Broadening the horizons for yellow fever: new uses for an old vaccine

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The vaccine against yellow fever is one of the safest and most effective ever developed. With an outstanding record in humans, has this live attenuated vaccine been overlooked as a promising vector for the development of vaccines against pathogens outside its own genus? Recent studies, including a report by Tao et al. on page 201 of this issue, have sparked renewed interest.

The yellow fever vaccine has already been used with great success to create vaccines against yellow fever's close relatives, the RNA-based flaviviruses. Recent successes with other yellow fever–based vaccines, combined with increased interest in the basic immunology of the virus, provide new hope that this nearly century-old vaccine vector may provide the basis for protecting against not just flaviviruses but also unrelated pathogens for which effective vaccines have yet to be developed.

Yellow fever basics

Yellow fever virus is a flavivirus that is transmitted between monkeys, humans, and mosquitos, and survives dry seasons in mosquito eggs—a transmission cycle that makes eradicating this virus virtually impossible. Causing illness and death at 1000-fold greater incidence than Ebola, with equally terrifying symptoms, yellow fever is one of the most lethal viral infections known to man. The virus, endemic to tropical Africa and South America, causes hemorrhage, multiorgan failure, and shock and is lethal in 20–50% of severe cases (1). Yellow fever virus infects cells of multiple organs, but beyond that knowledge of the basic biology of the virus is slim.

Development of the yellow fever virus vaccine began in 1930 when Max Theiler and his colleagues at the Rockefeller Foundation created the first attenuated strains of yellow fever by growing the virus in tissue culture for over 200 generations (2), an accomplishment that earned Theiler the Nobel Prize in 1951. Since the 1930s, Theiler's vaccine strain, known as 17D, has been administered to over 400 million individuals and caused only rare severe side effects or death (3). The efficacy of this vaccine is most evident in certain parts of Africa, where yellow fever virtually disappeared between 1939 and 1952 due to mass immunization programs.

After a single inoculation, the yellow fever vaccine elicits robust protection that is sustained for decades and possibly for life. Given its proven immunogenicity and safety, the yellow fever 17D strain seems an ideal choice as a vector for other vaccines. Although huge gaps in our knowledge remain, including the reasons why 17D is so effective, new studies are getting 17D plenty of attention.

Vector choice

What makes a good vector? The requirements include immunogenicity, safety, large cloning capacity, and genetic stability. In other words, an ideal vector stimulates a robust immune response that provides long-lasting protection against infection, does not make people sick, readily accepts the introduction of foreign sequences into its genome, and tolerates these genetic squatters over time without evicting them. From a clinical standpoint, it is also helpful if the vaccine can be administered in a single immunization to recipients who have minimal or no prior immunity to the vector. This combination is a tall order; for most if not all applications, the ideal vector doesn't exist. With each vector choice comes the challenge of balancing multiple factors and identifying the strengths and weaknesses inherent to each vector.

Immunogenicity and safety of 17D

Vaccines should provide large benefits with low risk. The yellow fever vaccine does just that by eliciting strong and long-lasting humoral and cellular immunity in humans with little risk to recipients. Neutralizing antibodies—the first line of defense against virus encountered upon reexposure—develop in 98–100% of yellow fever 17D vaccine recipients and can be detected for several decades after vaccination (1). Less is known about specific T cell responses, but total numbers of circulating $CD8^+$ T cells increase in vaccine recipients (4) and virus-specific $CD8^+$ T cell responses have been detected for up to 18 months after vaccination (5), suggesting that $CD8^+$ T cells are effectively primed by the vaccine.

The price paid for effective immunity is low, as severe complications of the yellow fever 17D vaccine are rare. Since 1945, postvaccine encephalitis has been reported in $\sim$1 in 20 million recipients,
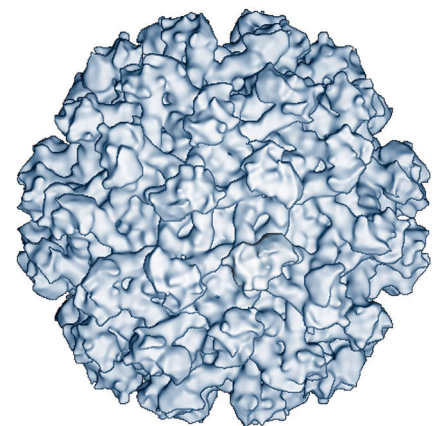
Cryo-electron microscope image reconstruction of an immature form of yellow fever 17D. Image provided by Richard J. Kuhn, Purdue University, West Lafayette, IN.

primarily young children. To avoid this, children younger than 9 months of age are not vaccinated. In the past 10 years, several fatal cases of viscerotropic disease that resembled natural yellow fever virus infection were reported after immunization, mostly among elderly people. It is unclear why this serious complication has cropped up recently, as sequencing of the virus from these patients failed to identify mutations that would suggest the emergence of a variant virus. These cases are a major concern, and understanding why these complications occur in some individuals may be a prerequisite to widespread use of a 17D-based vaccine.

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Other highly immunogenic vectors are even more risky. Vaccinia virus, an attenuated poxvirus used for centuries to vaccinate against smallpox, also elicits potent and possibly life-long immunity in humans. But the risks include overwhelming infection, encephalitis, and brain infection, and are considerably higher than those associated with the yellow fever vaccine. As a safer alternative, replication-deficient strains of vaccinia virus are now favored for use in humans. Replication-deficient vaccinia viruses carrying HIV antigens, for example, have been shown to be well tolerated and to stimulate virus-specific immune responses in primates. But the safety gain is outweighed by loss in immunogenicity, as these vectors raise only moderate neutralizing antibody and T cell responses in a fraction of recipients.

Adenovirus vectors are likely to prime strong immune responses, as they can replicate in many cell types including mucosal cells and professional antigen-presenting cells. Recombinant adenoviruses are highly immunogenic in animals but not in humans, where they face the formidable barrier of preexisting adenovirus-specific immunity, which causes the vector to be eliminated before an immune response to the foreign antigen can develop.

The immunogenicity and safety of many other live recombinant virus vec-

tors are still being evaluated. Even if these vectors prove to be safe and immunogenic in animals, a robust immune response in mice, or even monkeys, does not necessarily translate to a similar response in humans. For yellow fever, safety and immunogenicity are known commodities. "There has already been a huge experiment to test safety in humans, one that could never be repeated," says Raul Andino (University of California, San Francisco, San Francisco, CA). Rather than design a vaccine from scratch, Andino points out that it may be smarter to start with an existing vaccine that we already know works.

Capacity to carry new genes

Vector choice also hinges on the ability of a vector to carry foreign gene sequences without rejecting them over time and without losing infectivity. Unfortunately, the vectors that are the easiest to grow in the laboratory and will accept large amounts of foreign DNA are not necessarily the safest or most immunogenic in humans.

DNA viruses, such as poxviruses and adenoviruses, tolerate both the insertion of multiple large foreign genes and the loss of large pieces of their own DNA without losing infectivity. Both poxviruses and adenoviruses are also easy to grow in the lab and replicate in many different cell types. But Andino suggests that many scientists choose DNA vaccine vectors primarily because they are lab friendly, and thus overlook better choices.

RNA viruses are more resistant to the introduction of large foreign genes than their DNA counterparts, mostly because of their small size and relative instability. "Flaviviruses are very small and are not very tolerant of things outside the flavivirus genus," says Tom Monath (Acambis, Cambridge, MA). "Sometimes the genes get kicked out after a while." Many consider the size of the yellow fever virus to be its Achilles heel, although the limit on introducing genetic information into 17D—both in terms of location and amount—remains to be determined. Despite this, short foreign sequences have been expressed in yellow fever

and other small RNA viruses and have been shown to be stable over many passages. There are also ways around the limits on insertion size—including tricks like using bicistronic constructs containing internal ribosomal entry sites (IRES)—that may allow for the introduction of longer sequences.

The problem of antivector immunity

As previously mentioned, adenovirus suffers from the problem of preexisting immunity, and widespread vaccination against smallpox also burdens vaccinia virus as a vector. Similarly, a yellow fever–based vaccine carrying, for example, HIV epitopes might struggle in yellow fever endemic regions of Central and South America where immunization rates against 17D approach 90%. Such a vaccine could, however, be useful in the US (where only travelers are vaccinated) and Africa (where only 1–40% of people are vaccinated). A vaccine that would benefit African populations—especially one with the potential to immunize against both yellow fever and another endemic pathogen—would be particularly useful.

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It is unclear whether existing immunity to yellow fever, or a yellow fever–based vaccine, would preclude the use of another vaccine that has a yellow fever 17D backbone. Francis Ennis (University of Massachusetts Medical Center, Worcester, MA) and his group are actively addressing this question by administering new 17D-based vaccines to volunteers and comparing the responses of people who have or have not previously received a 17D-based vaccine.

Replication location

One reason 17D works so well is simply that it is a live attenuated virus. "Because they have the replication machinery of live viruses, they basically become virus factories," explains Ennis.

Replication location also matters. 17D probably replicates where the wild-type virus replicates and stimu-

lates the same kinds of immunity. Thus, it elicits an immune response that is well targeted against yellow fever virus itself. But if 17D is to be used as a vector for other microbial pathogens, we need to learn more about the possible match between 17D-generated immunity and the other infections, in terms of both replication sites and mechanisms. Potent mucosal immunity against a sexually transmitted pathogen like HIV, for example, would be most readily generated if the vaccine vector generates immune responses that target HIV-infected cells in the mucosa.

For yellow fever virus, much of this information is lacking. And although infectious virus can be measured in the blood of vaccine recipients for only a few days after immunization, no data exist on the persistence of viral antigens or the possibility of chronic infection. Rafi Ahmed (Emory University, Atlanta, GA) is now studying yellow fever virus in mice to determine the in vivo targets of infection. His group is also assessing innate immune signals that emanate from Toll-like receptors (TLRs) expressed on antigen presenting cells as a result of infection. Ahmed thinks that the ability of the virus to infect dendritic cells and stimulate TLR-dependent production of cytokines might in part explain why this vaccine stimulates such robust and durable T and B cell immunity.

Assessment of vaccine behavior depends on the ability to monitor and evaluate the immune responses that arise after vaccination. Virus-specific antibodies have long been detected in the blood using standard immunoassays and plaque assays. But tracking virus-specific T cells is more difficult and requires an understanding of the specificity of these cells.

Vaccination with 17D has been shown to activate and expand CD8^{+} T cells, but the details are sparse. Ennis' group identified the first T cell epitopes on 17D that human CD8^{+} T cells recognize on infected cells (5). After vaccination with 17D, CD8^{+} cells specific for these epitopes were expanded and could be detected for at least 18 months. Ahmed recently launched a large study of first-time vaccine recipients and is monitoring the kinetics of the virus-specific antibody and T cell responses induced by vaccination. His group has identified additional CD8^{+} T cell epitopes on the virus. Sorting of these cells will allow tests of their effector function and in vivo monitoring using peptide-based techniques.

17D and flavivirus vaccines
In 1989, Charles Rice (Rockefeller University, New York, NY) and colleagues cloned the entire yellow fever genome as cDNA into bacterial plasmids (6). This cDNA was then transcribed back into RNA and transfected into cells, resulting in the production of live viral offspring—a difficult feat considering the instability of the flavivirus genome in bacterial vectors. The development of this "infectious clone" technology allowed for easier manipulation of the virus genome and facilitated the use of 17D as a vaccine vector.

One way to make a vaccine, rather than inserting individual epitopes into or between existing viral proteins, is to put entire foreign coat structures around the replication machinery of a known vaccine vector. Several years after the infectious clone technology was developed, C.J. Lai and colleagues (National Institutes of Health, Bethesda, MD) made an infectious clone of Dengue virus (another flavivirus) and showed that the structural genes of one serotype of Dengue could be replaced by the structural genes of a different serotype, without robbing the virus of its ability to infect cells (7). Rice's group later used the same approach with the 17D virus and replaced the structural genes with those from Japanese encephalitis virus, another member of the flavivirus family (8). These recombinant vectors could then be used to generate neutralizing antibody responses against the native surface proteins of Dengue or Japanese encephalitis virus, and thus to protect against infection.

This "chimeric" virus approach, pioneered by Lai and extended by Monath and colleagues, was the basis for successful vaccines against Japanese encephalitis, Dengue, and West Nile virus (all flaviviruses). All of these vaccines provide protective immunity in mice and primates and have moved rapidly into clinical trials in humans. Monath stresses that these vaccines progressed rapidly through clinical trials largely because the yellow fever backbone is so reliable. "The balance between safety and immunogenicity is always the difficulty in developing a live vaccine," he says. "We can use the parent (17D vaccine) as a benchmark, which facilitates development and clinical testing."

Vaccines against other pathogens
With the success of the chimeric virus vaccines, why has yellow fever 17D not been widely used in the development of vaccines against other pathogens? "I do think that yellow fever has been neglected to some extent," says Rice. This may be changing, however. In the past few years, vaccines created by insertion of model antigens or gene fragments from nonflavivirus pathogens into yellow fever 17D have elicited robust immune responses in mice.

A group led by Ricardo Galler (Fiocruz, Rio de Janeiro, Brazil) performed one "proof of concept" experiment in collaboration with Ruth Nussenzweig (New York University, New York, NY). They inserted an epitope from the malaria circumsporozoite (CS) protein—the most abundant protein expressed on the parasite surface and a known target of protective antibodies—into the E protein of yellow fever 17D and showed that mice responded with neutralizing antibodies against malaria (9).

The 17D strain has also been used to create a model cancer vaccine in mice that works by activating cytotoxic T cell responses capable of eliminating tumor cells. Andino's group inserted a CD8^{+} T cell epitope from chicken ovalbumin between genes in 17D and showed that the CD8^{+} T cells activated by this vaccine in mice could fight off challenge with tumor cells expressing the T cell epitope, even if the tumor was growing before the vaccination was given (10).

Andino is now developing potential yellow fever–based HIV vaccines. He found insertion at several sites in the 17D genome to be stable and suc-
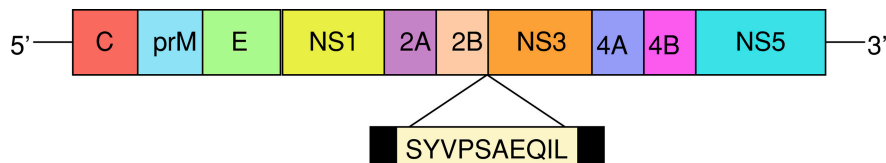
Yellow fever virus polyprotein showing structural (C, prM, E) and nonstructural (NS1-5) proteins. The location of the malaria CS epitope inserted by Rice and colleagues is shown.

cessful, although insertions at other sites killed the virus. The vaccine candidates have been shown to prime potent CD8+ T cell responses against HIV in mice and monkeys. The CD8+ response using a prime–boost approach in mice, he reports, is ~1000-fold higher than that achieved with poxvirus vectors alone. In previous work, prime–boost strategies using DNA and attenuated poxvirus vectors have shown some promising results in animal models, but have not worked well in humans, perhaps because the vectors are not replication competent. Although safety concerns with live vaccines are certainly warranted, Andino worries that the HIV vaccine field is allowing these concerns to trump the necessity for immunogenicity.

In a recent collaborative effort led by the Rice and Nussenzweig teams, which is reported on page 201 of this issue, a possible candidate malaria vaccine was developed using yellow fever 17D as a backbone (11). The authors targeted the same insertion position in 17D used by Andino's group but introduced a CD8+ T cell epitope from the CS protein of *Plasmodium yoelii*, a close relative of the *Plasmodium* species that causes human disease. Mice immunized with this vaccine developed malaria-specific T cell responses that could protect them against challenge with live parasites, even after a single dose. These T cells could be found in the circulation for as long as 24 weeks after immunization. "The most stunning thing was that the immune response and protection lasted so long—after only one immunization," says Victor Nussenzweig.

The authors hope to move these experiments into primates soon, and also plan to test the limits of the yellow fever genome by inserting multiple T cell epitopes or the entire CS protein, which may provide even better immunity.

They are also considering strategies to express the whole CS protein on the surface of the virus as a way of generating both antibody and T cell responses.

Malaria claims up to three million lives each year, and the vaccine problem is far from solved. A vaccine currently in clinical trials in Africa that also targets the CS protein was shown to elicit protective T cell and antibody responses, but only provided short-term protection in human volunteers (12). Immunization with malaria sporozoites lacking a gene required for liver-stage growth was recently reported to protect mice against infection and may provide an alternative approach to virus-based vaccine strategies (13). The authors of the current study hope that their vaccine might provide immunity to both malaria and yellow fever simultaneously in children who have not yet been vaccinated with the yellow fever vaccine. In this population, antivector immunity would also not be a factor.

The road ahead

There are many outstanding questions regarding the molecular mechanisms that govern yellow fever virus replication, the cellular targets of infections, and the requirements for generating long-lasting virus-specific immunity. The answers to these questions will not only increase our understanding of why this vaccine is so effective, but will also allow us to harness its power in search of better ways to protect against other deadly pathogens. As 17D is so effective at stimulating T cell immunity, it may turn out to be particularly useful for vaccination against other viruses—as well as cancer—where attempts to induce T cell immunity with other vaccines have failed.

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