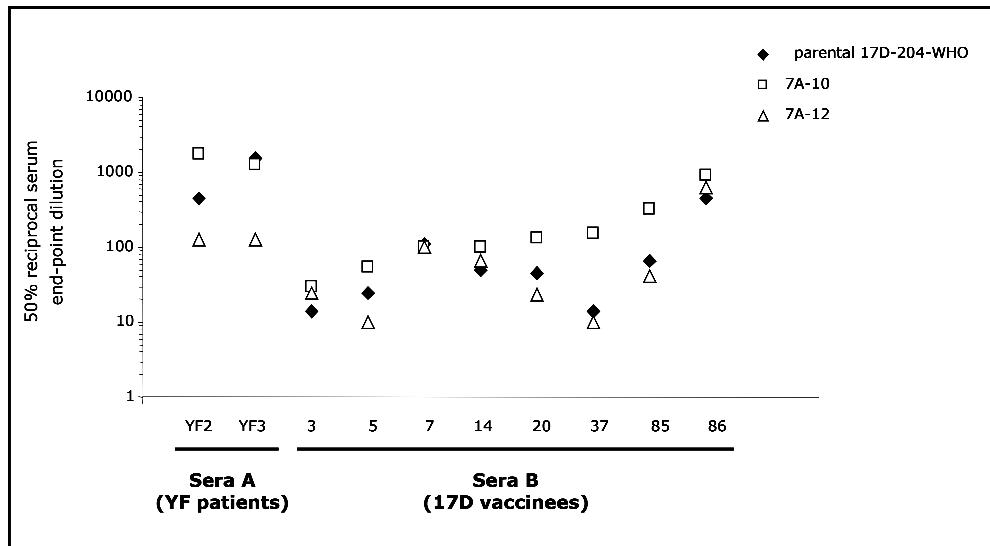

Fig 34: Sensitivity of escape mutants 7A-10 and 7A-12 to neutralization by sera from YF patients and from 17D vaccinees. As a positive control, the parental YFV-17D-204-WHO virus was used. 50% endpoint dilution represents the dilution of each serum at which 50% of the wells were protected from infection and are indicated as reciprocal dilution in a $\log_{10}$ scale. Each serum was tested in five replicates.

V-DISCUSSION

V-DISCUSSION

V-1 Quality of antibody libraries and results of selection.

V-1-1. Construction of two human immune antibody phage display libraries and their panning against purified YFV 17D-204-WHO virions.

The successful isolation of potent neutralizing antibody fragments by the phage display system is dependent on two critical parameters: the quality of the library and the quality of the antigen used for the screening. We used, as a source of RNA, peripheral blood lymphocytes (PBL) because they are easy to obtain. The PBL cells were harvested from patients who recovered of YFV infection six months after acute infection. At this time point, the majority of B-memory cells and long-lived plasma cells generated following YFV infection are believed to reside mainly in lymphoid tissues. Consequently, we may have have only a partial access to the antibody repertoire which was developed against the YFV. Dut to logistic problems in West Africa, it was not possible to obtain lymphocytes at the optimal time point, which is approx. four weeks after acute infection. The obtention of lymphocytes from bone marrow or spleen would circumvent this limitation but such a method is hardly feasible when biological materiel has to be harvested from humans, particularly in Africa. However, due to the affinity maturation process, only antibodies with high affinity (i.e. with high potency) were most likely isolated. Libraries are, therefore, not representative of the total antibody response against YFV, but of the long-lasting response which prevents reinfection.

Concerning the antigen, we used purified YFV particles from the YFV 17D-204-WHO vaccine strain. This antigen was used, as opposed to the wild-type YFV, to isolate antibody fragments which may be useful to identify yellow fever-type specific epitopes. Furthermore, YFV from the vaccine strain, in contrast to the wild-type strain, can be handled under biosafety level 2 without prior chemical or physical inactivation. Thus, sensitive conformational epitopes are preserved. We also preferentially used whole virions instead of recombinant YFV E proteins to ensure that antibodies will be selected against the E protein folded in its native conformation found on the viral surface. Sanchez et al. showed for WNV, by using three different immunization strategies in mice (inactivated whole virus, recombinant E protein and naked DNA), that the most potent neutralizing antibodies were generated following immunization with inactivated whole viruses whereas no neutralizing antibodies were isolated using a soluble form of the E protein (Sanchez *et al*., 2005).

V-1-2. Isolation of closely-related scFvs specific for the YFV E protein.

As expected, the scFvs we isolated in this study were all directed against the envelope E protein of the YFV. Interestingly, the three scFv (5A, 7A and R3(27)), exhibiting a potent neutralizing activity, were found to be genetically closely-related and to recognize the same or an overlapping epitope, suggesting that only one epitope specificity has been isolated from two different libraries. However, this result may be due to a technical bias inherent to phage display technology, particularly during the selection process. Washes are performed to remove unspecific phages but this step is critical since phages displaying scFv with a low affinity for the antigen may be lost and the enrichment will be done with phages bearing scFvs with the highest affinity. It cannot be excluded that others scFvs with a lower affinity or which recognize other epitopes could be isolated by modifying the selection conditions like the stringency of washes. To circumvent this problem, new methodologies have been developed. Recently, Kramer et al. isolated 147 unique antibody fragments specific for rabies virus (RV) from four libraries by performing selection procedures alternating on whole virions, on cells expressing RV glycoproteins and on recombinant RV glycoproteins (Kramer *et al*., 2005). An other alternative would be the use of the "substractive selection". To suppress the repetitive isolation of the same specificity of scFvs, the biopanning is performed as previously described except that one of this "frequent" scFv is incorporated during the biopanning. This process leads to the masking of the dominant epitope, resulting in the isolation of scFvs which are low abundant or have lower affinity (Ditzel *et al*., 1995; Tsui *et al*., 1996).

Another interesting point is that all three neutralizing scFvs have an identical VH-CDR3, and even an identical VH chain, and they differ from each other mainly by their VL-CDR3 region and by their relative neutralizing capacity (scFv-5A being the most potent one). Since these three neutralizing scFvs recognize the same or an overlapping epitope, this result strongly supports the fact that the VH-CDR3 region is probably the most important determinant of antigen specificity of antibodies as previously described (Kabat and Wu, 1991). These three scFvs could reflect the affinity maturation process which occurs during the development of the adaptive immune response. During affinity maturation, B-cells are clonally to the affinity of their expressed antibodies; the antibodies with the lowest Kd are selected and amplified. Thus, the three scFvs may represent three mutated versions of one original clone, each having a different affinity for the antigen. This is also supported by the fact that lymphocytes were harvested 6 months after acute infection, at which time, affinity maturation has already taken place. For the reasons outlined

before, this epitope specificity may represent the main type-specific antibody response induced by wild-type YFV, possibly also determining the major neutralizing epitope on the YFV vaccine.

V-2.Nature of the epitopes.

V-2-1. The epitopes are conformation-dependent and pH-sensitive.

The nature of the major neutralizing epitope has been found to be denaturation-dependent and related to the native structure of the E protein found on the viral surface, as shown by Dot blots, Western blots and RIPA experiments. This is further supported by the fact that the epitopes recognized by all scFvs are sensitive to low pH treatment. Since the treatment of flavivirus virions with low pH buffer has been shown to mimic the irreversible conformational changes of the E protein which take place during the fusion process, it is reasonable to assume that the epitopes of the scFvs are located in regions of the E protein which undergo pH-induced rearrangements during the fusion process.

V-2-2. The scFv-7A epitope is formed by amino acid residues from domain I (E-153, E-154 and E-155) and from domain II (E-71) in the E protein.

Using YFV 17D-204-WHO neutralization escape variants, we found that the aa residues located at position E-71, E-153, E-154 and E-155 of the E protein are associated with scFv-7A neutralization escape. This result suggests that those aa residues are essential parts of the epitope recognized by scFv-7A. The measurement of the distance between E-71 and E-153, E-154 and E-155 on one E monomer strongly suggests that the scFv-7A epitope could be a conformational epitope formed by two E proteins, each one contributing a different domain (E-71 of domain II from one monomer and E-153, E-154 and E-155 of domain I from the second monomer). The substitutions in a wild-type strain (Senegal 90) which is less sensitive to neutralization by scFv-7A were found to be in close vicinity or within the scFv-7A epitope, suggesting the great importance of this region for YFV neutralization. Interestingly, a study conducted on Dengue virus type 2 using Fab fragments has recently shown that the epitope recognized by one neutralizing Fab displays similar features than our epitope, being composed of aa residues at position E-317 in domain III of one monomer and E-106 at the tip of domain II of the other monomer (Goncalvez *et al*., 2004).

V-3. The scFv-7A epitope.

V-3-1. E-71 is extremely conserved among all sequenced YF strains and critical for neutralization.

We have identified one escape variant which is almost completely resistant to neutralization by scFv-7A. This virus differs from the parental 17D-204-WHO virus by one substitution at position E-71 in the E protein. This asparagine residue at position E-71 is 100% conserved among all wild-type and vaccine strains of YFV published to date, with the exception of one YFV strain isolated from mosquitos in 1979 in Trinidad, which has a N-D exchange at position E-71 (Fig 35). The high degree of conservation of the aa residue E-71 could lie in the fact that it is close to the postulated internal fusion peptide within domain II, which is predicted to span E-98 to E-110. Any mutation in this region is likely to alter fusion capacity and infectivity of the virus. The escape variant 7A-12 mutated at position E-71 showed much higher resistance to neutralization with scFV-7A than the escape variants mutated at E-153, E-154 and E-155 (95% vs. 25-50% in PRNT) showing that E-71 is the most critical amino acid residue in this complex YFV type-specific neutralizing epitope. Furthermore, in microneutralization experiments, the neutralizing titers of the sera from YF patients were up to 10 fold reduced with the variant 7A-12 supporting the fact that E-71 is an important determinant for YFV neutralization.

V-3-2. Aa residues E-153, E-154 and E-155 contribute to a lesser extent in the scFv-7A epitope.

The escape variants 7A-14, 7A-18 and 7A-10 which are mutated at positions E-153, E-154 and E-155 exhibit a lower neutralization resistance than the variant 7A-12, suggesting that those aa residues contribute to lesser extend to the scFv-7A epitope.

V-3-2-1. Role of E-155 in the scFv-7A epitope.

The variant 7A-10 mutated at position E-155 has an intermediate resistance to neutralization (50% in PRNT). The aspartic acid residue E-155 is, like E-71, highly conserved among YFV strains except the 17DD vaccine strain, which displays a serine residue at this position. Interestingly, the 17DD vaccine strains showed a slightly increased neurovirulence in animal models (Barrett and Gould, 1986). Moreover, a D155G mutation together with a G303K mutation in a 17D virus variant was isolated from a human case of vaccine-associated encephalitis

(Jennings *et al*., 1994). This case of YEL-AND might, therefore, be the consequence of the selective growth of one escape variant from the vaccine preparation. In this work, we found that the escape mutant 7A-10 substituted at position E-155 was efficiently neutralized by polyclonal sera from 17D immunized travelers. This result, therefore, underlines the fact that E-155 mutants are normally well controlled by immune responses induced by the YFV-17D vaccines and suggests a major role of the mutation G303K in the 17D-associated encephalitis case. The role of single mutations in the E protein of YFV-17D variants has to be interpreted carefully since YEL-AND and YEL-AVD are probably the result of a pluri-causative process. Thus, animal pathogenicity studies must be performed to evaluate the importance of these mutations. Interestingly, E-155 has been also reported together with other substitutions as a potential determinant for YFV viscerotropism and virulence attenuation. A D155A substitution was detected together with nine other aa substitutions in an attenuated YFV wild-type Asibi strain which was passaged six times in HeLa cells. This Asibi variant was found to be attenuated in newborn mice and had lost its mosquito competence (Dunster *et al*., 1999). A D155A mutation was also described in a hamster-adapted YFV Asibi strain. This variant was found to be viscerotropic for hamsters, a feature which is usually not observed in these animals following YFV Asibi infection (McArthur *et al*., 2003). These data strongly support an important role of E-155 in YFY tropism, virulence and neutralization. Nevertheless, due to the multiplicity of the phenotypic properties of the variants isolated in those studies and the multiplicity of substitutions occurring in the same time, the exact role of E-155 is difficult to interpret.

V-3-2-2. Role of E-154 in the scFv-7A epitope.

The T154A substitution in the virus variant 7A-14 has previously not been reported in a neutralization escape mutant but was found in human wild-type YFV isolates from Ecuador and Peru (Fig.35). Regarding the neutralization resistance activity of the variant 7A-14, the substitution at position E-154 does not play a very important role in neutralization and, therefore, contibutes only moderately to the scFv-7A epitope.

V-3-2-3. Role of E-153 in the scFv-7A epitope: potential N-linked glycosylation site at position E-151.

Like the variant 7A-14 which is mutated at position E-154, the variant 7A-18, which is substituted at position E-153, exhibits a low resistance to neutralization, suggesting a minor role of this aa residue in the scFv-7A epitope. However, a potential N-linked glycosylation site in the E protein is located at positions E-151

(N), E-152 (W) and E-153 (T) in several YFV strains. Post et al. have demonstrated that this N-linked glycosylation is only utilized by the 17DD and 17D-213 vaccine strains (exhibiting the NWT sequence) (Post *et al*., 1992). All African wild-type strains used in this study exhibit a NWN/K sequence at this position and are therefore not glycosylated at this position (Fig.35). Because the 17D-204 WHO strain used in this work displays a glycosylation sequence (NWT) and because this strain is derived from the 17D-213 strain, we assume that the YFV-204-WHO strain we used in this work is glycosylated at position E-151. However, because the YFV 17D-204 WHO strain and all African wild-type strains are all efficiently neutralized, it seems that the glycosylation site does not play a critical role for neutralization. Nevertheless, the escape mutants 7A-14 and 7A-18 display mutations at position E-153 or E-153/154. Those mutations modify the aa sequence from NWT to NWA and thus disrupt the predicted glycosylation site. Because variants 7A-14 and 7A-18 are weakly resistant to scFv-7A neutralization, it is reasonable to think that this glycosylation site may play a minor role in neutralization. The presence of sugars at this position may define the scFv-7A neutralizing epitope to some extent. The relationship between neutralization, antibody binding and glycosylation is not well understood. Although the glycosylation site at position E-153 is found only in few number of YFV strains, it is well conserved among several other flaviviruses. The presence of N-linked carbohydrates at position E-153 together with N-linked carbohydrates at position 67 which is unique to DV, has been shown for this virus to be required for recognition by DC-SIGN, a putative cellular receptor for DV (Navarro-Sanchez *et al*., 2003). Studies performed with other flaviviruses have shown that glycosylation of the E protein plays an important role in virus tropism (neuroinvasiness) and virus neutralization. It has been described for WNV that the loss of the glycosylation site at position E-153 of some isolates is correlated with the loss of neuroinvasiveness in mice (Beasley *et al*., 2004; Shirato *et al*., 2004). Studies of deglycosylated epitopes of JEV have shown a loss of interaction with well-characterized JEV-specific monoclonal antibodies (Lad *et al*., 2000). Interestingly, the adaptation of Dengue virus in mosquito cells result in the loss of the glycosylation site at position E-153 (Lee *et al*., 1997). A similar result was obtained with the Louping Ill virus for which the loss of neuroinvasiveness was correlated with the loss of glycosylation site at position 308 (Jiang *et al*., 1993). However, for YFV and for the mutants obtained in this study, the role of the N-linked carbohydrates at position E-153 is still obscure. On other hand, according to the weak neutralization resistance observed for the variants 7A-14 and 7A-18, it cannot be excluded that substitutions E-153 and E-154 may be responsible for minor conformational changes leading to the rearrangement of the scFv-7A epitope

which in turn promotes the destabilization of the interaction epitope-scFV. Interestingly, the YFV Senegal 90 strain, which is less efficiently neutralized by scFv-7A, displays a lysine residue at position E-153 which is not found in vaccine strains or in all other wild-type strains (see Fig.35).

V-4. Three isolated scFvs exhibit a broad and potent neutralizing activity in vitro.

Three scFvs (7A, 5A and R3(27)), which are genetically closely related, were able to neutralize six different YFV strains in vitro (one vaccine strain: the 17D-204-WHO and five African YFV wild-type strains representing three of the five known African genotypes: the East Africa/Central Africa (EA/CA), the West Africa I (WAI) and the West Africa II (WAII)). Interestingly, we observed that all scFvs exhibited a higher potency when PRNT experiments were performed using PS cells instead of Vero cells (Fig. 28 and 29). For example, 100 % neutralization was observed against the YFV Asibi strain using 0.07 µg/ml of scFv-5A in PS cells whereas the same neutralizating activity was obtained at a concentration of 5 µg/ml scFv in Vero cells. These differences may reflect virus tropism and its ability and its efficiency to infect different types of cells. Unfortunately, we were unable to test their neutralizing activity against the other vaccine strain 17DD or against the wild-type strains from South America. This experiment would be extremely interesting to see whether such antibody fragments could block all known YFV genotypes. To test whether the scFvs exhibit a cross-reactivity towards others flaviviruses, we conducted a PRNT experiment using WNV strain isolated in France in 2000. None of the neutralizing scFvs display any neutralizing activity against this virus, suggesting that the scFvs are YFV-specific. As WNV is not the most closely related virus to YFV, it could be interesting to test cross-reactivity of scFvs towards viruses which belong to the YFV sero-complex.

```
                                            E71                                  Fusion peptide
                                             |
17D-204-WHO             51 DRPAEVRKVCYNAVLTHVKINDKCPSTGEAHLAEENEGDNACKRTYSDRGWGNGCGLFGKG 110
17D-213                 51 ------------------------------------------------------------- 110
RKI-17D-112/95          51 ------------------------------------------------------------- 110
17D-204-ATCC            51 ------------------------------------------------------------- 110
17D-204-Pasteur         51 ------------------------------------------------------------- 110
17DD                    51 -----A------------------------------------------------------- 110
Asibi                   51 -G---A------------------------------------------------------- 110
85-82H                  51 -G---A------------------------------------------------------- 110
Nigeria87               51 -G---A------------------------------------------------------- 110
56205/Nigeria/1991      51 -G---A------------------------------------------------------- 110
Guinea/2000/            51 -G---A------------------------------------------------------- 110
Dar1279/Senegal/1965    51 -G---A------------------------------------------------------- 110
1914-81/Ecuador/1981    51 -G---A-----S---------------------------H--------------------- 110
Strain                  51 -G---A-----S---------------------------H--------------------- 110
1362/Peru/1977          51 -G---A-----S---------------------------H--------------------- 110
V-528A/Colombia/1979    51 -G---A-----S----N---------------E---------------------------- 110
AR350397/Brazil/1979    51 -G---A-----S----N---------------E---------------------------- 110
CAR86                   51 -G---A-----S------------------------D------------------------ 110
112/Sudan/1940          51 -G---A-----S------------------------D------------------------ 110
ArB8883/C.              51 -G---A-----S------------------------D------------------------ 110
Ethopia61               51 -G---A-----S------------------------D------------------------ 110
7914/Kenya/1993         51 -G---A-----S------------------------D------------------------ 110
SE7445/Uganda/1964      51 -G---A-----S------------------------D------------------------ 110
DaHB1504                51 -G---A-----S------------------------D------------------------ 110
788379/Trinidad/1979    51 --------------------D---------------------------------------- 110
Senegal90               51 ------------------------------------------------------------- 110
FNV                     51 ------------------------------------------------------------- 110
FVV                     51 -G---A------------------------------------------------------- 110
7A-10 escape mutant     51 ------------------------------------------------------------- 110
7A-12 escape mutant     51 --------------------K---------------------------------------- 110
```

```
                                                                        E153,154,155
                                                                           |
17D-204-WHO             111 SIVACAKFTCAKSMSLFEVDQTKIQYVIRAQLHVGAKQENWTTDIKTLKFDALSGSQ 168
17D-213                 111 --------------------------------------------------------- 168
RKI-17D-112/95          111 --------------------------------------------------------- 168
17D-204-ATCC            111 -----------------------------------------N--------------- 168
17D-204-Pasteur         111 -----------------------------------------N--------------- 168
17DD                    111 -----------------------------------------N-S------------- 168
Asibi                   111 -----------------------------------------N--------------- 168
85-82H                  111 -----------------------------------------N--------------- 168
Nigeria87               111 -----------------------------------------N--------------- 168
56205/Nigeria/1991      111 -----------------------------------------N--------------- 168
Guinea/2000/            111 -----------------------------------------N--------------- 168
Dar1279/Senegal/1965    111 -----------------------------------------N--------------- 168
1914-81/Ecuador/1981    111 -----------------------------------------NA-------------- 168
Strain                  111 --------------------------------------------------------- 168
1362/Peru/1977          111 -----------------------------------------NA-------------- 168
V-528A/Colombia/1979    111 -----------------------------------------N--------------- 168
AR350397/Brazil/1979    111 -----------------------------------------N--------------- 168
CAR86                   111 -----------------------------------------N--------------- 168
112/Sudan/1940          111 -----------------------------------------N--------------- 168
ArB8883/C.              111 -----------------------------------------N--------------- 168
Ethopia61               111 -----------------------------------------N--------------- 168
7914/Kenya/1993         111 -----------------------------------------N--------------- 168
SE7445/Uganda/1964      111 -----------------------------------------N--------------- 168
DaHB1504                111 -----------------------------------------N--------------- 168
788379/Trinidad/1979    111 ---------------------------------------D-K---------V----- 168
Senegal90               111 -----------------------------------------K--------------- 168
FNV                     111 -----------------------------------------K--------------- 168
FVV                     111 -----------------------------------------N--------------- 168
7A-10                   111 -------------------------------------------G------------- 168
7A-12                   111 --------------------------------------------------------- 168
```

Fig 35: Alignment of the partial sequence of the E protein for several YFV.

V-5. Homology of the scFv-7A with previously described YFV neutralizing epitopes of monoclonal mouse antibodies.

Three previous studies have identified neutralizing epitopes for YFV using murine monoclonal antibodies. These epitopes of YFV type-specific, neutralizing mouse monoclonal antibodies were mapped in domain I (E-155 and E-158), domain II (E-71 and E-72) and domain III (E-305/ E-325) of the E protein. Strikingly, a high correlation is observed between those murine neutralizing epitopes and results we obtained in this report since substitutions associated with neutralization escape in both works were found to be at position E-71 and E-155. Aa E-72, E-153, E-154 and E-158 are found to be adjacent to those aa. Interestingly, the data presented here might possibly reconcile all previous published data by assuming that E-71, E-72. E-153, E-154, E-155 and E-158 form together a major YFV neutralizing epitope.

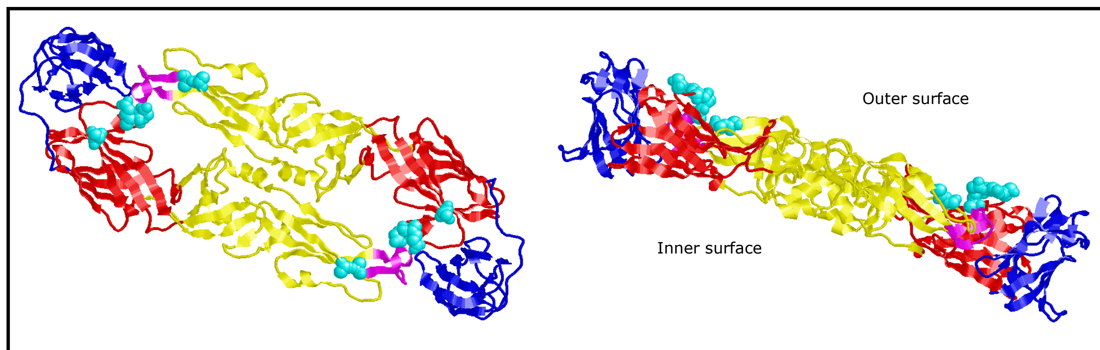

Fig 36: The putative major epitope of YFV. Aa residues E-71, E-72, E-153, E-154, E-155 and E-158 are coloured in cyan.

V-6. Neutralization of scFv-7A escape mutants generated from a 17D 204-WHO vaccine lot with sera from 17D-immunized travelers.

This work described the isolation of neutralization escape mutants which were grown under selection pressure using a recombinant antibody fragment and, thus, were originally present in the vaccine preparation. This result directly underlines the quasispecies nature of the 17D vaccine demonstrating that each vaccine lot is a heterogeneous mixture of viral subpopulations and may contain pre-formed neutralization escape variants. Consequently, it cannot be ruled out that naturally occurring neutralization escape mutants present in the vaccine preparation could carry a risk of neutralization escape in persons immunized with 17D vaccine (particularly travelers). This possibility has been previously raised by reports of

several YFV vaccination-associated disease cases. We have demonstrated here that 17D-escape variants were still sensitive to neutralization by polyclonal sera from travelers with minor drops in neutralizing titers. These data suggest that mutations at position E-71/E-153/E-154/E-155 do not carry a risk of neutralization escape in persons immunized with the 17D vaccine. Although the antibody response defined by scFvs-7A, 5A and R3(27) seems to be a major humoral response developed against YFV, this result reinforces the fact that multiple neutralizing epitopes exist for the YFV. Interestingly, the neutralizing titers of polyclonal sera from yellow fever cases were up to 10 fold lower for the 7A-12 variant (N71K mutation) whereas such differences were not observed when sera from 17D vaccines were used, suggesting that antibody responses raised against the wild-type YFV and the 17D YFV vaccine strain differ. Suprinsingly, the variant 7A-10 showed approx. 10 fold increased sensitivity to neutralization with half of the human sera. This result might be the consequence of conformational changes in the E protein induced by the mutation D155G, and, thus, resulting in the exposure of other neutralizing epitopes.

V-7. Neutralization of the YFV Senegal 90 strain.

We found that the wild type strain Senegal 90, isolated from mosquitos was four to ten times less sensitive to neutralization than all other wild types viruses tested. Because, in the E protein of this virus, mutations are found in close vicinity of E-71, E-153, E-154 and E-155, it is justified to consider the YFV Senegal 90 strain as a naturally occurring neutralization escape variant. The origin of the YFV strain Senegal 90 is quiet surprising. This virus has been isolated from mosquitos in 1990 in Senegal. The observed substitutions have never been described in other YFV so far. Interestingly, it has been previously shown that the expression of neutralizing epitopes differ between YFV propagated in mosquito and mammalian cells. (Barrett *et al*., 1990). A possible explanation is that such differential expression process may represent a evolutionary mechanism to avoid neutralization which could occur during the blood meal. This could explain why this variant is less efficiently neutralized by antibody fragments (7A, 5A and R3(27)) from human anti-YFV repertoire. Furthermore, these data reinforce the fact that the region formed by the scFv-7A epitope may represent the major human neutralizing YFV epitope.

V-8. Potential mechanism of the scFv neutralization.

Neutralization of viruses by antibodies is a complex process and the mechanisms involved remain controversial (Klasse and Sattentau, 2001). Generally speaking, the mechanism by which antibodies block virus infectivity is called neutralization. Specifically, antibody-mediated neutralization is usually referred to a process by which an antibody is able to bind to the viral surface, promoting a reduction of viral entry functions. There is still some debate on whether virus neutralization is due to the binding of a single antibody to one critical viral epitope ("single-hit neutralization") or due to the binding of a threshold number of viral epitopes ("multiple-hit neutralization or occupancy model"). The latter seems to be the mechanism which takes place in most of the viral neutralization processes reported so far (Burton *et al*., 2001).

However, because the virus replication cycle is a multi-step event, antibody neutralization may occur at each step of this process. Antibody binding to the surface of the virion may prevent virus attachment to target cells. This attachment blocking mechanism may be due to an antibody which binds viral epitopes needed to interact with the cellular receptor. Aggregation of virions could also be a critical process. All further steps inhibited by antibodies are classified as post attachment neutralization (PAN). Antibodies may neutralize viruses after they have attached to the target cells. As the replication cycle of most of viruses including flaviviruses require an endocytosis-mediated internalization step, neutralizing antibodies may also prevent the internalization process. Because endocytosed viruses require a fusion step between the viral and the cell membranes to promote the release of the viral genome into the cytoplasm, antibodies may act by preventing the fusion event, leading to the degradation of the internalized virus in the endosome. Theoretically, antibodies may also interfere after the capsid release by compromising later replicative steps. For flaviviruses, most of antibodies which exhibit a neutralizing activity are specific for the E protein and in some instances for the NS-1 protein. Antibodies binding to the E protein could prevent infection by blocking attachment of the virus to the cellular receptor and by interfering in later steps during fusion with cell membrane. Studies on the neutralization mechanism have revealed that antibodies against the domain III mainly act by preventing attachment of the virus to the target cells (Crill and Roehrig, 2001; He *et al*., 1995; Roehrig *et al*., 1998). This has been reinforced by a study (Thullier *et al*., 2001) which described that a monoclonal antibody specific for the domain III of dengue virus (E-306 to E-314) acts by preventing the association of this domain III epitope with the putative cellular receptor heparan-sulfate. Interestingly, the analysis of

sera from dengue-infected people suggest that dengue virus neutralization may occur primarily by preventing cellular attachment (He *et al*., 1995) On the other hand, monoclonal antibodies specific for the domain II have been shown to block virus-mediated cell membrane fusion probably by interacting with the fusion peptide (Roehrig *et al*., 1998) This potential role of antibodies interfering with the fusion process had been previously proposed for WNV (Gollins and Porterfield, 1986), and has been demonstrated for JEV (Butrapet *et al*., 1998). However, in the study performed by Crill et al., neutralizing antibodies specific for domains I and II were also found to block adsorption to Vero cells but to a lesser extend than those specific for domain III (Crill and Roehrig, 2001). The authors proposed that, by binding to domain I or domain II, such antibodies may block domain III-mediated virus-cell interaction through steric hindrance or by inducing some conformational changes in domain III, resulting in a loss of virus attachment capacity. Taken together, these studies strongly suggest that antibodies against domain III block viral attachment whereas those against domain II blocks the fusion process. The role of antibodies against domain I remains uncertain. Recently, Nybakken et al. described that a neutralizing Fab fragment specific for domain III was able to neutralize the WNV not by blocking virus attachment to its target cell as expected, but at a step after viral attachment (Nybakken *et al*., 2005). In this paper, analysis of the epitope recognized by this Fab in domain III showed that it is located in a region which is sensitive to conformational changes during pH shift at the junction between domain I and domain III. This strongly suggests that this Fab may play its role by interfering with the fusion process. Since the junction between domain I and domain III undergoes rearrangements following pH shift, the authors proposed a neutralization mechanism in which such antibodies may bind pH-sensitive regions of the E protein, resulting in the blocking of conformational changes of the E protein which are essential for the fusion process to take place. According to the structure of the pre and post-fusion states of the E protein, these regions are localized mainly on domain II, at the junction of domain I with domain II and at the junction of domain I with domain III. This is also supported by the published neutralizing mAbs, listed in Table 1. In this work, we found that the neutralizing scFv-7A epitope is formed by aa from domains I and II of the E protein, is conformation-dependent and is also pH-sensitive. Furthermore, the molecular localization of this epitope showed a close proximity with the fusion peptide. Thus, we can hypothesize that scFv-7A plays its neutralizing role by blocking the fusion process. However, it cannot be excluded that due the close vicinity of the scFv-7A epitope with domain III, recombinant antibody fragments may neutralize YFV by blocking attachment through induction of conformational changes as hypothesized previously.

Nevertheless, due to their small size, scFvs are unlikely to prevent virus attachment through steric hindrance. Furthermore, according to the proposed fusion mechanism for flaviviruses and the molecular location of these residues, it is tempting to speculate that scFv-7A may act by "clamping" the E protein homodimer and, thus, preventing its dissociation upon pH shift. As the dissociation of the E homodimer is a pre-requisite for trimerization and subsequently for the fusion process to take place, this binding might be sufficient enough to prevent fusion and therefore replication of the virus. A similar mechanism has been recently proposed for DV (Goncalvez *et al*., 2004). In their study, E-106 of the fusion peptide in domain I and E-317 in domain III have been described as forming an epitope for a DV E protein-specific Fab. A previous study of the E protein of TBEV showed the E-317 residue to be critical for the homodimer stabilization and the authors suggested that the region which includes E-317 may regulate the dissociation of the E homodimer upon pH shift during the fusion process, (Bressanelli *et al*., 2004; Goncalvez *et al*., 2004). Thus, the neutralization mechanism by which this Fab plays its role may take place through the prevention of the fusion process by blocking the transition of E dimers to E trimers. Thus, it is reasonable to suggest a similar neutralization mechanism for the scFv we isolated in this work.

V-9. Potentiel use of these scFvs as a therapeutical tool to treat YF.

Since the scFvs isolated here are able to neutralize six different strains of YFV in vitro and since YFV clearance is mainly antibody-mediated, our results raise the question whether those molecules could be a useful therapeutical tool to treat YF in humans. As scFvs can be easily expressed in bacteria, this system represents a cheap, easy and effective way to produce high amounts of human neutralizing antibodies.

However, it has been observed that in vitro neutralization potency may not be predictive of in vivo neutralization potency, and even in vitro non-neutralizing antibodies may confer in vivo protection (Brandriss *et al*., 1986; Iacono-Connors *et al*., 1996; Mozdzanowska *et al*., 1999). Thus, animal protection studies must be performed to evaluate the potency of our antibodies. Several studies have shown that antibody fragments (scFv or Fab) specific for Influenza virus, RSV, HSV-2 and VSV could protect mice upon challenge (Crowe *et al*., 1994; Kalinke *et al*., 1996; Mozdzanowska *et al*., 2003; Zeitlin *et al*., 1996). The major drawback of scFvs or Fab fragments as a therapeutical tool to treat viral diseases is their extremely short biological half-life in blood. It has been shown that the half-life of monomeric scFv

in the plasma does not exceed 10 min in mice and the molecules are rapidly cleared via the kidneys (Pavlinkova *et al*., 1999). As their plasma half-life directly correlates with their molecular weight, several studies have been performed to engineer those molecules into diverse formats like scFv dimers, trimers or tetramers resulting in an increase of their half-life (for review see Holliger and Hudson, 2005; Kontermann and Duebel, 2001). In contrast, recombinant antibody fragments in the IgG format have been described in a number of publications to protect animal models from challenge (for review, see ter Meulen and Goudsmit, In Press). These molecules are bivalent and, thus can bind two antigens conferring a higher avidity when compared to scFv. Intact antibodies contain the Fc domain which recruits cytotoxic effectors. Furthermore, the half-life of such molecules is enhanced through the interaction between the Fc domain and the FcRn/FcRp receptor by sequestrating from the degradative pathway (Kim *et al*., 1999). The absence of the Fc receptor in scFv molecule is one of the major drawback of the use of these molecules in the in vivo context. The preponderant role of the Fc domain to provide a protection against YFV in vivo has been emphasized by Schlesinger et al., who showed that Fab fragments derived from a protective E-specific monoclonal antibody failed to protect mice against YFV-induced encephalitis (Schlesinger and Chapman, 1995). Taken together, these data show that scFv molecules are very unlikely to confer in vivo protection. However, recent advances in antibody engineering may circumvent such limitations by generating scFv-Fc fused proteins which can be produced in mammalian cells. These molecules retain the antigen specificity and display higher stability, half-life and functionality and may provide a valuable tool to protect and treat against diseases (Ray *et al*., 2001).

V-10. Implications for the design of a cDNA-based YF vaccine.

As mentioned previously, the present live attenuated 17D-vaccine strains, even if they are considered as extremely safe, have several drawbacks which prevent the efficient control of yellow fever disease. The inherent problem of their quasispecies nature, which may be responsible for severe adverse effects, as well as its old method of production on embryonated eggs are obstacles to a modern method of vaccine mass-production. Therefore, the concept of a rational design of a new generation of YFV vaccines has been put foward. In 1989, Rice et al described the construction of an infectious cDNA clone for the YFV 17D-204 vaccine strain (Rice *et al*., 1989). Due to genetic instability of the cDNA, a two-step in vitro ligation approach allowed the creation of a full length YF cDNA that could be used for the in vitro transcription of infectious YF RNA. This was a milestone in flavivirus research

and in cDNA-based vaccine development. Recently, Bredenbeek et al reported the successful generation of a one step-cloning stable full length infectious YFV cDNA. The plasmid carrying the YFV cDNA was bacterially amplified, linearized and transcripts were produced through an SP6 RNA polymerase-dependent in vitro RNA transcription. Transcripts were transfected into mammalian cells, and YF viruses were obtained with titers and infectiousity comparable to the classically propagated YFV (Bredenbeek *et al*., 2003). Thus, this infectious cDNA clone-based technology may provide a valuable tool to produce high amounts of homogeneous YF virions which could be used as vaccine. One of the major advantages of this system is the diversity of molecular strategies which can be used to engineer a safer and more efficient live vaccine. This technology provides a platform to identify molecular determinants which govern virulence, attenuation and tropism for YFV. To this end, our data may serve for the engineering and the standardization of a new YFV vaccine based on an infectious cDNA clone, which could be used as a successor of the present live attenuated 17D vaccine.

VI-LITERATURE

VI-LITERATURE.

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VII-ANNEXES

VII-ANNEXES

VII-1. Figures & Tables.

Fig 1. Distribution of yellow fever in South America and Africa. 4
Fig 2. Resurgence of yellow fever as a major public health problem. 5
Fig 3. Phylogenetic relationships among genotypes of YFV from Africa and South America. 7
Fig 4. The flavivirus particle. 8
Fig 5. Proposed fusion mechanism for flaviviruses. 12
Fig 6. Schematic representation of the E protein on the viral surface. 12
Fig 7.Molecular location of neutralizing epitopes for several flaviviruses projected on the crystal structure of the homodimer envelope glycoprotein E of DV type 2. 16
Fig 8. Neutralizing epitopes identified for the YFV and their projection to the E protein crystal structure of DV type 2. 17
Fig 9. Schematic drawing of an IgG immunoglobulin. 25
Fig 10. Antigen-binding site and CDR regions. 25
Fig 11. Schematic representation of the somatic DNA recombination process. 27
Fig 12. Schematic drawing of a scFv molecule. 32
Fig 13. Representation of a recombinant phage expressing and displaying on its surface scFv-pIII fusion proteins. 33
Fig 14. Biopanning or selection step. 35
Fig.15. Schematic representation of the pHEN3 phagemid and the pAB1 plasmid. 41
Fig 16a. Overview of the phage libraries construction. 50
Fig 16b. Overview of the phage libraries construction. 51
Fig 17. PCR amplification of genes encoding for variable domains of light chains (Kappa and Lambda) and heavy chains. 77
Fig 18. Antigenicity of purified YFV-17D-204-WHO particles in ELISA. 79
Fig 19. Biopanning of YF-patient-derived antibody phage libraries against the YFV-17D-204-WHO antigen. 80
Fig 20. Reactivity of 58 monoclonal phages isolated from round 3 and round 4 during biopanning against purified 17D-204-WHO virions. 81
Fig 21. Expression and purification of scFvs in *E coli* cells. 83
Fig 22. scFv ELISA. 83
Fig 23. Alignment of the variable heavy (VH) and the variable light chains 84

(VL) of the YFV-specific scFvs.

Fig 24. Reactivity of scFvs with YFV proteins in Western Blot. 85

Fig 25. Immunoprecipitation of radiolabeled soluble YFV proteins. 87

Fig 26. Competition of scFv or E-specific mAbs with biotinylated scFv-7A for binding to YFV 17D-204-WHO virions. 88

Fig 27. pH sensitivity of the epitopes recognized by the scFvs. 89

Fig 28. Neutralization of the 17D-204-WHO (A) and the Asibi (B) strains of YFV by scFvs in plaque reduction neutralization test (PRNT) on Vero cells. 90

Fig 29. Neutralization of several wild-type strains of YFV by scFvs in PRNT in PS cells. 91

Fig 30. Neutralization sensitivity of YFV-17D escape mutants to scFv-7A. 92

Fig 31. Location of amino acid residues E-71, E-153, E-154 and E-155 associated with scFv-7A neutralization escape in the YFV E protein modeled onto the 3D structure of the DV type 2 E protein. 94

Fig 32. The scFv 7-A epitope. 95

Fig 33. Location of of residues of the YFV Senegal 90 strain which differ from other YFV wild-type strains. 96

Fig 34. Sensitivity of escape mutants 7A-10 and 7A-12 to neutralization by sera from YF patients and from 17D vaccinees. 97

Fig 35. Alignement of the partial sequence of the E protein for several YFV 106

Fig 36. The putative major epitope of YFV. 107

Table1. Location of neutralizing epitopes identified on the E protein for several flaviviruses. 14-15

Table2 Human neutralizing recombinant antibody fragments isolated against various viruses by phage display. 36

Table3 Diversity of monoclonal phages specific for YFV tested by BstN1 fingerprintings. 82

Table4 Reactivity of scFvs with YFV proteins solubilized under different reaction conditions. 86

Table5 Nucleotide and amino acid changes of the E protein for 17D-204-WHO variants which are associated with scFv-7A neutralization resistance. 93

VII-2. Abbreviations.

Aa	Amino acid
ADCC	Antibody-dependent cell-mediated cytotoxicity
Amp	ampicillin
APS	Ammoniumpersulfate
BSA	Bovine serum albumine
BSL	Biosafety level
C	celsius
cDNA	complementary Desoxyribonucleic acid
CDR	Complementary determining region
CPE	Cytopathic effect
DEPC	dethylaminepolycarbonate
DMEM	Dulbecco modified eagle medium
DNA	Desoxyribonucleic acid
DOC	Deoxycholate
DV	Dengue virus
E.coli	Escherichia coli
EDTA	Etylendiaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FCS	Fetal calf serum
FNV	French neurotropic virus
FR	Framework region
Fv	Fragment variable
FVV	French viscerotropic virus
HRP	Horseradish peroxidase
IMAC	Immobilized ion affinity chromotography
IPTG	Iso-propyl-thio-galactoside
JEV	Japanese encephalitis virus
kDa	kiloDalton
mAb	Monoclonal antibody
MES	2-N-Morpholinoethansulfonic acid
min	minute
MMLV	Murine moloney leukemia virus
moi	Multiplicity of infection
MW	Molecular weight
NCR	Non coding region
NK cells	Natural killer cells
NP-40	Nonidet 40
o.n.	overnight
dNTP	desoxynucleosidtriphosphat
ssDNA	Single stranded desoxyribonucleic acid
OD	Optical density
ORF	Open reading frame
PBL	Peripheric blood lymphocytes
PBS	Phosphate buffer saline
PEG	polyethylenglycol
PCR	Polymerase chain reaction
PFU	Plaque forming unit
Taq	Thermophilus aquaticus
pH	Potentiel hydrogenii
PRNT	Plaque Reduction Neutralization Test
RF	Replicative form
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction

scFv	Single chain Fragment variable
SDS	Sodium dodecyl sulfate
sec	second
TBEV	Tick borne encephalitis virus
$TCID_{50}$	50% tissue culture infectious dose
TEMED	N,N,N,N Tetramethyl-ethylendiamine
TNE	Tris-NaCl-EDTA
VL	Variable light chain
VH	Variable heavy chain
WHO	World Health Organization
WNV	West Nile virus
YEL-AND	Yellow fever-associated neurotropic disease
YEL-AVD	Yellow fever-associated viscerotropic disease
YF	Yellow fever
YFV	Yellow fever virus

VII-3. Publications, presentations and posters.

Publications.

Antibody responses against wildtype yellow fever virus and the 17D vaccine strain: characterization with human monoclonal antibody fragments and neutralization escape variants.
Daffis S, Kontermann RE, Korimbocus J, Zeller H, Klenk H-D, ter Meulen J (2005). Virology, 337(2):262-72.

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Presentations.

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Daffis S, Kontermann RE, Korimbocus J, Zeller H, Klenk H-D, ter Meulen J.
Departments of Medicine, Molecular Microbiology, Pathology and Immunology, 2005, St Louis, USA.

Mapping of neutralizing epitopes on domain I and II of the yellow fever virus envelope glycoprotein with human recombinant antibodies generated through phage display.
Daffis S, Kontermann RE, Klenk H-D, ter Meulen J.
International Center for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India.

Isolation of neutralizing recombinant antibody fragments against yellow fever virus by phage display–Mapping of neutralizing epitopes on the yellow fever virus envelope glycoprotein.
Daffis S, Kontermann RE, Klenk H-D, ter Meulen J.
INCODEV annual meeting, 2004, Paris, France.

Posters.

Mapping of neutralizing epitopes on domain I and II of the yellow fever virus envelope glycoprotein with human recombinant antibodies generated through phage display.

Daffis S, Kontermann RE, Klenk H-D, ter Meulen J.

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EHRENWÖRTLICHEN ERKLÄRUNG

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Humanmedizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel

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im Institut für Virologie, Klinikum der Philipps-Universität Marburg, mit Unterstützung durch Herrn Prof. Dr. Jan ter Meulen and Herrn Prof. Dr. H.-D. Klenk, ohne sonstige Hilfe, selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation aufgeführten Hilfsmittel benutzt habe.
Ich habe bisher an keinem in- und ausländischem Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.
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