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## A live, attenuated recombinant West Nile virus vaccine

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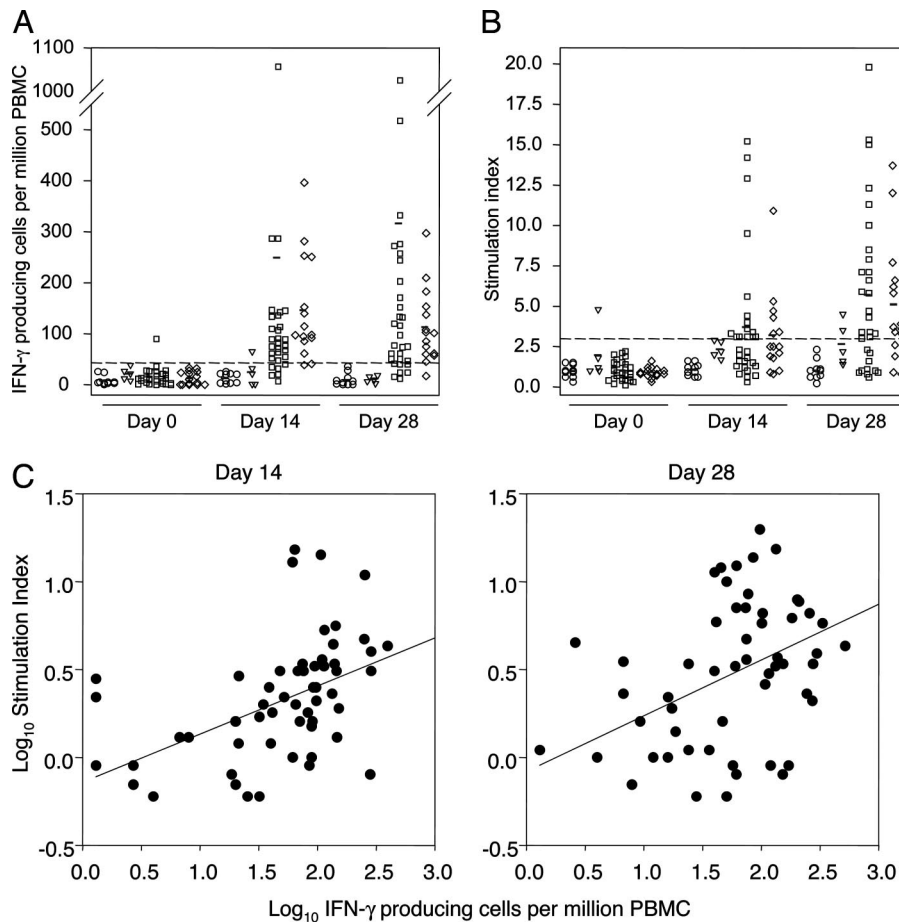
Monath TP, Liu J, Kanesa-Thasan N, Myers GA, Nichols R, Deary A, McCarthy K, Johnson C, Ermak T, Shin S, Arroyo J, Guirakhoo F, Kennedy JS, Ennis FA, Green S, Bedford P. (2006). A live, attenuated recombinant West Nile virus vaccine. *Infectious Diseases and Immunology Publications*. <https://doi.org/10.1073/pnas.0601932103>. Retrieved from [https://escholarship.umassmed.edu/infdis\\_pp/261](https://escholarship.umassmed.edu/infdis_pp/261)

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## Corrections

**MEDICAL SCIENCES.** For the article “A live, attenuated recombinant West Nile virus vaccine,” by Thomas P. Monath, Jian Liu, Niranjan Kanesa-Thanan, Gwendolyn A. Myers, Richard Nichols, Alison Deary, Karen McCarthy, Casey Johnson, Thomas Ermak, Sunheang Shin, Juan Arroyo, Farshad Guirakhoo, Jeffrey S. Kennedy, Francis A. Ennis, Sharone Green, and Philip

Bedford, which appeared in issue 17, April 25, 2006, of *Proc. Natl. Acad. Sci. USA* (103, 6694–6699; first published April 14, 2006; 10.1073/pnas.0601932103), the authors note that Fig. 5 did not include all vaccine study groups. The corrected figure and legend appear below. This error does not affect the conclusions of the article.



**Fig. 5.** Cell-mediated immune responses after vaccination with ChimeriVax-WN02 and YF-VAX vaccines. (A) WN virus-specific IFN- $\gamma$  producing T cells per million PBMC. Open circle, placebo; inverted open triangle, YF-VAX; open squares, ChimeriVax-WN02 vaccine 5.0 log<sub>10</sub> PFU; open diamonds, ChimeriVax-WN02 vaccine 3.0 log<sub>10</sub> PFU. Dashed line represents cut-off value. (B) T lymphocyte proliferation responses to inactivated WN virus antigen, represented as stimulation index. (C) Correlation between IFN- $\gamma$  producing WN virus-specific T cells and stimulation index in ChimeriVax-West Nile vaccines 14 and 28 days after immunization. Confidence intervals for Spearman's rank correlation of log<sub>10</sub> IFN- $\gamma$  producing PBMC per million and log<sub>10</sub> stimulation index were based on Fisher's transformation. On day 14, the correlation was 0.491 (95% CI, 0.231–0.686); on day 28, the correlation was 0.188 (95% CI: –0.112 to 0.456).

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**BIOCHEMISTRY.** For the article “The crystal structure of SdsA1, an alkylsulfatase from *Pseudomonas aeruginosa*, defines a third class of sulfatases,” by Gregor Hagelueken, Thorsten M. Adams, Lutz Wiehlmann, Ute Widow, Harald Kolmar, Burkhard Tümmler, Dirk W. Heinz, and Wolf-Dieter Schubert, which appeared in issue 20, May 16, 2006, of *Proc. Natl. Acad. Sci. USA* (**103**, 7631–7636; first published May 9, 2006; 10.1073/pnas.0510501103), the authors note that on page 7631, the first line of the second full paragraph in the right column appears incorrectly, due to a printer’s error. “We present the crystal structure of SdsA1 from *P. aeruginosa* strain PAO1 at 1.9 Å resolution, the class-III sulfatase” should read: “We present the crystal structure of the class-III sulfatase SdsA1 from *P. aeruginosa* strain PAO1 at 1.9 Å resolution.” This error does not affect the conclusions of the article.

[www.pnas.org/cgi/doi/10.1073/pnas.0604082103](http://www.pnas.org/cgi/doi/10.1073/pnas.0604082103)

# A live, attenuated recombinant West Nile virus vaccine

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West Nile (WN) virus is an important cause of febrile exanthem and encephalitis. Since it invaded the U.S. in 1999, >19,000 human cases have been reported. The threat of continued epidemics has spurred efforts to develop vaccines. ChimeriVax-WN02 is a live, attenuated recombinant vaccine constructed from an infectious clone of yellow fever (YF) 17D virus in which the premembrane and envelope genes of 17D have been replaced by the corresponding genes of WN virus. Preclinical tests in monkeys defined sites of vaccine virus replication *in vivo*. ChimeriVax-WN02 and YF 17D had similar biodistribution but different multiplication kinetics. Prominent sites of replication were skin and lymphoid tissues, generally sparing vital organs. Viruses were cleared from blood by day 7 and from tissues around day 14. In a clinical study, healthy adults were inoculated with 5.0 log<sub>10</sub> plaque-forming units (PFU) (*n* = 30) or 3.0 log<sub>10</sub> PFU (*n* = 15) of ChimeriVax-WN02, commercial YF vaccine (YF-VAX, *n* = 5), or placebo (*n* = 30). The incidence of adverse events in subjects receiving the vaccine was similar to that in the placebo group. Transient viremia was detected in 42 of 45 (93%) of ChimeriVax-WN02 subjects, and four of five (80%) of YF-VAX subjects. All subjects developed neutralizing antibodies to WN or YF, respectively, and the majority developed specific T cell responses. ChimeriVax-WN02 rapidly elicits strong immune responses after a single dose, and is a promising candidate warranting further evaluation for prevention of WN disease.

clinical trial | non-human primate | yellow fever

West Nile (WN) virus (family *Flaviviridae*) first appeared in the Western Hemisphere in 1999, causing an outbreak in New York City. In successive years, WN expanded its geographic range. Over 19,000 human cases have been reported in the United States (1). The spectrum of disease extends from a mild febrile exanthem to fatal encephalitis. Mosquito control has failed to stop progression of the disease. However, the introduction of vaccines for horses (2, 3) has controlled the veterinary disease. In late 1999, we initiated development of a vaccine for humans.

ChimeriVax-WN02 is a live, attenuated chimeric vaccine derived from an infectious clone of yellow fever (YF) 17D virus in which the premembrane and envelope proteins of YF 17D virus have been replaced by the corresponding genes of WN (4). The vector, YF 17D vaccine, is approved by regulatory authorities worldwide, and has a 70-year history of use in >400 million persons (5). Three mutations were introduced into the WN E gene at sites predicted to reduce neurovirulence (4, 6), resulting in a highly attenuated phenotype. The vaccine candidate was incapable of being transmitted by mosquitoes (7). ChimeriVax-WN02 vaccine protected hamsters against challenge with WT WN virus (8). In rhesus macaques, the vaccine caused a transient viremia, induced neutralizing (N) Ab, and protected against intracerebral challenge with WT WN virus (4).

Here, we report tests in monkeys that elucidate sites of virus replication *in vivo*, and the results of a clinical trial of a vaccine for prevention of human infection with WN virus.

## Results

### Safety, Immunogenicity, and Biodistribution in Non-Human Primates.

A study in cynomolgus macaques evaluated ChimeriVax-WN02 for possible toxicity, determined sites of virus replication *in vivo*, and determined the relationship between N Ab response and viral clearance. Groups of monkeys lacking detectable flavivirus hemagglutination-inhibiting Abs and Japanese encephalitis, WN, and YF N Abs were inoculated s.c. with 0.5 ml of ChimeriVax-WN02 (5 log<sub>10</sub> plaque-forming units, PFU), YF-VAX (5 log<sub>10</sub> PFU), or diluent. There were no clinical signs or changes in food consumption, body weight, serum chemistry, hematology, or coagulation parameters. Five animals per group were necropsied on days 7, 14, and 46. There were no histological changes in any organ. Cerebrospinal fluid from ChimeriVax-WN02 treated monkeys on days 14 and 46 were negative for WN IgM Abs by ELISA, whereas serum samples were positive for IgM Ab, indicating that the vaccine virus had not invaded the CNS.

ChimeriVax-WN02 virus was detected in sera of 14 of 15 (93%) monkeys. The mean peak viremia ( $\pm$ SD) was 474 ( $\pm$ 230.4) PFU/ml, and the mean number of viremic days was 3.7 ( $\pm$ 1.3). YF 17D virus was detected in sera of 7 of 15 (47%) monkeys. The mean peak viremia ( $\pm$ SD) was 67.3 ( $\pm$ 94.8) PFU/ml, and the mean number of viremic days was 1.4 ( $\pm$ 1.6). Although the viremia levels were low in both groups (Fig. 1A), the number of viremic days and the peak titer were higher in monkeys receiving ChimeriVax-WN02 than YF-VAX (*P* = 0.0002 and *P* < 0.0001, respectively; ANOVA). N Abs were detected on day 7 in a higher proportion of monkeys inoculated with YF-VAX [12 of 15 (80%) positive for YF N Abs] than in those inoculated with ChimeriVax-WN02 [6 of 15 (40%) positive for WN N Abs; *P* = 0.0604, Fisher's exact test, two-tailed]; however, by day 14 (as virus was clearing from tissues), 100% of the animals in both groups had N Ab responses against the virus inoculated (Fig. 1B).

To evaluate the safety implications of the earlier and higher viremia in monkeys, we used quantitative RT-PCR to determine sites of replication of the two viruses 3 days (*n* = 4 per group), and 7, 14, and 46 days (*n* = 5 per group) after inoculation. ChimeriVax-WN02 was detected earlier than YF-VAX (Table 1), but the sites of replication were similar, with a predilection for lymph nodes and spleen. ChimeriVax-WN02 was found in skin at the site of inocu-

Conflict of interest statement: Acambis is developing ChimeriVax-WN vaccine with the intent to sell and distribute in the United States and other countries.

Freely available online through the PNAS open access option.

Abbreviations: WN, West Nile; YF, yellow fever; N, neutralizing; PFU, plaque-forming unit; AE, adverse event; GMT, geometric mean titer; PBMC, peripheral blood mononuclear cell.

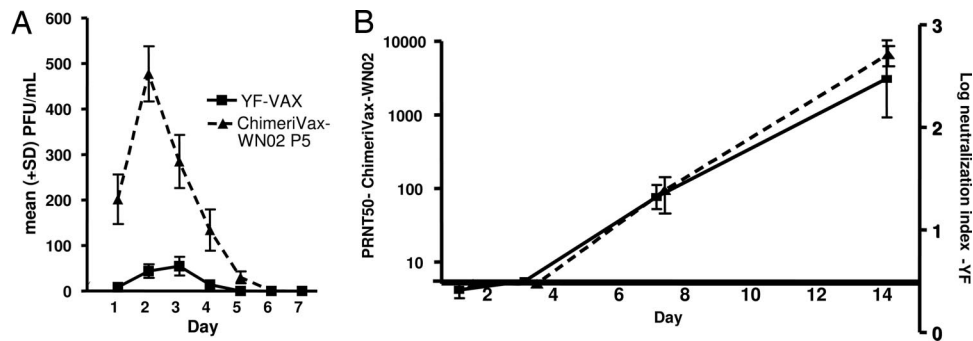
Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF196835).

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**Fig. 1.** ChimeriVax-WN02 or YF-VAX were inoculated s.c. into cynomolgus macaques. (A) Viremia mean ( $\pm$ SD) by day after inoculation of macaques with  $5 \log_{10}$  PFU ChimeriVax-WN02 or YF-VAX. (B) Neutralizing Ab GMT.

lation (but not in the contralateral arm) at high virus loads in three of four animals on day 3 (viremic period), and appeared to be (together with lymph nodes) the principal contributor to viremia. One ChimeriVax-treated animal had viral RNA in kidney on day 3. The higher viremia in monkeys inoculated with ChimeriVax-WN02 appeared to be due to the higher replication in skin and lymphoid tissues before the onset of immune clearance. On days 7 and 14, the viral burden in tissues of YF-VAX-treated animals exceeded that in ChimeriVax-WN02-treated animals. No virus was detected in liver, spinal cord, adrenal gland, or brain of any ChimeriVax-WN02 treated and most YF-VAX-treated animals. Virus RNA was detected on day 7 in the thymus, adrenal gland, and liver of one of the five YF-VAX-vaccinated monkeys. By day 14, virus RNA was present only in lymphoid tissues, and by day 46, clearance was complete for both viruses. Virus loads were generally low for both viruses, in the range of 50–500 PFU equivalents (eq/g). Plaque assays were consistent with quantitative RT-PCR (data not shown).

**Clinical Trial Comparing ChimeriVax-West Nile and YF-VAX.** A randomized, double-blind, placebo-controlled study in healthy male and female adults 18–40 years was performed under an Investigational New Drug application. An initial cohort of 15 subjects received  $5.0 \log_{10}$  PFU of ChimeriVax-WN02, whereas five controls received YF-VAX. A second cohort of 15 subjects received  $5.0 \log_{10}$  PFU of ChimeriVax-WN02, and 15 received placebo. In a third

cohort, 15 subjects received a lower dose ( $3.0 \log_{10}$  PFU) of ChimeriVax-WN02, and 15 subjects received placebo. Subjects were tested at baseline for N Abs to flaviviruses (WN, YF, St. Louis encephalitis, and dengue types 1–4). Immunogenicity was assessed in flavivirus-naïve subjects.

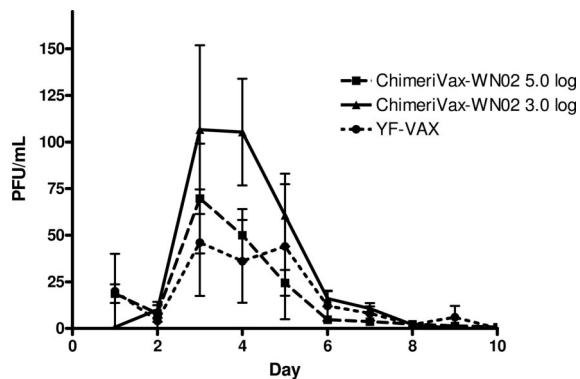
There were no significant differences between groups in demographic or baseline characteristics. Viremia was detected by plaque assay in 90%, 100%, 80%, and 0% of subjects in the ChimeriVax-WN02 5.0 log and 3.0 log, YF-VAX, and placebo groups, respectively. The mean duration of viremia was 5.1, 4.7, and 3.6 days in these groups, respectively (Fig. 2). Mean daily viremia levels for all three groups were low ( $\approx 100$  PFU/ml). The mean area under the curve for the low dose ( $3.0 \log$ ) ChimeriVax-WN02 group (311.7 PFU/ml per day) was statistically higher than for the high dose ( $5.0 \log$ ) group (173;  $P = 0.0288$ , ANOVA). Viremia was cleared by day 10 (Fig. 2).

Most subjects reported at least one adverse event (AE) (Fig. 3), but the incidence was similar across active and placebo recipients. There was no relationship between viremia level and the occurrence or severity of AEs. Only one subject (ChimeriVax-WN02 3.0 log group) had a mild elevation in body temperature ( $38.2^{\circ}\text{C}$ ) as an AE (on day 6). Two subjects with high elevations of creatine phosphokinase, one in the ChimeriVax-WN02 5.0 log and one in the YF-VAX group, were the subject of an intensive investigation concluding that the enzyme elevations were likely due to muscle injury from strenuous physical exercise rather than to the study vaccines (9).

**Table 1. Biodistribution of ChimeriVax-WN02 and YF-VAX in tissues of cynomolgus monkeys by day after inoculation**

Tissue	Day 3		Day 7		Day 14		Day 46	
	CV-WN <i>n</i> = 4	YF <i>n</i> = 4	CV-WN <i>n</i> = 5	YF <i>n</i> = 5	CV-WN <i>n</i> = 5	YF <i>n</i> = 5	CV-WN <i>n</i> = 5	YF <i>n</i> = 5
	Pos	Eq/ml	Pos	Eq/ml	Pos	Eq/ml	Pos	Eq/ml
Skin								
inoc	3	376, 408, 2850	1	78	0		0	0
contra	0		0		0		0	0
Spleen	0		0		4	59, 176, 234, 257	0	3
Lymph N								
Axillary	1	110	1	246	1	242	2	102, 167
Mandib	2	301,963	0		1	281	2	49, 164
Mesen	0		1	338	1	87	1	97
Thymus	0		0		0		1	3632
Bone marrow	0		1	75	1	650	2	52, 141
Liver	0		0		1	34	0	
Heart	0		0		0		0	
Kidney	1	399	0		0		0	
Pancreas	0		0		0		0	
Adr gland	0		0		1	25	0	
Skel muscle	0		0		0		0	
Brain	0		0		0		0	

CV-WN, ChimeriVax-WN02; YF, yellow fever 17D; Pos, number monkeys positive by PCR; Eq/ml, plaque-forming equivalents per ml; inoc, inoculation site; contra, contralateral arm; mandib, mandibular; mesen, mesenteric; skel, skeletal.



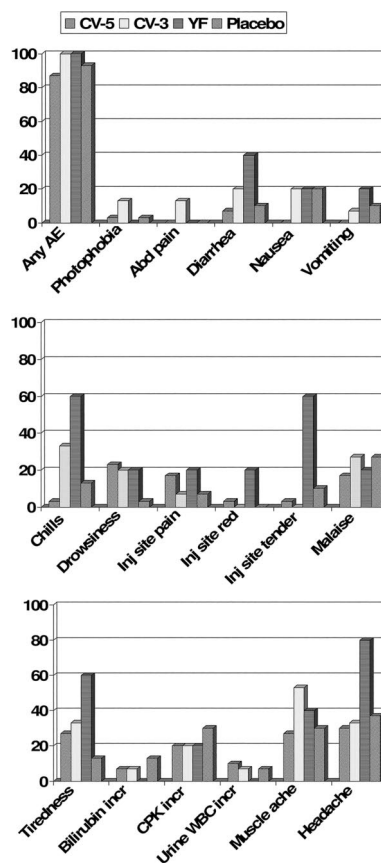
**Fig. 2.** Clinical trial of ChimeriVax-WN02: Viremia mean PFU/ml ( $\pm$ SD) by day after s.c. inoculation with 5.0 or 3.0  $\log_{10}$  PFU ChimeriVax-WN02 or YF-VAX.

On day 10, 7.1% (2 of 28) subjects vaccinated with 5.0  $\log_{10}$  PFU ChimeriVax-WN and none of those receiving 3.0  $\log_{10}$  PFU seroconverted. On day 21, the rate of seroconversion was 100% in both ChimeriVax-WN02 treatment groups and high N Ab titers were present (Fig. 4 and Table 2). In the ChimeriVax-WN02 3.0  $\log_{10}$  PFU group, one subject's low titer (40) on day 21 dropped to 10 and thus did not meet the definition of seroconversion on day 28. The geometric mean N Ab titers were, respectively, 6,241 and 11,392 in the 5.0 and 3.0  $\log_{10}$  PFU dose groups on day 21, and 1,280 and 1,218 on day 28. There was no significant difference in N Ab seroconversion rate ( $P = 1.0$ ) or geometric mean titer (GMT) ( $P = 0.914$ ) across the high and low dose groups on day 28. Moreover, there was no correlation between viremia (area under the curve) and N Ab response (data not shown). YF-VAX elicited a YF-specific N Ab response in five of five (100%) of subjects, with a GMT of 3,880 on day 28. YF-VAX failed to elicit a cross-reactive WN N Ab response except in one subject, who developed a low titer (20) on day 21, but was seronegative by day 28.

Serum samples were obtained 3, 6, and 12 months after vaccination. Thirty-five of 36 (97.2%) of ChimeriVax-WN02 vaccinated subjects tested at 12 months were seropositive and retained high titers of N Ab (Fig. 4). One subject (in the ChimeriVax-WN02 3.0  $\log_{10}$  PFU group) seroreverted between day 28 and 3 months; this subject was still seronegative at 6 months, but had a N Ab titer of 1,280 at 12 months.

**T Cell Responses.** Nearly all recipients of ChimeriVax-WN02 vaccine [28 of 30 (93.3%) and 15 of 15 (100%) in the 5.0 log and 3.0 log dose groups, respectively;  $P = 0.55$ , ANOVA] developed specific IFN- $\gamma$  producing cells in ELISPOT assays after restimulation with WN E peptide pools. In contrast, only 1 of 5 YF-VAX and 0 of 10 placebo recipients responded to WN ( $P \leq 0.001$  vs. ChimeriVax-WN02 dose groups). High geometric mean levels of IFN- $\gamma$ -producing cells per million peripheral blood mononuclear cells (PBMCs) were found in the ChimeriVax-WN02 5.0 and 3.0 log dose groups on days 14 [78.1 (95% CI, 55.3–110.4) and 118.0 (95% CI, 80.8–172.2), respectively] and on day 28 [93.8 (95% CI, 63.8–137.9) and 91.9 (95% CI, 62.3–135.6)] (Fig. 5A). Interestingly, a later maximal IFN- $\gamma$  response was associated with a longer duration of viremia and greater number of days of viremia, but showed no correlations with maximal viremia titer, area under the viremia curve, or N Ab titer.

WN-specific T cell proliferative responses were detected in PBMC from 25 of 30 (83%) and 13 of 15 (87%) of ChimeriVax-WN02 5.0 log and 3.0  $\log_{10}$  PFU recipients, 3 of 5 (60%) of YF-VAX recipients, and 0 of 10 placebo recipients (Fig. 5B). The maximal SI was measured on day 14 in 8 of 25 (32%) and 4 of 13 (31%), and on day 28 in 17 of 25 (68%) and 9 of 13 (69%) of ChimeriVax-WN02 5.0 and 3.0 log dose vaccinated subjects with



**Fig. 3.** Incidence of AEs occurring at rate  $>10\%$  in the ChimeriVax-WN02 or placebo groups.

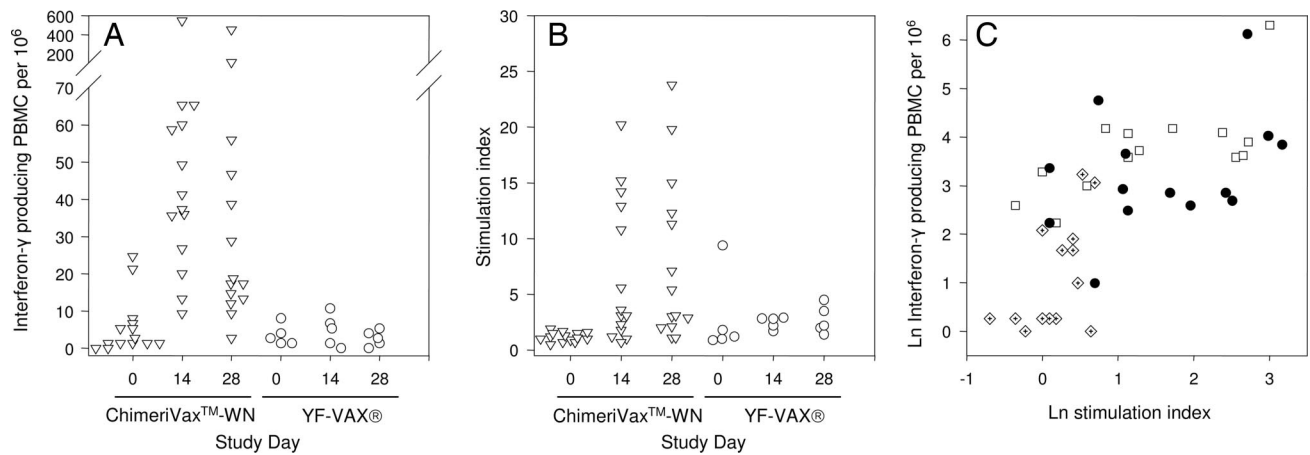
detectable T lymphocyte lymphoproliferation responses. Individuals with a later maximal proliferation response had higher maximal viremia levels, higher area of viremia under the curve ( $280.58 \pm 306.44$  for day 28 maximal responders vs.  $102.92 \pm 105.39$  for day 14;  $P = 0.02$ , Kruskal–Wallis Rank Sum), and a greater number of viremic days. T lymphocyte proliferative responses were detected in one YF-VAX vaccinated subject on day 0. This response was not detectable on day 14, but reappeared on day 28 for this individual. Similarly, one ChimeriVax-WN02 5.0 log dose recipient who was Ab seronegative had detectable IFN- $\gamma$  responses to WN virus peptides on day 0. This volunteer developed WN-specific T cell proliferative responses on day 28 (SI = 12.3). It is possible that these donors might be immune to a flavivirus that was not tested in Ab assays.

Concordance of WN virus-specific IFN- $\gamma$  ELISPOT and T lymphocyte proliferation assays in ChimeriVax-WN02 recipients was stronger on study day 14 (9 of 14 volunteers, 64%) than on study day 28 (4 of 14 volunteers, 29%) [ $r = 0.491$  (95% CI, 0.231–0.686) and  $r = 0.188$  (95% CI, –0.112 to 0.456), respectively] (Fig. 5C). T cell responses were detected by both assays on either day 14 or 28 in 25 of 30 (83%), 13 of 15 (87%), and one of five (20%) subjects in the 5.0 and 3.0  $\log_{10}$  PFU dose ChimeriVax-WN02 vaccine and YF-VAX groups.

## Discussion

The *in vivo* replication of virulent, WT YF virus has been studied in monkeys (10). WT virus spreads from Kupffer cells to parenchyma of the liver, with severe coagulative necrosis of hepatocytes. In contrast, YF 17D vaccine does not cause liver damage. There are no previous data on the sites of replication of YF 17D vaccine. Studies of other flaviviruses suggest that the initial site of replica-





**Fig. 5.** Cell-mediated immune responses after vaccination with ChimeriVax-WN02 and YF-VAX vaccines. (A) WN virus-specific IFN- $\gamma$  producing T cells per million PBMC. Open diamonds, ChimeriVax-WN02 vaccine 5.0 log<sub>10</sub> PFU; open squares, ChimeriVax-WN02 vaccine 3.0 log<sub>10</sub> PFU; inverted open triangle, YF-VAX; open circle, placebo. (B) T lymphocyte proliferation responses to inactivated WN virus antigen, represented as stimulation index. (C) Correlation between IFN- $\gamma$  producing WN virus-specific T cells and stimulation index in ChimeriVax-West Nile vaccines 14 and 28 days after immunization. Confidence intervals for Spearman's rank correlation of log<sub>10</sub> IFN- $\gamma$  producing PBMC per million and log<sub>10</sub> stimulation index were based on Fisher's transformation. On day 14, the correlation was 0.491 (95% CI, 0.231–0.686); on day 28, the correlation was 0.188 (95% CI: –0.112 to 0.456).

tains all viral structural and nonstructural proteins. Based on experience with other viruses, such as HIV (27, 29), the WN virus E peptide-specific IFN- $\gamma$  responses may largely represent WN-specific CD8<sup>+</sup> T cells, although this was not specifically demonstrated. These results are consistent with studies of lymphocytic choriomeningitis virus in mice and vaccinia in humans demonstrating that effector CD8<sup>+</sup> T lymphocytes expand upon antigen stimulation and contract again after the acute phase of infection (30, 31).

The structural genes of divergent mosquito-borne flaviviruses such as WN and YF viruses have a lesser degree of homology (32). However, the inactivated WN antigen preparation contains WN nonstructural proteins with both specific and cross-reactive epitopes that induce a CD4<sup>+</sup> T lymphocyte response (33, 34). As a result, the T lymphocyte proliferation assays are probably detecting not only WN virus-specific CD4<sup>+</sup> responses, but also cross-reactive responses against the more conserved nonstructural regions of WN and YF viruses. Therefore, it is not surprising that we found some disparity between the WN virus E specific IFN- $\gamma$ -producing T cells and the T lymphocyte proliferative responses in the two vaccine groups.

We detected a significant association between the duration of viremia and maximal IFN- $\gamma$  and proliferation responses in recipients of ChimeriVax-WN02 regardless of vaccine dose. Later maximal T cell proliferative responses were also associated with higher maximal viremia levels and area under the viremia curve. CD8<sup>+</sup> T cells play an important role in destroying infected cells, and CD4<sup>+</sup> T cells help Ab producing B cells and CD8<sup>+</sup> T cells to proliferate. Immunization with ChimeriVax-WN02 may lead to the generation of a spectrum of WN virus-specific T cell repertoires of differing avidity, which control infection with vaccine virus and later, after challenge with WN virus, in nature.

In summary, ChimeriVax-WN-WN02 vaccine induces high levels of N Abs and CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses against WN virus in nearly all human volunteers within 14–28 days of vaccine administration. Such a vaccine could be useful in preventing illness and limiting outbreaks of WN virus infection. Further clinical trials to define vaccine safety and immunogenicity are warranted.

## Materials and Methods

**Viruses and Cell Lines.** The construction of ChimeriVax-WN02 vaccine has been described (4). Mutations were introduced into the WN E codons E107 (L → F), E336 (A → V), and E440 (K → R).

Chimeric RNA was transfected to Vero cells by electroporation. Progeny virus was amplified under serum-free conditions, and supernatant fluid was harvested to produce a master virus seed [passage 3 (P3)], production seed (P4), and vaccine (P5) according to current good manufacturing practices. The P5 vaccine lot was manufactured in a 100-liter bioreactor using cells grown on micro-carrier beads. The full genomic sequence of P4 and P5 verified that all three introduced E protein mutations were intact and that no other genetic changes had occurred. The virus was purified and concentrated by nuclease digestion (to cleave host cell DNA), depth filtration, ultrafiltration, and diafiltration.

WT WN virus used in animal challenge studies is the NY99 strain (NY99–35262-11 flamingo isolate, Centers for Disease Control, Fort Collins, CO) with two additional passages in Vero E6. YF 17D commercial vaccine (YF-VAX, Sanofi-Pasteur, Swiftwater, PA) was used without passage (human and monkey studies) or after one passage in Vero cells for T cell and N Ab tests.

**Viremia and Neutralizing Antibody Measurements.** Virus in serum and tissues was measured by plaque assay in Vero cell monolayers in 12-well plates. After inoculation, cells were overlaid with methyl cellulose, incubated at 37°C for 5 days, fixed with formaldehyde, and stained with 1% crystal violet. Fifty-percent plaque reduction N tests were performed in Vero cell monolayers as described (35). In the study of cynomolgus monkeys, the YF N test was a constant serum, varying virus assay in Vero cell monolayers. The result is expressed as a log<sub>10</sub> neutralization index with a cut-off for a positive result of 0.7.

**Quantitative Assessment of Tissue Virus by PCR.** The study was conducted to Good Laboratory Practices. Monkeys received a single s.c. injection and were evaluated for clinical signs, changes in food consumption and injection site appearance, and changes in body weight. Blood samples were collected on day 0, and on days 2, 4, 6, 10, 14, 28, and 46 for chemistry, hematology, and coagulation tests. Serum was collected on days 0–10 for viremia, and on days 7, 14, 30, and 46 for N Ab tests. At necropsy on days 7, 14, and 46, cerebrospinal fluid was collected, the monkeys were perfused with sterile isotonic saline, and a complete necropsy performed.

Monkey tissues were homogenized in 300  $\mu$ l of PBS (for plaque assays) or Buffer RLT (Qiagen, Valencia, CA) for quantitative PCR. Virus RNA was detected in duplicate or triplicate reactions by quantitative PCR assay using virus RNA standards made from



infected lysate of Vero cells. Standards were amplified in triplicate along with test samples on each reaction plate. The primer and probe set was designed to anneal to sequence shared by ChimeriVax-WN02 and YF 17D viruses. The forward primer was at nucleotide positions 340–362, and the reverse primer bounded to nucleotides 424–445. The probe labeled with FAM at the 5' end and TARMA at the 3' end annealed to the virus genome at nucleotides 376–403 of YF-17D and ChimeriVax-WN02 virus. Reactions were carried out as one-step quantitative PCR reactions using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) in 1× TaqMan Universal PCR Master Mix without AmpErase UNG, 1× Multiscribe and RNase Inhibitor, 100 μM probe, 200 nM forward primer, and 200 nM reverse primer. After the initial reverse transcription reaction at 48°C for 30 min and an intermittent incubation at 95°C for 10 min, reactions were completed by 40 cycles of amplification at 95°C for 15 s and 60°C for 1 min. PFU equivalent was calculated according to the virus RNA standards run on the same reaction plate. A signal level between negative and lowest point of the standard curve was considered nonquantifiable (“equivocal”).

**T Cell Assays.** WN virus antigen was prepared as a single lot in Vero cells as described (36). Control antigen was prepared in a similar fashion by using uninfected Vero cells.

**Quantitation of WN-Specific IFN-γ Producing T Cells.** IFN-γ ELISPOT assays were performed with modifications to the procedure described in refs. 27 and 37. Briefly, cryopreserved PBMCs were thawed, washed, plated (250,000 cells per well), and stimulated in triplicate with four pools of 20-mer peptides (Mimotopes, Victoria, Australia) overlapping by 10 amino acids corresponding to the E sequence of WN NY-99. Plates were read by a single reader on a microscope or by automated ELISPOT reader. A cutoff value of 43 spots per million PBMC was calculated as mean ± 2 SD of the sum of the day 0 E pool responses. The mean day 0 response for each peptide pool was low (five spots per million PBMC).

**WN-Specific T Lymphocyte Proliferation Assay.** Assays were performed in replicates of five as described (36) using inactivated WN virus antigen as stimulant, phytohemagglutinin as positive control, and mock-infected Vero cell lysate as negative control. Stimulation indices were calculated after eliminating the high

and low value for each replicate of five as follows: mean cpm WN virus antigen/mean cpm control antigen. A positive response was a stimulation index ≥ 3.

**Statistical Methods.** Differences in mean log<sub>10</sub> peak IFN-γ responses and mean log peak stimulation indices as well as associations between IFN-γ responses and T lymphocyte proliferation responses and parameters related to viremia and neutralizing Abs were assessed by using a Kruskal–Wallis Rank Sum test. For the clinical trial, statistical comparison of treatment groups used logistical regression methods for seroconversion rates and ANOVA for GMTs.

**Clinical Trial.** The double-blind trial was performed at a single center (PRA International, Lenexa, KS) under a protocol approved by an Institutional Review Board. Exclusion criteria included history of military service or travel to tropical flavivirus-endemic areas, immune suppression, egg allergy, positive hepatitis or HIV tests, pregnancy, lactation, significant medical or psychiatric disorders, and abnormal baseline clinical laboratory tests. On day 0, eligible subjects received a s.c. inoculation of 0.5 ml of ChimeriVax-WN02, YF-VAX, or placebo (50 mM Tris/0.85% NaCl/10% sorbitol in water for injection) (see *Results*). Subjects took daily oral temperature, completed a symptom diary, and returned to the clinic on days 1–14, 21, and 28 for determination of AEs. Blood was taken on days 0–14 and 21 for viremia, on days 0, 10, 21, and 28 for Ab tests, and on days 0, 14, and 28 for T cell studies. Viremia duration was calculated as the last visit date on which the subject had detectable viremia minus the first date of detectable viremia (plus 1 day); area under the curve for the period days 1–14 was calculated by using the linear trapezoidal method. The primary efficacy endpoint was the proportion of subjects who were seronegative at baseline and who developed ≥ 4-fold rises in N Abs on day 28. Secondary endpoints were GMT and T cell responses.

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