Advances and controversies in yellow fever vaccination

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Abstract: Ever since its development in 1937, the live-attenuated 17D yellow fever (YF) vaccine has been one of the most effective vaccines available to man. In this review we highlight the major steps in the development of 17D YF vaccine. We discuss the use of neutralizing antibodies as a surrogate marker for protection, and explore the strengths and weaknesses of the current plaque reduction neutralization test (PRNT), a technique developed in the 1960s that continues to be superior to every modern test in both sensitivity and specificity. The neutralizing antibodies demonstrated by the PRNT can be detected for several decades after vaccination, possibly even for the remainder of the recipient's natural life. We review the available evidence on the duration of protection after primary vaccination, a topic that has been the subject of controversy over the last few months. For persons who are immunocompromised due to disease, medication or advancing age, the duration of protection may be shorter: they should always have their vaccine response checked by PRNT. Due to the higher risk of severe adverse events after vaccination with 17D YF in this group, the development of a new, inactivated vaccine will have substantial benefits in this population.

Keywords: 17D, correlates of protection, duration of protection, immunocompromised, immunogenicity, neutralizing antibodies, plaque reduction neutralization test, protective immunity, vaccination, yellow fever

Introduction

Yellow fever virus (YFV) is an enveloped single-stranded, positive-sense RNA virus and member of the genus Flavivirus. Transmission of YFV involves nonhuman primates and various species of *Aedes* mosquitoes. Upon entry of its target, the dendritic cell, the viral RNA molecule is translated by the host cell to form a polyprotein [Mukhopadhyay *et al.* 2005]. After proteolytic processing, three structural and seven nonstructural proteins are formed. The structural capsid, (pre)membrane (prM) and envelope (E) proteins constitute the virion. The nonstructural proteins are involved in the replication and assembly of the viral particle [Murray *et al.* 2008].

Based on phylogenetic analysis of the *prM* /*E* gene region it is estimated that currently circulating strains of YFV arose in East Africa within the last 1500 years. YFV was subsequently introduced, along with its vector *Aedes aegypti*, from West Africa into the Americas during the slave trade, 300–400 years ago. "YFV then spread westwards across the continent and persists in the jungle of South America to this day" [Bryant *et al.* 2007]. Sequence data support that viral epidemiology in both Africa and South America is dominated by quiescent persistence in mosquito eggs through transovarial transmission and periodic amplification through sylvatic transmission [Bryant *et al.* 2007; Sall *et al.* 2010].

"Yellow fever (YF) was one of the most feared epidemic diseases from the 15th to 19th centuries, when large scale outbreaks in port cities of North and South America, Africa and Europe caused devastating mortality" [Bryant *et al.* 2007]. When disease develops after an infectious mosquito bite, patients become febrile and viraemic with titres up to 10^6 infectious particles per millilitre of blood during the first days of their illness [Monath, 2001]. Timing of symptoms suggests that injury to the organs responsible for the YF syndrome is most likely immune mediated, as most of the virus has been cleared by then through neutralizing antibodies (NAs) [Monath, 2001]. In

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survivors, the damage to liver and kidney is completely reversible.

The history of the creation of the 17D YF vaccine is eloquently described by Frierson [Frierson, 2010]. We only highlight the major steps in this fascinating history of pioneering research.

In 1900 the fourth US Army Yellow Fever Commission (Walter Reed, James Carroll, Aristide Agramonte and Jesse Lazear) proved in groundbreaking experiments with human volunteers that *A. aegypti* mosquitoes were able to transmit the causative agent of YF 12 days or more after biting a YF patient during the first days of their illness. Carroll showed that contaminated blood remained infectious even when passed five times through bacterial filters, alluding to the viral nature of the causative agent.

In 1927 a fortunate coincidence of an outbreak of YF near Accra, the arrival of Indian *Macacus rhesus* monkeys for experimental infection and the prepared minds of Stokes, Bauer and Hudson resulted not only in the identification a suitable laboratory animal to study YF, but also in the final proof of the viral origin of YF [Stokes *et al.* 2001]. YFV from a patient named Asibi with mild YF symptoms was successfully transmitted from monkey to monkey. Serum of infected *M. rhesus* monkeys remained infectious after passage through V and N grades of Berkefeld filters.

In the 1930s, Soper shattered the dream of eradicating YF from the Americas through antimosquito measures. He showed that jungle YF was a "permanent source of virus" maintained through a nonhuman primate–mosquito transmission cycle, increasing the need for a vaccine to control the disease [Soper, 1937].

Building on methods of growing and attenuating virus through repeated passage in nonhost nervous tissue, Theiler succeeded in creating the first attenuated strain cultured in mouse brain tissue with diminished liver (viscerotropic) damage, but increased neurologic (neurotropic) side effects [Theiler, 1930]. YFV was subsequently grown on mouse and later chicken embryo tissue, and to reduce neurotropism, nervous tissue was removed.

The next major breakthrough was in 1937 when a chance mutation at the 100th subculture of the Asibi YFV strain in nervous-tissue-deprived chicken embryo (passage 176) yielded a virus strain that failed to kill rhesus monkeys when injected intracerebrally, and protected these nonhuman primates after injection "by any route" against infection by fully virulent strains [Smith *et al.* 1938]. This 17D strain, harvested from a 3-day culture in 7-day-old developing chick embryos, diluted in human serum, centrifuged, filtrated and freeze dried was characterized and used by the subcutaneous route in a large field study in Brazil [Smith *et al.* 1938]. At the end of 1938, 59,532 people, including children (>2 years old) and pregnant women, had been vaccinated, and "No serious reactions of any kind were observed" [Smith *et al.* 1938].

In July 1941, severe cases of encephalitis were observed in Guanhães, Brazil, after routine vaccination with a series of vaccine lots derived from a single 17D substrain. Apparently, this substrain had undergone further unwanted modifications during a short series of 20 tissue cultures and 3–5 chick-embryo passages of the parent 17D strain. This resulted in increased neurovirulence when inoculated intracerebrally in rhesus monkeys, and the occurrence of encephalopathic reactions in 2% of recipients (especially in the age groups below 15 years) in a prospective comparative vaccination study [Fox *et al.* 1942]. As a result, the number of passages was strictly limited by the World Health Organization (WHO) to no more than two by the use of a 'virus master seed lot' from which a 'virus working seed lot' is prepared for the inoculation of embryonated chicken eggs since 1945 [WHO, 1957].

Small amounts of human serum were used to stabilize YFV during cultivation, and sporadic cases of delayed jaundice after vaccination were reported since 1939. To meet the requests for very large quantities of YF vaccine during the Second World War (WWII), the US Laboratories of the International Health Division required 8–10 litres of human serum every week for vaccine production. Most of the serum was obtained from medical students, interns, nurses and laboratory personnel of the John Hopkins School of Hygiene and Public Health. In 1942, reports were received of "considerable amounts of jaundice" in US military personnel which were associated with certain lots of YF vaccine. In 1942, a total of 49,233 cases of hepatitis occurred among military personnel, nearly all attributable to YF vaccination. Rates differed depending on the period of 1942 that was studied, varying between 5.45 and

11.43 per 1000. Between 1942 and 1945 the total hospital admissions for 'infectious and serum hepatitis' among US troops amounted to 182,283 [Thomas *et al.* 2013]. Investigations pointed to human serum as the source of the icterogenic agent and that this agent was probably a virus which could induce chronic carriership in humans [Sawyer *et al.* 1944]. As a result, human serum was banned from YF vaccine production from April 1942 onwards [Seeff *et al.* 1987], after which cases of postvaccination jaundice decreased significantly [Thomas *et al.* 2013]. Modern formulations of stabilized vaccines in lyophilized state can withstand 37°C for 14 days without losing minimally required potency [Monath, 1996].

Correlates of protection

There is no direct correlate of protection in humans, since the challenge studies needed to establish such a correlate are not ethical in humans. The protective efficacy of 17D YF vaccine is supported by challenge studies in nonhuman primates and the observation that during outbreaks YF only occurred in unvaccinated persons [Staples *et al.* 2010]. Only 10 cases of YF after vaccination with 17D vaccine are known to have occurred since the introduction of the vaccine. A definitive explanation for these failures was never established, and could include failure to respond to the vaccine, vaccine deterioration and nonvaccination. [Monath *et al.* 2008].

NAs against YFV are considered to be the best surrogate marker for protection against YF. In experiments conducted almost a century ago [Stokes *et al.* 2001], passively immunized primates were completely protected against challenge with wild-type YFV. More recently, Julander and coworkers confirmed this principle by passively immunizing hamsters with sera derived from hamsters inoculated with the inactivated and live attenuated YFV vaccines [Julander *et al.* 2011].

Historically, the neutralizing activity of human serum after YF vaccination was determined by protection of monkeys [Beeuwkes *et al.* 1930] and later of white mice [Sawyer and Lloyd, 1931; Theiler, 1931] against a lethal challenge with YFV. In these animal assays, YFV was incubated with the recipient's serum and administered to the animals, after which the survival of the animals would give an indication of the neutralizing capacity of the human serum. With the development of a plaque reduction neutralization test (PRNT) a sensitive, reproducible and less costly method to measure NAs became available.

The PRNT is a functional assay that measures the ability of serum to neutralize YFV [De Madrid and Porterfield, 1969; Spector and Tauraso, 1968]. Employing a cell culture and single agar-overlay procedure, the neutralizing activity was determined in a 1:2 dilution of unheated serum against serial dilutions of 17D YFV (constant serum–varying virus PRNT). The neutralizing capacity was calculated from virus dilutions yielding 10–50 plaque-forming units of virus (PFUVs). PFUVs are defined as the smallest quantity of virus that will produce a plaque in the monolayer. The difference in the amount of PFUVs between the virus–serum mix with serum taken before and 28 days after vaccination was expressed as the neutralization index (NI = $\log_{10}$ [number PFUVs in serum before vaccination] – $\log_{10}$ [number PFUVs in serum 28 days after vaccination]).

In a small YF challenge study conducted in rhesus monkeys in 1973 it was found that more than 95% of previously immunized monkeys survived a direct lethal subcutaneous challenge with the Asibi strain if their NI in the constant serum–varying virus PRNT was above 0.7 (this equates to an 80% reduction in numbers of PFUV). In contrast, 80% of previously immunised monkeys died if they had a NI below 0.7 [Mason *et al.* 1973]. This study forms the basis for the cut-off for protection in the constant serum–varying virus PRNT.

Owing to the relatively high amount of serum needed for the constant serum–varying virus PRNT described above, the modern-day PRNT is a serum dilution–constant virus plaque reduction method [Monath *et al.* 2008]. In this assay, a standardized amount of 17D YFV yielding 50–100 PFUVs is added to serial dilutions of serum. This approach reduces the amount of serum required for the assay at least fivefold. The cut-off for protection is set at 50% or 80% reduction of PFUVs in the 1:10 dilution in the varying serum–constant virus PRNT [Julander *et al.* 2011; Lang *et al.* 1999; WHO, 1998]. Some laboratories also use the 90% cutoff. Some have replaced the traditional 6-well PRNT with micro-PRNT assay in 96-well plates, an assay which is more commonly used for other flaviviridae such as dengue and West Nile virus. The microneutralization assay has not been extensively studied for YF. For this reason, Simoes and colleagues [Simoes *et al.* 2012] recently compared the YF

micro-PRNT to standard PRNT in sera of persons vaccinated with 17DD virus and found a sensitivity of 100% and specificity of 94.7% for the micro-$PRNT_{90}$. The micro-$PRNT_{50}$ was also sensitive at 91.1% but suffered in specificity (72.9%). The microneutralization technique needs further improvements, but may be advantageous when large numbers of samples need to be analysed.

Although the PRNT is the gold standard for determining NAs against YFV, methodological differences between laboratories using the serum dilution–constant virus PRNT may influence the sensitivity of the assay and make a comparison difficult [Monath *et al.* 2008]. As noted previously by Domingo and colleagues, both negative and positive control samples should be included in every assay in order to calculate the percentage of neutralisation and for quality control [Domingo *et al.* 2012a].

Therefore, the authors strongly recommend the use of an international reference YF immune serum to report titres in international units (IU/ml) to allow standardization of the results [Ferguson and Heath, 2004]. This is especially true if PRNT is used for clinical applications such as determining protection against YFV in travellers after vaccination. Results obtained by other methods for determining protective immunity against YF, such as the indirect immunofluorescence assay (IIF/IFA), correlate poorly with the titres obtained from PRNT testing [Domingo *et al.* 2012a] (and also from the current authors' unpublished data).

Modern techniques for a more accurate quantification of virus neutralisation (reverse transcriptase polymerase chain reaction [RT-PCR], flow cytometry [Domingo *et al.* 2012b; Hammarlund *et al.* 2012]) may reduce intertest variability and assay time (currently at least 5–6 days). These adaptations to the original PRNT remain to be investigated.

Irrespective of PRNT particulars, we believe that NAs should be measured in the following situations: (1) after vaccination of HIV-infected persons with detectable viral load and/or CD4 count below 500 cells/ml [Pacanowski *et al.* 2012; Veit *et al.* 2009]; (2) after inadvertent administration of 17D YF vaccine to individuals on immunosuppressive medication; (3) in order to avoid revaccination in individuals who were started on immunosuppressive medication after their initial vaccination. In women who were pregnant at the time of vaccination, measurement of NA titres should also be considered due to the possibility of poor response [Thomas *et al.* 2012].

Duration of protection

Currently, according to the WHO International Health Regulations (IHRs), the certificate of immunization (International Certificate of Vaccination or Prophylaxis [ICVP]) for travellers is only valid for 10 years after the most recent YF vaccination. However, there are strong indications that the actual duration of protection against YF after vaccination is much longer. It might even be lifelong, analogous to other well-known live vaccines such as smallpox [Hatakeyama *et al.* 2005; Kim *et al.* 2007; Wrammert *et al.* 2009].

During and shortly after WWII, servicemen from all branches of the United States military were vaccinated with 17D YF vaccine upon deployment. Rosenzweig and colleagues [Rosenzweig *et al.* 1963] found that 100% of Navy servicemen and Marines, vaccinated 17–19 years previously, were protected in mouse assay. Poland and colleagues [Poland *et al.* 1981] determined the persistence of YF NAs in a cohort of WWII veterans from the Navy and Air Corps. More than 30 years after vaccination, 97% of these veterans were still protected in plaque assay, although the cutoff for protection was not specified. In 1958, Groot and Riberiro [Groot and Riberiro, 1962] investigated neutralizing activity of sera from 108 people, vaccinated 17–18 years before. A total of 97% tested positive in the weanling mouse neutralization test using the intracerebral technique. Recently, Coulange Bodilis and colleagues [Coulange Bodilis *et al.* 2011] analysed 84 elderly subjects who were vaccinated >10 years ago (average time since last vaccination 14 years, range 10–60 years), and found 100% of certified recipients were still protected, even going back 60 years (protection was defined as 80% neutralization in a 1:10 serum dilution in a serum dilution–constant virus setup).

Therefore, like smallpox vaccine [Wrammert *et al.* 2009], the current 17D YF vaccine provides long-term protection far beyond the 10-year revaccination interval mandated by WHO IHR, a fact also recognized by the United States Advisory Committee on Immunization Practices (ACIP) [Staples *et al.* 2010]. Based on the findings detailed above, the authors favour a substantial extension

of the official WHO revaccination interval beyond the current 10 years. Recently, WHO's Strategic Advisory Group of Experts on immunization (SAGE) went even further and issued a recommendation to the effect that protection against YF after vaccination with 17D should be considered to be lifelong [WHO, 2013]. SAGE noted that further study is needed in those who may mount a suboptimal response (i.e. immunocompromised persons). We propose that further study is also very much required in those who are fully immune competent, to demonstrate continued long-term protection beyond 30 years. SAGE's recommendation will be discussed with the countries issuing the IHRs for implementation, but for now the IHRs remain unchanged and booster vaccination after 10 years is still officially required.

The exact mechanism behind the long-lasting immunity induced by 17D and other successful vaccines remains unknown, but increasing evidence points to the way the innate immune system influences the adaptive response: long-lasting immunity could very well be the result of early strong activation of the innate immune system [Kasturi *et al.* 2011; Monath, 2012], most likely through a number of dendritic cell subsets which are activated by 17D YFV via multiple Toll-like receptors that result in synergistic production of pro-inflammatory cytokines [Kawai and Akira, 2010; Napolitani *et al.* 2005; Pulendran and Ahmed, 2006; Querec *et al.* 2009; Querec and Pulendran, 2007].

Inactivated YF vaccine

Recently, Monath and coworkers showed the efficacy of a new, inactivated YF vaccine, which elicits a protective immune response in healthy 18- to 50-year-old individuals. Adverse events were mild and comparable to vaccination with the live attenuated YF vaccine [Monath *et al.* 2011]. In this double-blind, controlled, dose escalation phase I clinical study, Vero cell cultured YFV was inactivated by β-propiolactone and adsorbed to aluminium hydroxide. Participants were vaccinated twice with 21 days between the first and second vaccination. Of those who received the highest antigen dose (4.8 μg antigen), all showed protective NAs 10 days after the second vaccination in a serum dilution–constant virus PRNT with at least 50% plaque reduction in 1:10 serum dilution. A tenfold reduction of antigen (0.48 μg antigen) resulted in a protective NA response in 88% of participants. In a previous study, hamsters which were immunised with this inactivated vaccine were protected against challenge with wild-type YFV infection [Monath *et al.* 2010].

In addition to the whole virion inactivated YFV vaccine, two mouse studies with inactivated YF vaccination were reported. In 2008, Gaspar and colleagues showed promising results with vaccination of 17D YFV inactivated by hydrostatic pressure [Gaspar *et al.* 2008]. In 2011 Schafer and coworkers developed a YF vaccine consisting of a nonreplicating vaccinia virus transfected with the gene encoding the precursor of the membrane and envelope (prME) protein of the 17D YFV strain. One intramuscular inoculation protected mice against intracerebral challenge of a lethal dose of wild-type YFV, and pre-existing immunity against vaccinia virus did not alter this outcome [Schafer *et al.* 2011]. These inactivated YF vaccines are awaiting further evaluation in clinical trials, and should be investigated especially in immunocompromised individuals. An additional factor to take into account is the dependence on cold chain logistics, a concern that is most relevant in developing countries.

Immunocompromised persons

The live-attenuated YF vaccine has always been regarded as one of the safest and most effective vaccines, with more than 600 million administrations worldwide so far, leading to protective NAs in 99% of recipients 30 days after vaccination and only mild adverse events in 10–20% of recipients [Monath *et al.* 2008]. However, vaccination in patients with impaired immunity may lead to uncontrolled virus replication and cause encephalopathy or YF-like disease. For example, one HIV-infected vaccine recipient with a $CD4^+$ T cell count of 120/mm^3 was reported to have died from vaccine-induced myeloencephalitis [Kengsakul *et al.* 2002], and several persons with a history of thymectomy developed YF vaccine-associated visceral disease [Eidex and Yellow Fever Vaccine Study Working Group, 2004], leading to an absolute contraindication for administration of YF vaccine in these individuals. It should be noted that the guidelines regarding vaccination in these groups are based on very small numbers of serious adverse events (SAEs) after YF vaccination, and the nature of the available data makes an accurate estimate of the risk difficult. As pointed out by Thomas and colleagues in their excellent systematic review [Thomas *et al.* 2011], the studies that have been performed to date suffer from heterogeneity in study design, in nature of data collection

(active *versus* passive surveillance), and in differences in definitions and certainty about the exact cause of registered SAEs. Also, there is likely to be a bias from underreporting due to inadequate medical care in many remote areas in YF endemic countries. Calculating incidence rates of SAEs after YF vaccination is currently not appropriate due to the small numbers concerned and the insecurities in the data [Thomas *et al.* 2012].

The absolute contraindications to YF vaccination are based on altered immune status and include immunosuppressive medication, haematological malignancies, chemotherapy and irradiation of the thymus. Most of these contraindications are based on theoretical grounds due to the difficulties outlined above. SAEs rarely occur, even when on theoretical basis one might expect them. Azevedo and colleagues analysed retrospectively [Azevedo *et al.* 2012] 19 solid organ transplant recipients who accidently received live attenuated YF vaccine while on immunosuppressive medication, and of whom 18 had never been vaccinated against YF before. The vaccine was administered from 3 to 241 months after transplantation and the intensity of immunosuppressants varied widely, but none developed a YF vaccine neurotropic or viscerotropic disease. Unfortunately no NA titres were measured.

No adverse events were reported in recent publications on the administration of live attenuated YF vaccine in HIV patients with $CD4^+$ cell counts >200/mm^3, but NA titres were insufficient for protection (90% neutralization in 1:20) in 8% of vaccine recipients [Sidibe *et al.* 2012]. Interestingly, it was noted that inadequate NA titres were independently associated with higher HIV viral loads [Pacanowski *et al.* 2012; Veit *et al.* 2009]. In the trial reported by Veit and colleagues, higher $CD4^+$ cell count was predictive of higher NA titre, and 7 of 102 individuals had $CD4^+$ counts below 200/mm^3, none of whom developed serious adverse events. An absolute contraindication to YF vaccination now exists for a $CD4^+$ count <200/mm^3, but caution is advised with a $CD4^+$ count 200–500/mm^3. In addition, the duration of protection may be shorter than for non-HIV-infected persons.

A person's age at the time of YF vaccination remains an important consideration for administration of the vaccine: children below 9 months of age, or below 6 months of age during an epidemic, should never be vaccinated (absolute contraindication). A possible explanation for the increased occurrence of YF vaccine-associated neurotropic disease (YEL-AND) in infants could be the immaturity of the blood–brain barrier, higher or more prolonged viraemia or immune system immaturity [Gershman and Staples, 2012]. Persons aged 60 years or older who have no history of YF vaccination are considered to have a relative contra-indication for YF vaccination [Monath *et al.* 2008; Thomas *et al.* 2012]. The increased occurrence of YF vaccine-associated viscerotropic disease (YEL-AVD) in persons aged 60 years or older [Khromava *et al.* 2005] could be due to a delayed antibody response and higher viraemia after a first-time YF vaccination [Roukens *et al.* 2011].

The complex problem of balancing the risk of severe vaccine-related complications *versus* the risk of actual YF infection would disappear with the introduction of an effective inactivated vaccine. This would benefit particularly patient groups in whom vaccination with live attenuated vaccine is currently contraindicated.

Conclusions

Despite a long history of safe and efficacious YF vaccination, sporadic case reports of SAEs and the need to protect immunocompromised persons against YFV keep this field in transition. A new, inactivated vaccine has been developed and awaits further clinical evaluation. If the live-attenuated 17D vaccine could be replaced by this new vaccine in the near future, it would end the difficult act of balancing risks of using a live vaccine in immunocompromised persons. The PRNT, developed in the 1960s, is still the gold standard for determining anti-YFV neutralizing antibodies. Renewed efforts to replace it with a less laborious method are needed, since the demand for quantification of protective antibody levels in immunocompromised individuals will only increase. Neutralizing antibody levels should be reported in international units to facilitate interlaboratory comparisons and standardize the interpretation of test results. Even though the authors favour a substantial extension of the official revaccination interval, the recent statement by SAGE that protection after 17D YF vaccination is lifelong needs further study.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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