Vaccine 29 (2011) 7229–7241

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

From research to phase III: Preclinical, industrial and clinical development of the Sanofi Pasteur tetravalent dengue vaccine

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ARTICLE INFO

Article history:
Available online 13 July 2011

Keywords:
Dengue
Vaccine
Human
Development
Safety
Immunogenicity

ABSTRACT

Dengue vaccine development has reached a major milestone with the initiation, in 2010, of the first phase III clinical trial to investigate the Sanofi Pasteur CYD tetravalent dengue vaccine (TDV). The CYD TDV candidate is composed of four recombinant, live, attenuated vaccines (CYD-1–4) based on a yellow fever vaccine 17D (YFV 17D) backbone, each expressing the pre-membrane and envelope genes of one of the four dengue virus serotypes. The vaccine is genetically and phenotypically stable, non-hepatotropic, less neurovirulent than YFV 17D, and does not infect mosquitoes by the oral route. In vitro and in vivo preclinical studies showed that CYD TDV induces controlled stimulation of human dendritic cells, and significant immune responses in monkeys. Scale up and industrialization are being conducted in parallel with preclinical and clinical development to fulfill the needs of phase II/III trials, and to anticipate and facilitate supply and access to vaccine in the countries where the dengue disease burden makes it an urgent public health priority. The vaccine has now been administered to more than 6000 children and adults from dengue endemic and non-endemic areas and no safety concerns have arisen in any of the completed or ongoing trials. A three-dose vaccination regimen induces an immune response against all four serotypes in the large majority of vaccinees. Preexisting flavivirus immunity favors quicker and higher immune responses to CYD TDV, without adversely effecting clinical safety or increasing vaccine viremia. The observed level and nature of the cellular immune responses in humans are consistent with the good safety and immunogenicity profile of the vaccine. Preliminary results of an ongoing, proof-of-concept efficacy and large scale safety study in Thai children are expected by the end of 2012. Here we discuss the different steps and challenges of developing CYD TDV, from research to industrialization, and summarize some of the challenges to the successful introduction of a dengue vaccine into immunization programs.

1. Introduction

All four serotypes of dengue virus cause clinical manifestations ranging from self-limiting dengue fever (DF) to severe disease such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Dengue disease represents a growing threat, with more than one hundred countries affected and more three billion people at risk (for a review see Ref. [1]). This growth has made the development of an effective vaccine an international health priority.

Vaccine development programs in academic laboratories and pharmaceutical companies have investigated a variety of technologies, including live, attenuated vaccines (LAVs), recombinant virus vectors, recombinant proteins, and DNA vaccines (see Ref. [2] and articles presented in this special issue of Vaccine). At Sanofi Pasteur, efforts are focused on a tetravalent dengue vaccine (TDV) comprising four recombinant, live, attenuated dengue viruses (CYD-1–4) based on the yellow fever 17D vaccine strain (YFV 17D). This technology originated at the US National Institutes of Health and St Louis University [3,4] and was further developed at Acambis, now part of Sanofi Pasteur [5]. The live-attenuated and chimeric nature of these vaccine viruses necessitated extensive preclinical and clinical characterization, from the early stages of research through to the ongoing phase III clinical program and industrial development, taking into consideration the specific regulations pertaining to genetically modified organisms (GMO). Of note, a chimeric vaccine against Japanese encephalitis virus (JEV), which is similarly based on the YFV17D vaccine, has recently been licensed (IMOJEV™, Sanofi Pasteur, Lyon France).

The challenges of introducing a safe and efficacious dengue vaccine are not restricted to research and development. The production process must be scaled up sufficiently to supply vaccine for clinical phase III and for the subsequent launch of the vaccine in case

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doi:10.1016/j.vaccine.2011.06.094

of licensure. Furthermore, the introduction of dengue vaccination into existing national vaccination schedules must be planned for as there are a number of hurdles to be anticipated and addressed. Building upon previous reviews [6,7] here we discuss the numerous challenges inherent to the expected success of this candidate dengue vaccine, from early research, to large scale phase III clinical development, industrialization, and vaccine introduction.

2. Sanofi Pasteur CYD tetravalent dengue vaccine: Construction and overall development strategy

Each of the four recombinant dengue vaccine viruses, one per serotype, were constructed by substituting genes encoding the premembrane (prM) and envelope (E) proteins of YFV 17D with those from a wild-type (wt) dengue virus [5,8]. These wt viruses were the PUO-359/TVP-1140 Thai strain for serotype 1, the PUO-218 Thai strain for serotype 2, the PaH881/88 Thai strain for serotype 3, and the 1228 (TVP-980) Indonesian strain for serotype 4. The CYD TDV is produced by combining the four CYD viruses into a single vaccine preparation containing 5 log 10 CCID50 of each serotype ('5555 formulation'). The vaccine is freeze-dried and contains no adjuvant or preservative. It is presented in a single-dose vial or in a 5-dose multi-dose vial.

Preclinical evaluation was carried out in vitro on primary and transformed cells, including human cells, and in vivo in non-human primates (NHP). Studies were designed to provide information on the phenotypic and genotypic stability of CYD-1–4, as well as on the protective immunity provided by vaccination against all four circulating viral serotypes. Non-clinical safety (NCS) studies have been, and continue to be conducted to assess toxicity, biodistribution, and shedding.

One particular paradigm that was used in the research, development and industrialization of this vaccine was the early constitution and continuous incrementation of a development risk management plan (RMP). This reference document summarizes the risks identified, however hypothetical, and tracks how risks are detected, assessed and managed during vaccine development. It will be used throughout clinical development until registration.

Another innovation was the parallel pursuit of development and industrialization pathways. Indeed, scale-up and industrial development were initiated before the start of efficacy evaluation and will continue in parallel so that, in case of a favorable outcome, vaccine can be produced consistently, thus mitigating the supply risk and allowing vaccination against dengue disease to commence where it is most urgently needed.

Clinical evaluation has included reactogenicity, viremia, humoral and cellular immunogenicity, and genetic stability. The functional role of antibodies was investigated using neutralization assays (PRNT50). The potential consequences of antibody dependent enhancement (ADE) have been considered throughout the development program, and included the early development of a reproducible in vitro assay. The evaluation of efficacy and long-term safety is ongoing.

The environmental risk evaluation has included the assessment of the vaccine viruses' tropism, structure, and ability to replicate and be transmitted by arthropod vectors. All of these aspects directly or indirectly affect safety and immunogenicity.

The various dimensions of Sanofi Pasteur's dengue vaccine development program are illustrated in Fig. 1.

3. Preclinical evaluation

3.1. Genetic stability

The YFV 17D vaccine genome is remarkably stable, both in vivo and in vitro [9], which may be attributed to the low error-rate of the viral RNA polymerase [10]. As this enzyme is also responsible for the replication of the CYD viruses, it was expected that the stability of the CYD-1–4 viruses would be similarly high. The full sequence of each of the CYD-1–4 genomes was established at various stages throughout the production of GMP vaccine lots, from the first passages, to premaster seed lots (PMSL), master seed lots (MSL) and bulk, and ultimately at a later step in the process (bulk + 10 passages) [11]. A few mutations present early on in the process appeared during the scale change between PMSL and MSL, and were then stably conserved throughout the process, likely reflecting adaptation to Vero cells. After further passages, the four viruses displayed very high genetic stability, with no change between master seed lots (MSL) and bulk lots despite significant changes to the process, including cell culture scale-up, removal of serum from the culture medium, and growth in bioreactors at later stages. Only a few genetic variations were observed beyond bulk, which were often partial (mixture of the initial sequence and the new one), and appeared after a relatively high number of virus replication cycles. Variations in prM/E were determined to have no impact on neurovirulence in mice, and none of the variations located in the non-structural and capsid genes inherited from YF17D were at attenuation positions [12].

Sequencing the vaccine viruses isolated from humans after vaccination would be the logical next step in documenting the genetic stability of CYD-1–4. However, consistent with the favorable safety profile observed in clinical trials, viremia after CYD vaccination is infrequent in humans, only rarely exceeding the level of PCR detection. So far, only four samples from viremic participants have been analyzed. The partial sequences that could be obtained from the very low amounts of CYD viral RNA extracted from serum showed no mutations compared with the original bulk sequences, with the exception of one silent mutation in serotype 4 (unpublished data).

3.2. Phenotypic stability

Despite its importance in the assessment of genetic stability, consensus genome sequencing cannot detect minor quasi-species in a vaccine seed or batch, neither can it provide significant information about the potential biological consequences of a given mutation. However, mutations affecting the efficiency of infection or the growth and transmission of the virus in cell culture generally modify the plaque phenotype [13,14]. We therefore monitored the consistency of plaque phenotype throughout vaccine lot production using a plaque size phenotype assay. Plaque size phenotype was found to be stable at all production steps, for all four CYD viruses [11].

Phenotypic stability was further assessed in animal models. Mouse models of neurovirulence have been used to discriminate between the neurotropism of dengue vaccine candidates and that of their parental viruses. One such model in suckling mice has been shown to be an acceptable alternative to non-human primate (NHP) models [15]. After intracerebral inoculation in both mice and NHPs, all four CYD viruses were seen to be significantly attenuated, even compared with the parental YFV 17D vaccine [15]. The suckling mouse model is now routinely used for in-process control testing during the manufacturing process of Sanofi Pasteur's YFV 17D-based flavivirus vaccines.

3.3. In vitro immunogenicity

Skin dendritic cells (DCs) are among the first cells to encounter a viral inoculum and are also the most efficient antigen-presenting cells (APC) implicated in the primary immune response [16]. Interactions between human DCs and wild-type dengue viruses are well documented [17,18]. DNA microarrays have been used to show that human gene expression patterns differ in the case of DF versus DHF

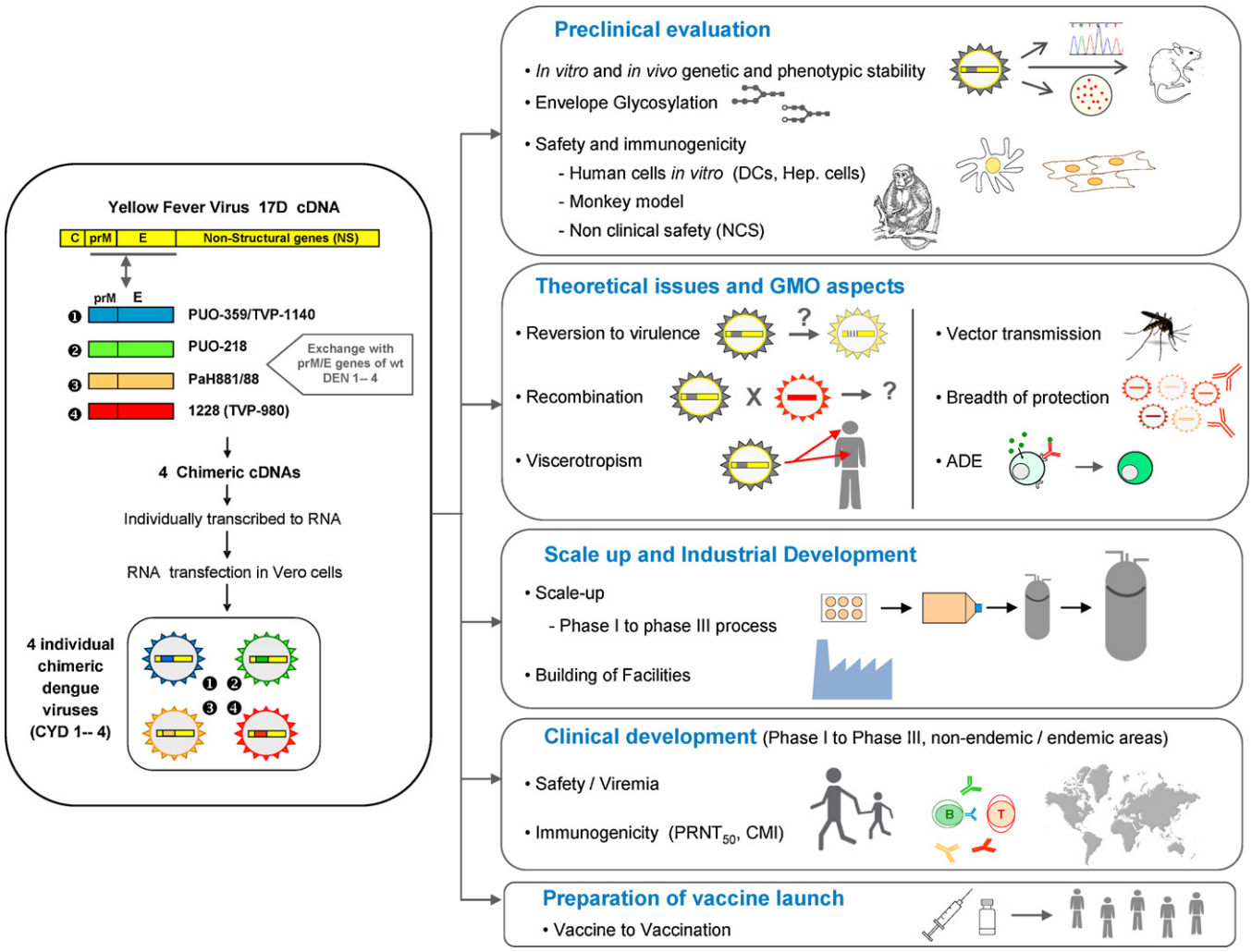

Fig. 1. Construction of the Sanofi Pasteur dengue vaccine and overall development strategy.

[19,20]. Patients with DF displayed a typical but transient antiviral signature, whereas patients with severe dengue displayed a blunted response which may have in fact followed a strong innate or adaptive response that was no longer present at the time of analysis [19]. While kinetic analysis would be needed to provide a complete picture, it is probable that early innate events, including APC and neutrophil activation [20], play a major role in shaping the subsequent evolution of the immune response and the disease presentation. We compared the immunological consequences of infection with the CYD viruses versus their wt parents by investigating the infectivity of CYD-1–4 in monocyte-derived human DCs [21], and determined the consequences of infection in terms of cellular activation and maturation, and the secretion of pro- and anti-inflammatory cytokines, chemokines and type I interferons [22]. The CYD-1–4 viruses were seen to induce DC maturation and a controlled response, accompanied by limited inflammatory cytokine production and consistent expression of anti-viral type I IFN, in agreement with their good clinical safety profile and immunogenicity (see below).

These results were confirmed and expanded upon using DNA array profiling [23]. Microarrays were used to assess the innate gene signature in human mDCs infected with CYD-1–4 (either alone or in combination), a wt dengue serotype 3 virus, and a classically attenuated serotype 3 virus (VDV3) that had been shown to be reactogenic in humans [24]. We observed a highly reproducible signature for each of the four CYD viruses, involving stimulation of Type I IFN genes and associated genes (ISGs), together with genes encoding chemokines and other mediators involved in the initiation of adaptive responses. In addition to the well-know role of type I IFNs, several genes upregulated by CYD infection, such as RIG-I, MDA5, IFITM1, TRAIL, have been identified by other authors as critical for a protective response against dengue and other flaviviruses [25–27]. As expected, the gene signature observed in the case of CYD-infection showed similarities but also some differences with the findings of similar studies using YFV 17D vaccines [28]. The CYD gene profile was again consistent with clinical trial observations of safety and immunogenicity. In contrast, the gene profile of the virulent wt DENV-3 strain was in agreement with previous reports with other wt dengue viruses, and consisted of a strong inflammatory profile with the induction of chemokines involved in neutrophil attraction. Interestingly, while initial studies into a limited number of parameters [24] found a similar pattern for the VDV3 and CYD viruses (and similar expectations regarding safety), the use of DNA arrays showed that the profiles were markedly different. The reactogenic VDV3 virus induced an almost exclusively 'antiviral' profile, with only limited induction of genes involved in early innate or subsequent adaptive immune responses. We hypothesized that VDV3 induced a weak, early immune response that was insufficient to prevent or control a second, higher round of viral replication, and that it is this second round that contributed to the high reactogenicity of VDV3 observed in human volunteers, concomitantly with peak viremia at Day 8 [24].

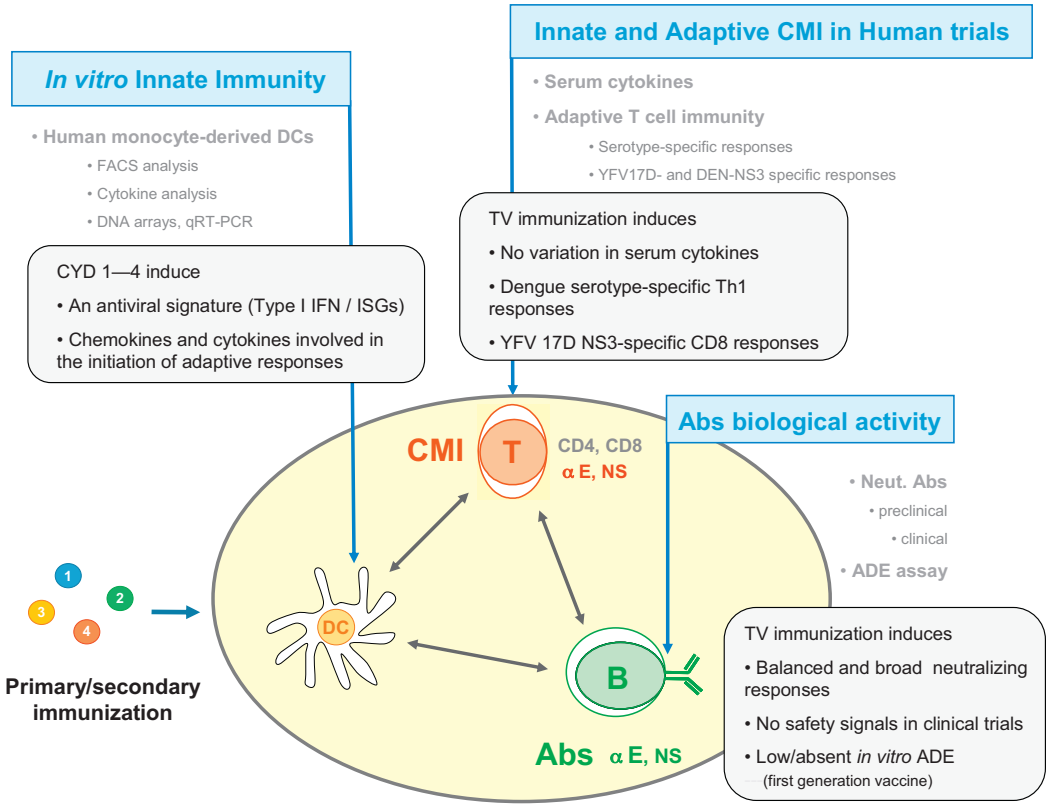

Fig. 2. Synthesis of in vitro and in vivo immunogenicity data from preclinical and clinical studies.

These DNA array data support the safety and immunogenicity of the CYD dengue vaccine candidate and highlight the interest of determining a broad innate signature in preclinical studies to better predict the outcome in humans. These studies are part of a broad 'systems biology' approach, and the experiments that we developed at both early preclinical and clinical stages more than 10 years prior to this review, are in agreement with the newly defined concept of 'systems vaccinology' [28,29].

Fig. 2 summarizes the immunogenicity data obtained in vitro and in vivo.

3.4. Other in vitro assays

A number of other in vitro assays, which will not be described here, have also been used to characterize the CYD vaccine candidates. These include: electron microscopy to assess viral maturity; SDS/PAGE analysis to assess the consistency of the protein content and profile of the vaccines; replication in insect C6/36 cells; temperature sensitivity assays; replication in DC SIGN-transfected cell lines to assess the ability of vaccine candidates to interact with this molecule and subsequently effectively enter cells, and glycosylation status. In this latter assay, it was observed that the dengue envelope had the expected glycosylation pattern for each considered serotype (Dubayle et al., in preparation).

As each monovalent dengue virus vaccine is based on YFV17D, the evaluation of hepatotropism has received particular attention, both in vitro and in vivo. These assays are discussed in the following paragraphs.

3.5. In vivo immunogenicity and viremia

Some NHPs, including rhesus (*Macaca mulatta*) and cynomolgus monkeys (*Macaca fascicularis*), are sensitive to dengue infection, and while infection in these species remains asymptomatic, viremia can be used to assess the attenuation of vaccine candidates by comparing vaccine virus viremia with that of the wt parental strains. Additionally, the absence of viremia after a wt viral challenge in vaccinated animals compared with that in unvaccinated controls can be used as an indicator of protection. Viremia can thus be considered as both a direct indicator of tropism and an indirect indicator of safety since it has been identified as one of the factors associated with virulence and disease severity in humans [30].

NHP studies can also be used to provide information on the ability of dengue vaccine candidates to elicit neutralizing antibodies. Studies have shown that primary immunization with CYD TDV induces short-lived, low-level viremia whereas booster immunizations do not. One or more vaccinations conferred immunity against the four serotypes of wt virus, as well as almost complete protection against upon subsequent wt challenge [8].

As with any multivalent vaccine, dengue vaccine development is complicated by the potential for interference between serotypes which can result in a dominant immune response against only one or two serotypes. In monkeys, we observed interference after vaccination with a CYD TDV and identified several potential mitigation approaches: (i) simultaneous administration of two complementary bivalent vaccines at separate anatomical sites drained by different lymph nodes; (ii) sequential administration several weeks apart of two complementary bivalent vaccines; (iii) pre-immunization against another flavivirus; (iv) reformulation of the tetravalent vaccine with a reduced dosage of the immunodominant virus (in this case, CYD-4), and (v) re-vaccination at 1 year [31]. These studies also showed that immunizations should be spaced several months apart to favor a better induction of memory and prevent negative interference, possibly due to short-lived, cross-reactive (IgM) antibodies, cross-reactive T cells, or innate immunity. These regimens have also been tested in humans. Results confirm the importance in a 3 dose regimen of a vaccination at 1 year and highlight differences between

species, such as the optimal interval between immunizations (in preparation).

Fig. 2 summarizes the types of immunogenicity data obtained in vitro and in vivo.

4. Non clinical safety

Several studies have been performed, are ongoing or are planned to assess the non clinical safety and biodistribution of CYD TDV. No toxicity linked to the vaccine has been observed in any of the studies performed so far (unpublished data).

5. Scale up and industrial development

Scale up and industrialization were initiated very early on in the development program—in parallel with the preclinical phase and clinical phase I—to ensure that the demand for vaccine for clinical phase III and the future demand for licensed vaccine can be met.

The four vaccine viruses are produced from four virus seed lots using an identical manufacturing process for each serotype. Banking systems for serum-free Vero cells have been established to produce master and working viral seeds and cells, allowing reliable and consistent supply of virus and cells respectively. Vaccines and cells are characterized and tested for safety in accordance with WHO, European and US guidelines [32–37]. All tests are part of a control strategy designed to ensure product quality and consistency. It includes quality control specification, product characterization, adherence to good manufacturing practices (GMP), validated manufacturing process, raw material testing, in process testing, and stability testing. The quality control (QC) specification is typical for a live, attenuated, viral vaccine, based on current regulations and guidelines, assessing mainly purity, safety and potency of the vaccine. Due to the use of Sanofi Pasteur serum-free Vero cell banks for both cell and viral culture, the CYD TDV manufacturing process includes no raw materials of animal origin; neither does the vaccine contain any preservatives, adjuvants, or antibiotics. A proprietary stabilizer is present in the finished product which has been shown to have excellent stability: accelerated stability studies found that vaccine from the phase III lots of CYD TDV (unidose presentation) was stable up to 1 month at $25 \pm 2\,^{\circ}C$, and that the viral titer decreased by less than 0.5 log 10 CCID50 after 7 days at $+37 \pm 2\,^{\circ}C$. Reconstitution vaccine was found to be stable for up to 6 h at $+5 \pm 3\,^{\circ}C$.

Three new dedicated facilities (Utilities, QC and Production) are being built at a new vaccine production site in Neuville sur Saone, France (Fig. 3). The considerable investment that this represents is consistent with the continuous efforts made by the Sanofi Pasteur teams over the past 15 years towards the development of a safe and efficacious dengue vaccine.

6. Clinical development

6.1. Clinical development challenges

The principal challenge for of the clinical development of a dengue vaccine is to demonstrate that, when given with a suitable vaccination regimen, the candidate vaccine safely elicits adequate and balanced immune responses against the four dengue serotypes and that, in absence of an established correlate of protection, these responses translate into clinical efficacy.

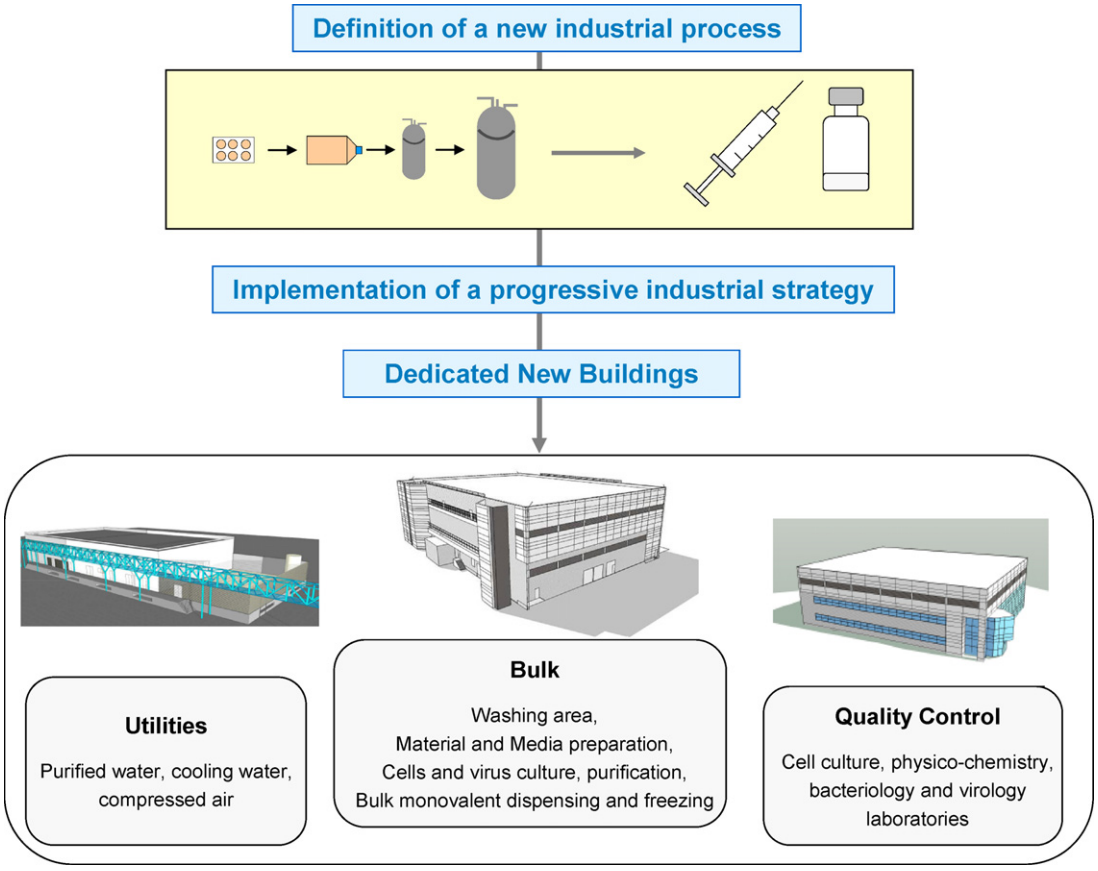

Fig. 3. Major achievements of the dengue vaccine industrial development.

Dengue is endemic in regions with very different flavivirus immunological and epidemiological backgrounds: Japanese encephalitis virus and vaccination in Asia; yellow fever virus and vaccination in Latin America; and differing dengue epidemiology. The efficacy and safety of the dengue vaccine candidate must therefore be demonstrated in both these regions, potentially with different co-administered vaccines. Clearly the priority is to develop a vaccine for dengue endemic countries of Asia-Pacific, Latin America and the Caribbean to address the unmet medical need for children and adults, but travelers and military personnel from non-endemic countries may also benefit from dengue vaccination. Consequently, clinical trials are also needed in non-endemic populations from Europe, USA and elsewhere. The durability of immune responses and long term safety must also be demonstrated. The plaque reduction neutralization test (PRNT) is considered to be the laboratory standard for assessing neutralizing antibodies against dengue virus [38], and when applied to the assessment of immunity against Japanese encephalitis, a 50% PRNT titer of 1:10 is considered as a reasonable surrogate of protection [39]. For the development of the dengue vaccine candidate, and pending the availability of efficacy data, the same PRNT titer threshold is being used as a marker of seropositivity.

The clinical development program has been developed in accordance with the WHO guideline for the clinical evaluation of Dengue Vaccines in endemic areas [40]. As of April 2011, more than 6000 volunteers had received at least one dose of TDV, from children aged 12 months to adults up to 60 years, and in both dengue-endemic (Brazil, Colombia, Honduras, Malaysia, Mexico, Philippines, Puerto Rico, Thailand, and Vietnam) and non-endemic (Australia, Mexico City, and USA) areas.

Fig. 4 summarizes the clinical safety and immunogenicity findings obtained so far, and presents the objectives of ongoing and future studies.

6.2. Phase I evaluation

6.2.1. Safety and humoral immunity

The first clinical evaluation of a CYD vaccine candidate was with a monovalent serotype 2 CYD virus (CYD-2) in healthy, flavivirus-naïve, US adults [41]. The safety profile of CYD-2 was reported to be similar to that of the yellow fever control vaccine (YF-VAX®, Sanofi Pasteur, Swiftwater, PA) and low levels of transient CYD-2 viremia were observed. Most vaccinees seroconverted to the dengue 2 wild-type virus (strain 16681), and prior YFV 17D immunity was found to result in stronger, broader (cross-protective), and longer lasting anti-dengue immune responses.

A subsequent study with a tetravalent formulation containing 5 log10 CCID50 of each serotype—the formulation currently under phase III evaluation—demonstrated complete seropositivity (serotype-specific titers ≥10 in a PRNT50 assay) against the four dengue serotypes after three vaccinations [42]. This study was also conducted in dengue-naïve US adults who received, in a 0–3.5–12-month regimen, 3 injections of TDV or 1 injection of placebo then 2 injections of TDV. A favorable safety profile and low levels of viremia (measured using a CYD-specific, quantitative, real-time PCR assay), mainly CYD-4, were observed after the first vaccination. Viremia was even lower and more infrequent after the second TDV vaccination compared to the first, with more than 85% of vaccinees having no detectable CYD-1, 2, or 3 viremia.

The implications of this finding for the vaccine's safety are significant: the first TDV vaccination which elicited a predominantly serotype 4 immune response did not sensitize participants, as the second TDV vaccination in the presence of anti-serotype 4 antibodies was not associated with either increased viremia of the 3 other serotypes or an exacerbation of safety outcomes. After having received three TDV vaccinations, all participants seroconverted to all four WHO reference dengue strains tested. Immune responses

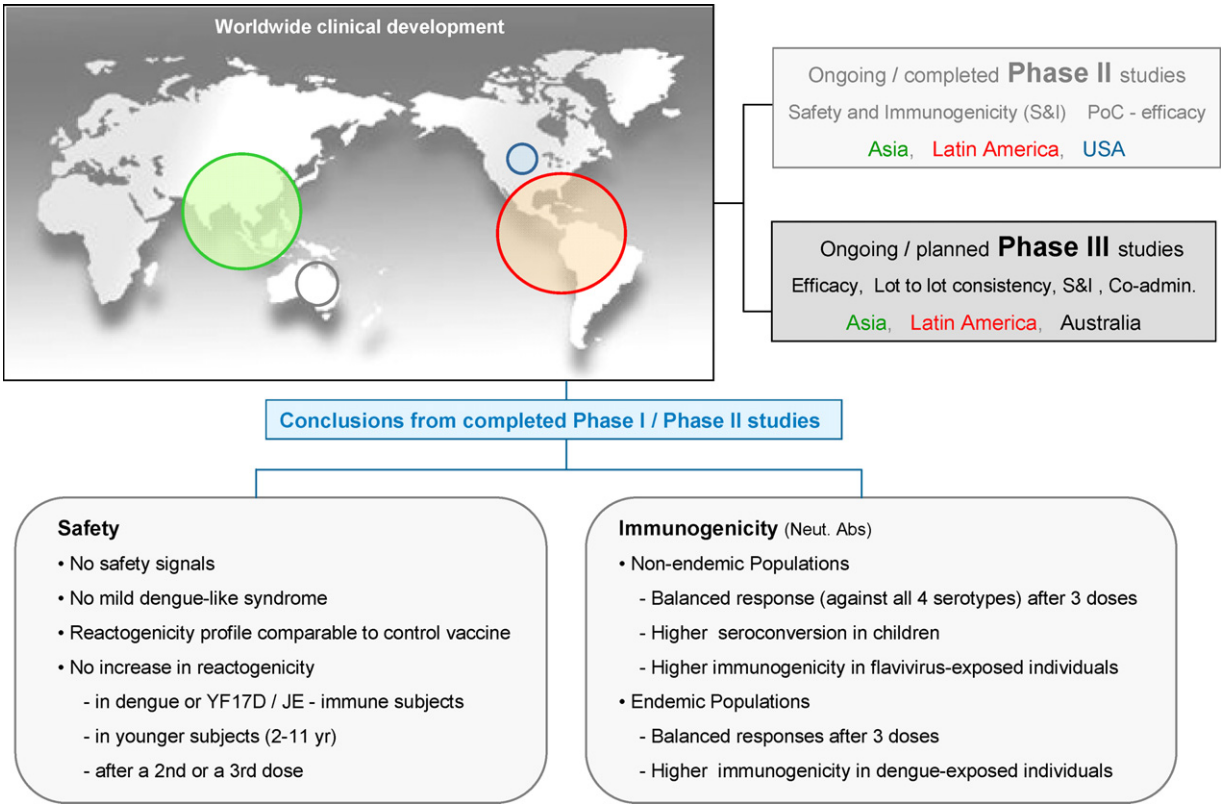

Fig. 4. Worldwide map of phase II/II dengue clinical trials, and major results obtained so far in humans.

were seen to increase incrementally with each vaccination in terms of both geometric mean titer (GMT) and the proportion of seroresponders. Four weeks after the third TDV vaccination, GMTs were 67, 538, 122 and 154 against serotypes 1, 2, 3 and 4 respectively.

This study also provided the first indication that the three- to four-month interval between the first two vaccinations might be suboptimal. Volunteers in the control group appeared to mount higher titers after their first two TDV vaccinations which were given eight to nine months apart, compared with those in the active group given three to four months apart. Cellular immune responses were also monitored in this trial [43]. The level and nature (cytokine profile, CD8/Th bias, serotype dominance) of the observed innate and adaptive cellular responses were in good agreement with both the favorable safety profile and humoral immunogenicity data, as will be discussed in Section 6.2.2.

Following on from the tetravalent study in US adults, two other phase I studies were performed with the same 0–3.5–12-month vaccination regimen and the same 5555 formulation, one in an area of dengue endemicity (Philippines), the other in a non-endemic area (Mexico City) [44,45]. These studies, which were the first CYD studies to enroll children, included four age groups: adults aged 18–45 years; adolescents aged 12–17 years; children aged 6–11 years, and young children aged 2–5 years. Findings from these studies were consistent with those reported by Morrison et al., confirming the immunogenicity of the vaccine and its ability to induce a balanced response against all 4 serotypes when given with a 3 dose regimen. There were no vaccine-related serious adverse events, other significant clinical adverse events or clinically significant trends in biological safety. Reactogenicity was not higher after the second and third TDV vaccinations than after the first, and was not higher in children than in adults. Beyond the individual study results, the observed reactogenicity profile and safety conclusions from two studies were similar, despite the differences in immunological status of the two populations, providing further evidence that pre-existing flavivirus immunity does not adversely affect the safety of CYD TDV, and echoes the observation that reactogenicity was no higher after the second and third TDV vaccination, compared to the first. In agreement, another study in Australian adults showed that prior immunity against either DENV-1 or 2 (induced by vaccination with conventionally attenuated dengue vaccine candidates developed by Mahidol University, Bangkok, Thailand) resulted in a strong and broad response against all four serotypes after a single TDV vaccination, without any adverse effect on reactogenicity [46]. These data are also in agreement with a recent report on the influence of prior heterologous immunity on the outcome of vaccination with other dengue vaccine candidates [47].

CYD viremia was evaluated as an indicator of safety in each phase I study and in each case findings were similar to those reported by Morrison et al. for US adults: low levels of viremia (mostly below the RT-PCR's lower quantitation limit) were detected in a minority of vaccinees, mainly after the first TDV vaccination. When detected, viremia was mostly CYD-4.

In summary, phase I studies showed us that the CYD TDV is well tolerated and immunogenic for all four serotypes in both adults and children as young as 2 years old, irrespectively of whether they reside in dengue-endemic or non-endemic areas. The observed safety profile was considered to be good and consistent with progression to larger studies. We therefore decided to initiate clinical phase II with a three-dose, 0–6–12-month regimen.

6.2.2. Cell mediate immunity

It has been shown that heterologous, cross-reactive responses tend to trigger TNF-α while homologous responses trigger IFN-γ [48–50]. Similarly, subclinical dengue infection in school children has been observed to be associated with higher frequencies of IL2-and IFN-γ-producing T cells, compared with symptomatic infections [51]. It has also been suggested in human a challenge model that sustained IFNγ responses were linked to protection [52]. Cellular immune responses to vaccination should therefore include high-avidity, homologous responses against all serotypes. These responses should be Th1-biased, and dominated by IFN-γ, not TNF-α.

We assessed the CD4 and CD8 responses elicited by CYD TDV vaccination against the parental YFV 17D and dengue viruses in volunteers with or without pre-existing flavivirus immunity [43]. We detected no changes in serum pro-inflammatory cytokines after vaccination, regardless of the baseline immune status. Significant YFV 17D NS3-specific CD8 responses and DENV serotype-specific T helper 1 responses were observed and were dominated by IFN-γ over TNF-α. The corresponding antibody responses were initially dominated by a serotype 4-specific response in baseline-naïve individuals. Subsequent vaccinations then broadened the response to the other serotypes. A similarly broad response was seen after primary CYD TDV vaccination in participants with preexisting dengue serotype 1 or 2 immunity. The original antigenic sin hypothesis suggests that suboptimal, heterologous, cross-dengue serotype, anti-NS3 CD8 responses may be involved in the severity of secondary heterologous infection [53], although the late appearance of these responses in the course of disease calls this into question. [54]. In any case, little or no cross-reactivity has been seen between YFV 17D and dengue NS3-specific CD8 responses. As the CYD viruses express YFV 17D NS3, they would not elicit potentially deleterious, cross-dengue serotype, anti-NS3 responses [48–50].

A scientific consultation on cell mediated immunity (CMI) in dengue and dengue vaccine development, convened by the World Health Organization Initiative for Vaccine Research, stressed the interest of documenting the cellular response to a dengue vaccination during clinical phase III to better understand the short- and long-term safety and immunogenicity of the vaccine candidates [55]. However, the volume of blood (35–50 ml) typically required for the CMI analyses discussed above has limited their application to adult volunteers. We are exploring the feasibility of performing such analyses using low volume blood samples (up to 3 ml).

Fig. 2 summarizes the cellular immunity findings from these clinical trials.

6.3. Phase II evaluation

The objectives of clinical phase II include: documenting the immunogenicity and safety of the tetravalent vaccine candidate with the 0–6–12 month schedule in populations with different flavivirus exposure and vaccination histories; investigating co-administration with another live virus vaccine in toddlers (measles, mumps, rubella vaccine), and exploring potential alternative CYD dengue vaccine formulations and vaccination scenarios. Phase II trials were therefore initiated in a number of countries, particularly in the USA, Latin America and Asia (Fig. 4).

The first phase II study to provide safety and immunogenicity data for children was conducted in Peru (ClinicalTrials.gov NCT00788151). Preliminary data from this study, where 2–11 year-olds with a history of YF vaccination 1–7 years earlier received three TDV vaccinations or three control vaccinations at months 0–6–12, were consistent with the phase I study findings in children discussed above, and showed that the vaccine candidate had a good safety profile and was immunogenic in this population (Lanata et al., manuscript in preparation).

During phase I, pre-existing immunity against YF was seen to result in quicker and broader immune responses to CYD-TDV vaccination, without adversely affecting safety and without increasing CYD viremia [41,44]. An exploratory, phase II study in Mexican adults included an assessment of whether pre-existing immunity against another flavivirus—JEV—would have a similar effect (Clin-

icalTrials.gov. NCT00740155). Preliminary data from this study showed that the dengue-specific seropositivity rates and GMTs were higher in participants vaccinated with one dose of inactivated JE vaccine (JE-VAX, Research Foundation for Microbial Diseases of Osaka University) followed 3.5 months later by one dose of CYD TDV, than in participants vaccinated with two doses of CYD TDV, 3.5 months apart. The safety profile of the first dose of TDV was seen to be unaffected by the prior dose of JE-VAX, as was CYD viremia which was low and infrequent in both groups [56].

It is not yet known whether the observed positive effect of prior flavivirus immunity on the immunogenicity of subsequent CYD TDV vaccination will translate into a benefit in terms of protection, nor is it known whether the anti-dengue antibody persistence will be equivalent in these different scenarios. The critical finding here is not that there is an apparent beneficial effect on the immunogenicity of the candidate dengue vaccine, but rather that there is no adverse effect of prior routine vaccination against JE or YF on safety and reactogenicity of CYD TDV.

In summary, the CYD vaccine candidates have so far demonstrated a satisfactory safety profile. No serious adverse events (SAE) related to vaccination have been identified in the studies mentioned above. Reactogenicity has appeared similar to that of the control vaccines. Reactogenicity was not increased by the presence of baseline immunity to either dengue or yellow fever, nor was it increased after the second or third vaccination than after the first.

6.4. Efficacy proof of concept

In parallel with the phase II safety and immunogenicity studies in various populations, a proof of concept efficacy and large scale safety trial was initiated among 4000 Thai children aged 4–11 years (ClinicalTrials.gov NCT00842530). Participants have received 3 subcutaneous injections of either CYD TDV or a placebo or active control at months 0–6–12 and are currently being followed to assess efficacy against virologically-confirmed dengue disease, regardless of severity. Results from this trial are expected by the end of 2012.

6.5. Phase III clinical evaluation

The most obvious objective of this final phase of clinical development before registration is to demonstrate whether the candidate vaccine protects against disease. While clinical trial data show that CYD TDV vaccination elicits neutralizing antibodies against all four serotypes in the majority of vaccinees, only efficacy trials will be able to demonstrate whether this correlates with protection against disease.

The first phase III clinical trial of CYD TDV, and indeed of any dengue vaccine, was initiated in 2010. The objectives of this placebo-controlled trial (ClinicalTrials.gov NCT01134263), in Australian adults, are to assess lot-to-lot consistency, safety and immunogenicity. A second phase III trial is ongoing to assess safety and immunogenicity in Malaysian children (aged 2–11 years). Among the phase III trials to commence in 2011, two multinational studies in endemic areas of Latin America and Asia will assess the vaccine's efficacy (Fig. 4).

Some of the key elements of the design of these efficacy studies are listed below:

The required sample size for a vaccine efficacy trial is inversely proportional to the disease incidence in the population studied. Performing efficacy trials in populations with the highest incidence therefore reduces the sample size and/or the duration of follow-up required to conclude. It also means that efficacy is evaluated in the population that stands to benefit most from a safe and efficacious vaccine. In the case of dengue, the disease incidence and burden are particularly high among children, especially in Asia. Dengue efficacy trials will therefore be conducted in children.

The primary endpoint will be the prevention of laboratory-confirmed, clinical dengue, regardless of severity. Given that dengue can present as non-specific febrile illness, laboratory confirmation of suspected cases will be essential for the accurate estimation of the vaccine's efficacy.

Active surveillance will be important to ensure that all potential dengue cases are rapidly identified, allowing blood samples for laboratory analysis to be drawn as close as possible to the onset of illness.

Given earlier observations that pre-existing immunity against yellow fever in Latin America, Japanese encephalitis in Asia, and dengue in both regions can affect the vaccinee's immune response to dengue vaccination, flavivirus immune status will be documented in a subset of volunteers to allow the potential impact on efficacy to be determined.

To prepare the trial sites for these efficacy trials, in particular to assess whether potential dengue cases can be identified and whether the diagnostic algorithm is applicable in the field, prospective, active-surveillance studies were initiated in two cohorts in Asia (ClinicalTrials.gov NCT01218906) and Latin America (NCT01293331). Furthermore, healthcare systems were mapped to determine where patients go for their healthcare, to ensure a robust system for case capture.

There is a theoretical possibility that vaccine-induced immunity (antibody and/or cellular responses) may lead to an increased severity of subsequent wt dengue infection in the vaccinated group than would otherwise occur. It is therefore critical to assess the severity of the potential dengue cases to identify any such enhanced disease and to distinguish between this and vaccine failures. For this purpose, the efficacy trials will be closely monitored by Independent Data Monitoring Committee (IDMC) that will assess the severity of potential dengue cases and follow-up closely on any severe cases, as well as monitoring the safety of the study population as a whole.

As an additional measure to assess the safety of CYD TDV, a number of trials (including the two efficacy trials) will feature an extended follow up period of 3–5 years during which time the occurrence of severe dengue cases will be closely monitored. A subset of participants will be assessed for antibody persistence during this extended period to determine whether there is a need for booster vaccinations.

Finally, another noteworthy objective of phase III will be to determine whether the dengue vaccine can be co-administered with widely used pediatric vaccines.

7. Environmental risk assessment

As the CYD-1–4 viruses are genetically-modified, live flaviviruses, we assessed the risk that they could be transmitted by arthropod vectors, recombine with a circulating virus, or revert to virulence. This environmental risk assessment program has been described elsewhere in detail and will only be discussed briefly here [6].

7.1. Transmission by arthropod vectors

For an arthropod vector to disseminate a CYD virus, it must first become infected by feeding on a vaccinated host, which requires the vaccinated host to have a sufficiently high level of viremia. However, as discussed above clinical trial data show that CYD viremia is very low to inexistent, and short-lived [42].

After infecting the vector, the virus must be able to replicate in the vector. The ability of the CYD viruses to replicate in

Aedes albopictus mosquito cell culture (C6/36) and in Ae. aegypti mosquitoes—the principal vectors of YF and dengue viruses—was evaluated in comparison with the parental YF17D and wt dengue viruses [57]. The CYD viruses were shown to be incapable of orally infecting either species or replicating in midgut tissue after intrathoracic inoculation. In this respect, the CYD viruses are even more attenuated than YFV-17D in these species.

The risk of vector transmission was further assessed using species other than the usual Aedes species of mosquito. Specifically, given that some flaviviruses are tick-borne, the ability of CYD-1–4 to replicate in females of two species of hard tick, Ixodes ricinus and Rhipicephalus appendiculatus after intra-thoracic inoculation was assessed in comparison the the parental DEN-1–4 and YFV 17D viruses (Kazimirova et al., in preparation). The presence of virus in tick salivary glands was then assessed at different points after inoculation. Unlike TBEV, viral clearance instead of amplification was observed after innoculation of CYD viruses. Furthermore, and again in contrast with TBEV, there was no transmission of CYD viruses between co-feeding infected females and uninfected nymphs.

In summary, the low levels of viremia observed after vaccination in humans, combined with the lack of transmission by arthropod vectors safeguard against the dissemination of CYD viruses in the environment.

7.2. Recombination

It was hypothesized that live flavivirus vaccines might in theory recombine with other flaviviruses or other RNA viruses [58]. Although this hypothesis was based on an analogy with non-flaviviruses, and on theoretical assumptions which have since been challenged [59,60], the possibility that new viruses might emerge from such a recombination event has been evaluated together with the potential consequences of such an event.

The likelihood of intermolecular recombination between flaviviruses was addressed using replicon pairs derived from TBEV, Japanese encephalitis virus (JEV) and West Nile virus (WNV) [61]. The very few recombination events detected for JEV only (none for TBEV or WNV), were aberrant recombinations resulting in virus with impaired growth properties, confirming that flaviviruses have a low propensity for homologous recombination.

To determine the potential consequences of such an event should it ever occur, worst-case recombinants between wt DENV-4 or YFV 17D and the wt Asibi strain of YF virus were investigated [62,63]. Compared with the wt parental viruses, these recombinants were found to be highly attenuated in terms of replication in, and transmission by mosquitoes, and in terms of blood parameters and clinical outcomes in NHP. Findings from these studies suggested that the chimerization process itself contributed to the attenuation of the CYD viruses.

Thus, not only is the recombination of the CYD vaccine viruses with a wt flavivirus extremely unlikely, any recombination would be unlikely to cause disease or be disseminated.

7.3. Reversion to virulence

Reversion to virulence has been raised as a potential concern. However, the reversion of a CYD virus into a virulent YF virus is highly improbable given (i) the genetic stability of the CYD viruses, (ii) the absence of the YFV17D preM or E genes, and (iii) the numerous attenuating residues located in the YFV17D nonstructural genes that are inherited by CYD-1–4 [11]. Reversions in all of these would be required for virulent virus to emerge, which is virtually impossible.

8. Other potential safety risks

8.1. Antibody dependent enhancement

Humoral and cellular immune responses are associated with protection against dengue, but have also been implicated in the immunopathology of severe dengue disease, the etiology of which appears to be multi-factorial (for reviews see [48], and Whitehorn et al. in the present issue of Vaccine). The antibody dependent enhancement (ADE) phenomenon—the enhancement of viral replication by heterotypic, non-neutralizing antibodies from prior infection, via the Fc receptor on mononuclear leukocytes—was hypothesized to be one of the multiple factors responsible for severe disease [64,65]. Although it is still a matter of debate whether or not ADE plays a critical role in vivo, the consensus is that clinical development of dengue vaccines should not be forestalled by hypothetical safety concerns [40].

The risk that dengue vaccination could result in ADE was nevertheless taken into consideration and evaluated from the start of Sanofi Pasteur's dengue research program. A sensitive and reproducible in vitro assay, developed using FcγRII positive-K562 cells and flow cytometry, was applied to sera from Thai children immunized with first-generation, live, attenuated dengue vaccine candidates. We found no, or only minimal, ADE activity in vitro despite the diversity of the vaccinees' immune profiles which included both low and high PRNT antibody titers against one or several dengue serotypes [66]. In particular there was no in vitro ADE in the presence of broad neutralizing responses against all four DENV serotypes. Thus, whatever the role of ADE in the etiology of severe dengue in vivo, a vaccine able to induce sustained neutralizing responses against all four serotypes should circumvent the issue. Dengue cases, and severe dengue cases in particular, are closely monitored in clinical trials. Due to the rarity of such severe outcomes, however, it is likely that only phase IV trials and post-marketing surveillance will provide a definitive answer as to whether ADE constitutes a risk for vaccinees.

8.2. Virus tropism and potential serious adverse events

Vaccination with YFV 17D vaccine is associated with the extremely rare occurrence (estimated incidence: 0.3–0.4 per 100 000 vaccinated individuals) of acute viscerotropism disease [67]. As the CYD viruses are based on YFV 17D, there is a perceived risk that acute viscerotropism disease may also occur after CYD vaccination.

Viral tropism is known to be largely linked with the virus' E protein, and in the case of the CYD viruses the E gene is one of the two genes (with preM) inherited from a dengue virus. The CYD viruses are therefore incapable of expressing the E protein of YF17D, and are consequently unlikely to display the same tropism.

We verified this in vitro by determining the growth kinetics of CYD-1–4 and their DENV-1–4 and YF17D parents in three hepatic cell lines (HepG2, Huh7 and THLE-3) as a potential marker of viscerotropism. Compared with YFV 17D, the replication of CYD-1–4 viruses was markedly lower in HepG2 and THLE 3 cells, but not in Huh7, suggesting that the CYD-1–4 viruses are less hepatotropic that YF17D virus vaccine in humans [21]. Differences between cell lines may be explained by the fact that Huh7 cells are permissive to viral replication, irrespective of attenuation phenotype. Further evidence of the lower hepatotropism of CYD compared with YFV 17D was provided by in vivo studies in monkeys, in which no liver infection was observed following inoculation with CYD-4, while a few foci were present following YFV 17D inoculation [68].

These findings are consistent with the in vitro and preclinical in vivo experiments showing the viscerotropism and neurotropism of the CYD viruses to be significantly attenuated compared with YFV 17D. It is thus reasonable to anticipate the safety profile of CYD

TDV to be improved compared with that of YFV 17D. Monitoring for potential acute viscerotropic disease (AVD) and acute neurotropic disease (AND) is conducted in clinical trials. However, due to the rarity of these conditions, it will not be possible to draw a definitive conclusion as to the risk for vaccinees until after licensure.

8.3. Breadth of protection

To address the risk that a circulating virus escapes vaccine-induced immunity, we assessed the capacity for monkey sera raised against the CYD vaccine viruses to cross-neutralize a large panel of wt strains collected for each serotype from different areas of dengue endemicity [69]. Results suggest that vaccine-induced antibodies provide broad coverage against geographically diverse strains, by cross-reacting against a panel of approximately 20 different circulating strains per serotype. Similar analyses are ongoing with human sera.

9. Vaccine introduction

The development and production of a safe and efficacious vaccine are the first steps to ensuring the protection of populations at risk from dengue. However, a number of other challenges—including epidemiological, economic, regulatory, and logistical ones—must also be met to ensure the successful introduction of the vaccine in the field (Fig. 5).

One of the main challenges is determining the true burden of dengue disease. Indeed, accurate country specific surveillance data will be required to guide the introduction of dengue vaccine and to assess the value of vaccination programs. Almost all available data are from the national surveillance systems in place in many endemic countries. The objectives of these systems are to follow the incidence of dengue, to measure the morbidity and mortality of the disease across age groups, to rapidly detect important epidemiological events and assess the impact of control measures. They are not, however, organized to provide accurate estimates of disease incidence or a complete picture of the disease at either regional or national levels. The weaknesses of such systems include the following: case notification is not always mandatory, access to healthcare is variable and can be poor in certain areas or segments of the population, non-specific febrile illnesses caused by dengue can be misdiagnosed, and laboratory capacity can be insufficient to confirm all suspected cases [70]. It should also be mentioned that funding can be insufficient to maintain and improve these systems.

Estimates of disease incidence and burden that rely solely on the number of reported cases will inevitably underestimate the magnitude of the problem. Several authors have confirmed this 'underreporting' by comparing national surveillance system data with data obtained either via prospective studies or enhanced surveillance [71–73]. Underreporting impacts not only the assessment of disease incidence, but also any subsequent assessment of the cost of disease or the impact of control measures such as vaccination (for a review, see [74]). Improved surveillance systems will be needed to quantify the medical value of the dengue vaccination programs in studies of effectiveness and vaccination coverage.

The epidemiology of dengue varies considerably, both geographically and temporally. For example the peak age-specific disease incidence differs between Asian (highest incidence seen in children) and Latin American (highest incidence seen in young adults and adolescents) countries and has evolved in recent years

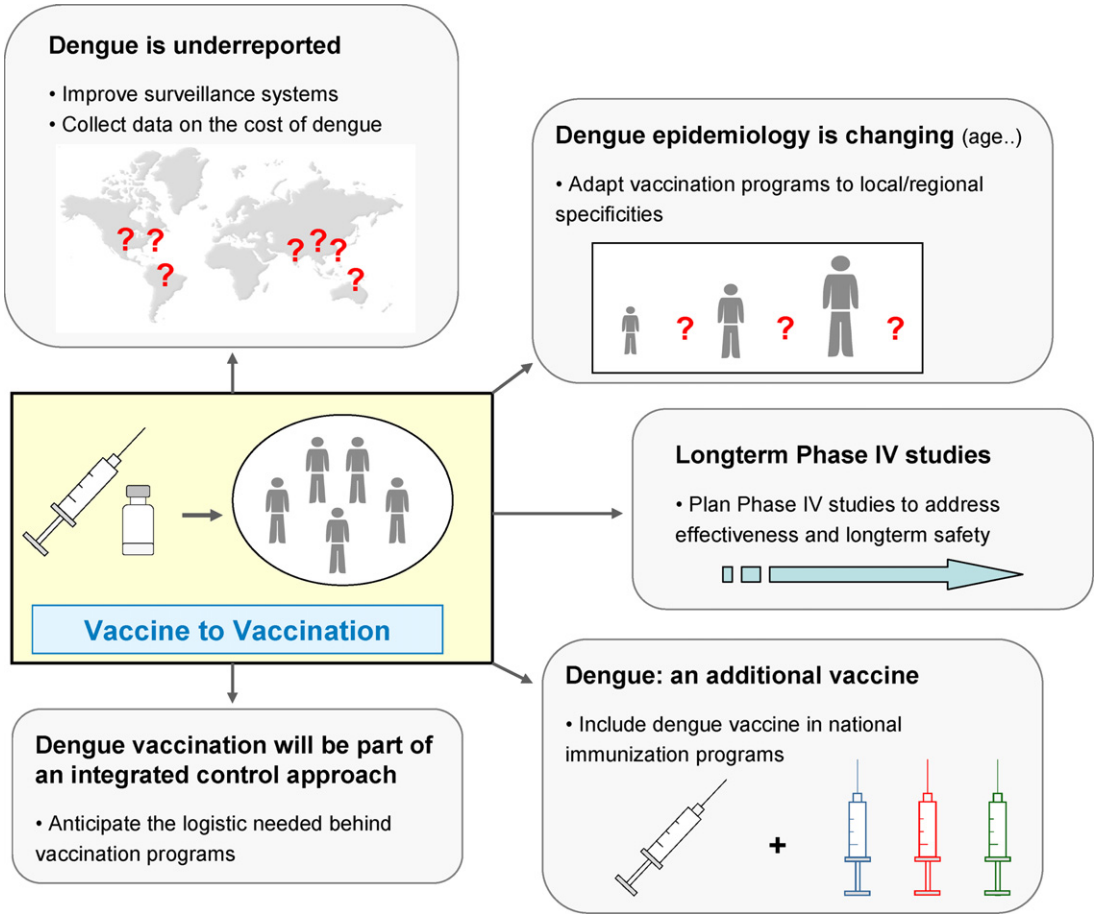

Fig. 5. Challenges associated with the successful introduction of the dengue vaccine.

[75–77]. Such epidemiological specificities may require vaccination programs to be tailored regionally or nationally. Existing immunization programs represent another national specificity that must be accounted for as the introduction of dengue immunization must not be at the cost of existing programs, and the need to return for additional vaccinations at additional visits can be logistically and financially challenging. The 'programmatic feasibility' of dengue immunization will therefore depend on these issues, as well as the existing infrastructure, vaccination strategy, and the need for effective communication strategies.

Dengue immunization should be considered as part of a wider, integrated strategy with community involvement, surveillance, case management, vector and outbreak control. Governments will need to anticipate budget needs for routine dengue vaccination, catch-up programs, consumables, infrastructure, training, and surveillance. Alternative funding mechanisms will be needed to finance vaccination programs in some countries located in endemic zones.

It is likely that the initial introduction of dengue vaccination will be accompanied by long-term phase IV studies that should be planned in collaboration with national authorities, and will serve to demonstrate the medical value (including effectiveness and safety) and feasibility of vaccination [78].

To meet these challenges and successfully introduce dengue vaccination in endemic countries a global, coordinated approach will be required, involving the immunization community, potential funders, national health authorities in endemic countries, and non-governmental organizations. Within this context, the Dengue Vaccine to Vaccination (v2V) initiative was founded in 2009 to develop guidance for the successful introduction of dengue vaccination [79]. Another group, the Dengue Vaccine Initiative (DVI), was established in 2010 to build upon the work of the Pediatric Dengue Vaccine Initiative and to increase awareness of the need to support the development and use of dengue vaccines [80]. The DVI's goal is to accelerate the introduction of safe and broadly protective vaccines into the national immunization programs of endemic, developing countries.

10. Conclusions

The Sanofi Pasteur CYD tetravalent dengue vaccine candidate, developed according to a systematic 'systems vaccinology' approach, has demonstrated satisfactory safety and immunogenicity in in vitro and in vivo preclinical tests, as well as in clinical trials in both flavivirus-naïve and immune individuals. Potential risks, however unlikely, hypothesized as being associated with the vaccine's recombinant technology or the immunopathology of severe dengue disease, have been assessed in detail using a number of methods. All study findings are consistent with the continued investigation of the CYD candidate vaccine in phase III trials to assess the vaccine's efficacy and safety in large cohorts of children in Asia and Latin America. An effective vaccine is urgently required and coordinated efforts to facilitate the introduction of a dengue vaccine into the national immunization programs of endemic countries have started. With the initiation of large scale efficacy trials and due to unprecedented industrial and clinical development, this dengue vaccine candidate provides hope that protection is now within reach.

Acknowledgements

The authors would like to acknowledge Grenville Marsh for excellent editorial assistance, all Sanofi Pasteur and former Acambis team members, as well as external collaborators involved in preclinical, clinical and industrial development, in particular Farshad Guirakhoo, Konstantin Pugachev, Tom Monath, Veronique Barban, Nathalie Mantel, Yves Girerd-Chambaz, Anke Harenberg, Jean Dubayle, Carine Navarro, Patrice Riou, Christophe Fournier, Guillaume Ravel, Remi Forrat, Denis Crevat, Gustavo Dayan, Derek Wallace, Anh Wartel-Tram, Betzana Zambrano, Enrique Rivas, Yanee Hutagalung, Thelma Laot, Alain Bouckenooghe, Simonetta Viviani, Vincent Canouet, Laurent Chambonneau, Rafaela Dumas, Chantal Rotario, Lyn Morgan, Jean-Philippe Linard, Sophie Gervais, Emanuelle Trannoy, Nicolas Burdin, Jeffrey Almond, Steve Higgs, Charles McGee and, Sutee Yoksan. We would also like to acknowledge all investigators, in particular Drs Dennis Morrison, Maria Rosario Capeding, Jorge Luis Poo, J Qiao, C Lanata and D Shaw, as well as the volunteers involved in the clinical trials.

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