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A Randomized, Double-Blind, Controlled Trial of the 17D Yellow Fever Virus Vaccine Given in Combination with Immune Globulin or Placebo: Comparative Viremia and Immunogenicity

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Abstract. We evaluated whether coadministration of the yellow fever (YF) virus vaccine with human immunoglobulin (Ig) that contained YF virus-neutralizing antibodies would reduce post-vaccination viremia without compromising immunogenicity and thus, potentially mitigate YF vaccine-associated adverse events. We randomized 80 participants to receive either YF vaccine and Ig or YF vaccine and saline placebo. Participants were followed for 91 days for safety and assessments of viremia and immunogenicity. There were no differences found between the two groups in the proportion of vaccinated participants who developed viremia, seroconversion, cluster of differentiation (CD)-8⁺ and CD4⁺ T-cell responses, and cytokine responses. These results argue against one putative explanation for the increased reporting of YF vaccine side effects in recent years (i.e., a change in travel clinic practice after 1996 when hepatitis A prophylaxis with vaccine replaced routine use of pre-travel Ig, thus potentially removing an incidental YF vaccine-attenuating effect of anti-YF virus antibodies present in Ig) (ClinicalTrials.gov identifier: NCT00254826).

INTRODUCTION

Yellow fever (YF), caused by the YF virus, is a potentially fatal flavivirus infection endemic in sub-Saharan Africa and tropical South America, and it is preventable by a relatively safe and highly effective vaccine. The live, attenuated 17D YF vaccine was developed in the 1930s, and it is approved for use in persons > 9 months of age.¹ In the past decade, there have been increased numbers of reports of systemic adverse events, namely YF vaccine-associated neurotropic (YF-AND) and viscerotropic (YF-AVD) disease in primary vaccinees.^{2–5} A review of adverse events reported to the US Vaccine Adverse Event Reporting System (VAERS) from 2000 to 2006 estimated the risks of YF-AND and YF-AVD at 0.4 per 100,000 and 0.8 per 100,000 doses, respectively.⁶ YF-AND has a bimodal distribution, with infants and elderly being at highest risk.⁷ YF-AND may include post-vaccinal encephalitis, autoimmune disease with central or peripheral nervous system disease, or Guillain–Barre syndrome.⁸ The mechanism of YF-AND is unclear, but it has been proposed that it may be related to levels of post-vaccination viremia and/or host response factors. YF-AVD resembles wild-type disease, with vaccine virus dissemination and replication in multiple organs.^{9,10} Advanced age, thymus disease, and other immunosuppressive conditions are major risk factors associated with YF-AVD.^{3,11–13}

One explanation for the increased reports of vaccine-associated adverse events is increased surveillance and reporting. Interestingly, before the approval of hepatitis A

vaccine in 1996, the YF vaccine was commonly given simultaneously with Ig (for hepatitis A prophylaxis) during pre-travel immunizations. In the past, human Ig usually contained YF-specific neutralizing antibodies, because it was produced from plasma of military recruits who were routinely vaccinated with YF vaccine.¹⁴ Early trials of YF vaccine that included insufficiently attenuated strains used passive–active immunization with concomitant administration of immune serum to reduce vaccine side effects in monkeys and humans.^{15,16} With knowledge of these facts, one of the authors (M.C.) hypothesized that the coadministration of the YF vaccine and pre-travel human Ig for hepatitis A prophylaxis—which was often done in travel clinics before 1996—would reduce post-vaccination viremia and thus, potentially reduce adverse events. We then designed and conducted a randomized clinical trial to assess the effects of Ig on YF vaccine-associated viremia and immunogenicity.

MATERIALS AND METHODS

Participants. The study participants were healthy adults ages 18 to 40 years without a history of previous YF vaccination or travel or residence in YF-endemic areas. Informed consent was obtained from all subjects, and the study was approved by the Institutional Review Boards at Emory University and the Centers for Disease Control (CDC). Volunteers recruited from the Atlanta metropolitan area were enrolled at two clinical research sites: the Hope Clinic of Emory Vaccine Center and the Emory Children’s Center Vaccine Research Clinic. During the screening period (days –30 to –1), eligibility criteria were assessed, and they are listed at www.clinicaltrials.gov (study number NCT00254826). Baseline laboratory tests (complete blood count [CBC] with differential, chemistry, liver function tests, and urinalysis) were performed at the screening visit and on days 5 and 11 post-vaccination.

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Participants were reported as lost to follow-up if they did not complete the treatment regimen as allocated or missed both the day 30 and day 91 visits.

Study design. The study was conducted as a randomized, double-blind, controlled trial. The randomization was stratified by race and sex based on a prior clinical trial that noted differences in neutralizing antibody titers by race and sex.¹⁷ Block randomization was used to ensure balance between the numbers of participants assigned to treatment. The primary objectives were to (1) compare the proportion of participants who developed viremia after immunization with YF vaccine in the Ig versus saline groups; (2) compare the proportion of participants who seroconverted; and (3) characterize the dynamics of T-cell activation and cytokine responses in each group. The secondary objectives were to (1) compare the levels of viremia; (2) compare the levels of neutralizing antibodies; and (3) compare the dynamics and magnitude of T-cell activation and innate immune responses.

Clinical procedures. Eligible participants were immunized with the Food and Drug Administration (FDA)-approved 17D YF vaccine, YF-VAX (Sanofi Pasteur, Swiftwater, PA), subcutaneously into the deltoid region on day 0. The first 64 participants received the YF vaccine from one lot (UE804AA); the remaining 16 participants received vaccine from another lot (UE984AA), because the first lot expired during the trial. Immediately after the administration of the YF vaccine subcutaneously into the deltoid, immunoglobulin (Ig) or saline injections were administered intramuscularly into the upper outer quadrants of the buttocks. Human Ig (lot number 26N6RJ1, GamaSTAN S/D; Talecris Biotherapeutics, Inc., Research Triangle Park, NC) from a single lot was given at a dose of 0.06 mL/kg body weight. This dose is recommended by the manufacturer for pre-travel prophylaxis of hepatitis A. The placebo for Ig was sterile saline (0.9% sodium chloride injection, USP; Hospira Inc., Lake Forest, IL) administered similarly. Participants received two to three injections of Ig or saline depending on their weight (maximum of 2 mL per injection). Participants returned on post-vaccination days 1, 2, 3, 5, 7, 9, 11, 14, 30, and 91 for determination of viremia and immunological and safety assessments. Safety and reactogenicity were assessed by collecting solicited adverse events. Participants completed a post-vaccination report card at 28 days post-vaccination, where they recorded the body temperature, severity of injection site reactions (swelling, redness, and pain or tenderness), and systemic signs and symptoms (fever, headache, and myalgia).

Laboratory assays.

- (1) Flavivirus enzyme-linked immunosorbent assay (ELISA). During screening, volunteers were excluded if the ratio of their serum IgG ELISA optical density to a negative control was more than two for antibodies to YF, West Nile, or dengue viruses.¹⁸
- (2) Plaque reduction neutralization test (PRNT). Serial titers of YF virus-specific neutralizing antibodies by PRNT with a 90% cutoff value (PRNT₉₀) were determined on days 2, 7, 9, 11, 14, 30, and 91 as described previously.¹⁹ During screening, volunteers were excluded if they had a PRNT₉₀ titer of $\geq 1:10$.
- (3) Reverse transcription-polymerase chain reaction (RT-PCR) assay. Baseline and post-vaccination plasma viremia were determined on days 0, 1, 2, 3, 5, 7, 9, 11, and 14

by using a quantitative Taqman real-time RT-PCR assay (Applied Biosystems, Foster City, CA) as described previously²⁰ except for the following modifications. RNA extraction was performed on 1 mL plasma with the Nuclisens Isolation Kit (BioMerieux Inc., Durham, NC), and it was eluted in 85 μ L elution buffer. The 100 μ L RT-PCR reaction contained 30 μ L RNA, 0.1 μ M YF virus forward primer, 0.2 μ M YF virus reverse primer, and 0.1 μ M fluorogenic probe. PCR was carried out for 40 cycles of denaturation at 93°C for 30 seconds and annealing at 59.5°C for 60 seconds. Day 0 samples were used as negative controls for RNA extraction, and water was used as a PCR negative control.

- (4) Viral infectivity. Plasma YF virus plaque assays were performed on days 1, 2, 3, 5, 7, 9, and 11 as previously described.²¹
- (5) T-cell assays. The magnitudes of activated CD4⁺ (on days 0, 11, 14, 30, and 91) and CD8⁺ (on days 0, 3, 7, 11, 14, 30, and 91) T cells were determined by staining of whole blood with fluorescently labeled antibodies to Human Leukocyte Antigen (HLA)-DR, CD38, Ki-67, and B-cell lymphoma (Bcl)-2 and performing flow cytometry as previously described.²⁰
- (6) Cytokine assays. Multiplex cytokine assays were performed on thawed plasma samples stored at -80°C with Beadlyte technology (MPXHCTYO-60K; Millipore, Billerica, MA) with the Bio-Plex Luminex-100 Station (Bio-Rad, Hercules, CA). Human bead mates were used to determine the levels of several cytokines with the Human Multi-Cytokine Flex Kit using the detection protocol B (Upstate, Billerica, MA) and duplicate standards and samples. Cytokine concentrations were calculated using Bio-Plex Manager 4.1 software with a five-parameter logistic curve-fitting algorithm applied for standard curve calculations.

Statistical methods. A sample size of 40 participants per treatment arm provided 82% power to detect a difference of 24% in the proportion of participants with viremia (two-sided *Z* test with pooled variance; significance level = 0.05). This power calculation assumed that 93% of participants receiving YF vaccine and saline exhibited viremia and a reduction of 24% (to 69% of participants) in the Ig group. The primary analysis of the data was performed as an intent-to-treat analysis. The proportion of vaccinated participants that developed post-vaccination viremia was compared between the treatment groups with Fisher exact tests.

PRNT₉₀ titers were highly skewed, but a data transformation (\log_2 [PRNT/10]) provided an ordinal set (titers of 10, 20, 40, 80, 160, 320, 640, and 1,280 ... were transformed to values 0, 1, 2, 3, 4, 5, 6, 7 ...).²² Undetected PRNT₉₀ titers were set to five (i.e., \log_2 [PRNT/10] = -1). This transformation ensured that the titers met the asymptotic normal properties required by general linear models. Maximum \log_2 (PRNT/10) titers, the area under the curve (AUC) for \log_{10} viremia, and the maximum \log_{10} viremia were compared between treatment groups (and separately between the two vaccine lots) with a two-sample *t* test.

Repeated-measures analyses of \log_2 PRNT/10 titers, activated CD8⁺ and CD4⁺ T cells, cytokines (\log_{10}), and clinical laboratory data were done using a means model with SAS Proc Mixed (version 9), providing separate estimates of the means by time on study and treatment groups. Each

model included treatment, time on study, and the statistical interaction between the two. An unstructured variance-covariance form among the repeated measurements was assumed for each outcome, and estimates of the standard errors of parameters were used to perform statistical tests and construct 95% confidence intervals. The model-based means are unbiased with unbalanced and missing data as long as the missing data are non-informative (missing at random). All statistical tests were two-sided, and a P value ≤ 0.05 was considered statistically significant.

Supplemental data analysis. Each of the 11 solicited adverse events was counted only one time per participant as the most severe level reported (maximum intensity) aggregated across severity (mild, moderate, or severe) and time on study and compared between treatment groups with a χ^2 or Fisher exact test. The generalized estimating equation (GEE) approach was used to analyze the repeated binary data (positive or negative) for viral infectivity.

Data obtained at baseline and subsequent time points were compared between the Ig and saline groups by performing a GEE analysis using SAS Proc Genmod with an exchangeable correlation binomial-logit model.

RESULTS

Trial participants. During the screening period, 121 volunteers were evaluated for eligibility (Supplemental Figure 1); 41 volunteers were excluded: 10 individuals based on flavivirus serology (7 individuals were positive, and 3 individuals were indeterminate) and 31 individuals for a variety of reasons (abnormal safety laboratories [$N = 3$], did not show for vaccination day or declined to participate [$N = 12$], hepatitis C virus antibody-positive [$N = 2$], underlying chronic medical conditions [$N = 10$], poor venous access [$N = 3$], and prior travel to YF-endemic region [$N = 1$]). Seventy-four of eighty participants completed the final visit on day 91 (93% retention rate). The participants in the two treatment arms were well-randomized and balanced with respect to age, sex, race, and ethnicity (Supplemental Table 1). Clinical laboratory evaluations, such as hemoglobin, white blood cell (WBC) counts, creatinine, and liver function tests, remained within normal limits in both groups.

Viremia. The proportion of participants that developed post-vaccination viremia, a coprimary outcome, did not differ between the treatment groups (38/40 and 40/40 in the Ig and saline groups, respectively = 0.49) (Figure 1). The median

viremia levels (Figure 1) and the mean AUC were similar in the Ig and saline groups [11.95 (95% confidence interval [CI] = 0–25.20) and 12.02 (95% CI = 1.57–22.47)], respectively. In addition, we used a standard plaque assay to determine viremia by infectivity. Overall, 54 of 80 (67.5%) participants were infectivity-positive, and there was a small and not statistically significant difference in the proportion positive between the Ig and saline groups (Ig = 72.5%; saline = 62.5%; $P = 0.47$). Furthermore, analysis of the plaque assay data over serial time points showed that there was no significant difference between the Ig and saline groups (Supplemental Figure 2) ($P = 0.2$). Similar to a previous study,²³ we also noted that the overall proportion with viremia detected by infectivity ($N = 54/80$) was lower than the proportion detected by RT-PCR ($N = 78/80$).

Seroconversion. All participants in both groups seroconverted by day 14, which was shown by a PRNT₉₀ titer ≥ 10 , a coprimary outcome (Figure 2). YF virus-neutralizing antibodies were first detected on post-vaccination day 9, continued to increase at day 30, and declined by day 91 (Figure 2). There was no difference in the maximum PRNT₉₀ titers (\log_2) achieved in the Ig or saline groups (8.30 ± 2.15 in the Ig group versus 8.90 ± 1.96 in the saline group; $P = 0.20$). There were no differences in the maximum PRNT₉₀ titers (\log_2) based on race ($P = 0.59$) or sex ($P = 0.88$).

T-cell activation. Activated CD8⁺ T cells, the third coprimary outcome as determined by Ki-67⁺/Bcl-2^{low} phenotype, peaked on day 14 after vaccination, representing 3–4% of the total CD8⁺ T cells. They returned to near baseline by 30 days post-vaccination (Figure 3). Similar results were obtained for the HLA-DR⁺/CD38⁺ CD8⁺ T cells (data not shown). The mean percent activated CD8⁺ T cells were similar between the two treatment groups ($P = 0.42$), and their kinetics were also similar.

Similarly, activated CD4⁺ T cells, as determined by the Ki-67⁺/Bcl-2^{low} phenotype, peaked between 11 and 14 days after vaccination, represented ~2% of the total CD4⁺ T cells, and returned to near baseline by 30 days post-vaccination (Figure 4). Again, similar results were obtained with HLADR⁺/CD38⁺ markers on CD4⁺ T cells (data not shown). The mean percent activated CD4⁺ T cells were similar for the two treatment groups ($P = 0.85$), and their kinetics were also similar.

Cytokines. The levels (\log_{10} scale) of cytokines, such as interferon gamma-inducible protein (IP)-10 ($P = 0.76$), monocyte chemoattractant protein (MCP)-1 ($P = 0.47$), Regulated on Activation Normal T Cell Expressed and Secreted (RANTES) ($P = 0.22$), granulocyte-macrophage

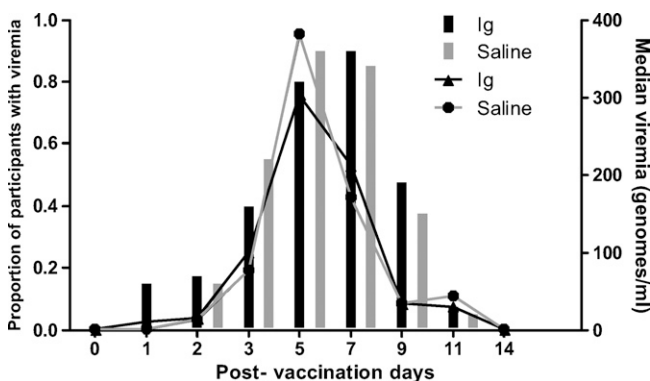


FIGURE 1. 17D YF vaccine-induced viremia (measured by RT-PCR) on post-vaccination days 0–14.

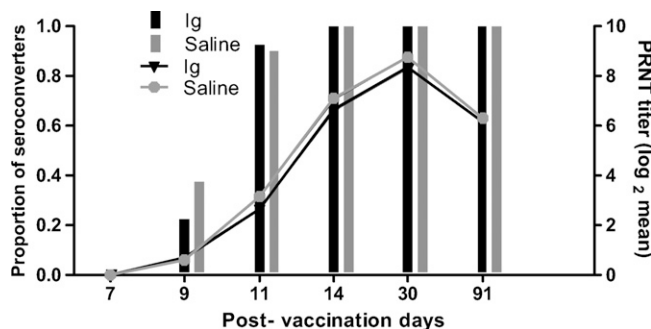


FIGURE 2. Proportion of seroconverters and their PRNT₉₀ titers after immunization with the 17D YF vaccine.

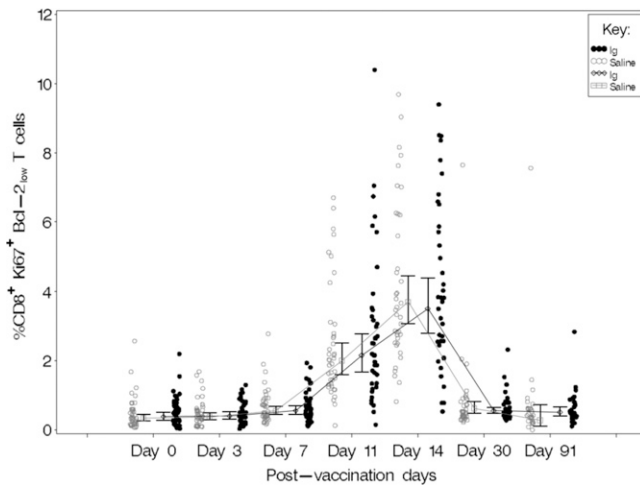


FIGURE 3. Activated CD8⁺ T cells after immunization with 17D YF vaccine and Ig or saline.

colony-stimulating factor (GM-CSF) ($P = 0.65$), Eotaxin ($P = 0.88$), and tumor necrosis factor- α (TNF α ; $P = 0.69$), were not different between the Ig and saline groups. IP-10 and MCP-1 showed significant post-vaccination peaks between days 7 and 9 in both groups ($P < 0.001$ for IP-10 and $P < 0.01$ for MCP-1) and returned to near baseline by day 14. Significant post-vaccination changes in TNF α were not detected in either of the two study groups ($P = 0.47$; test for time on study effect).

Post-hoc analyses of the effect of vaccine lot. The two vaccine lots had different titers of YF vaccine virus ($5.86 \log_{10}$ PFU/0.5 mL versus $6.40 \log_{10}$ PFU/0.5 mL), but there were no statistically significant differences observed for maximum \log_{10} viremia (mean \pm SD = 2.79 ± 1.02 and 2.77 ± 0.76 for vaccine lots 1 and 2, respectively, $P = 0.9$), viremia AUC (mean \log_{10} = 11.66 ± 6.18 and 13.29 ± 5.47 for vaccine lots 1 and 2, respectively, $P = 0.3$), and \log_2 PRNT₉₀ titers (mean = 8.79 ± 2.06 and 7.88 ± 2.03 for vaccine lots 1 and 2, respectively, $P = 0.1$). Before the initiation of clinical activities, the initial human Ig lot was confirmed to have PRNT₉₀ titers of 1:20 and 1:40 in two separate Ig vials. A PRNT titer of ≥ 20 is considered protective.⁷

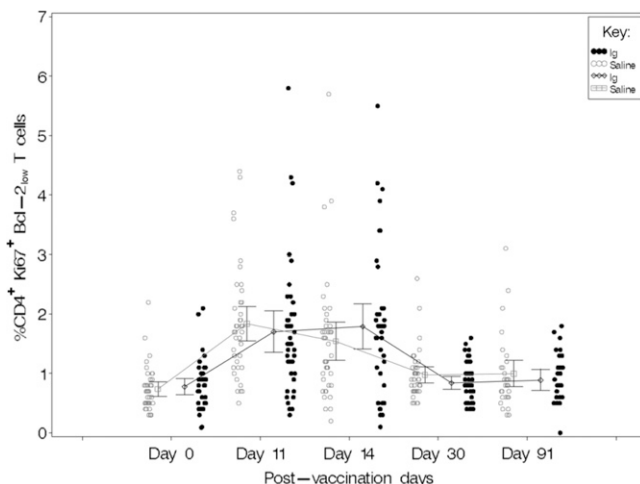


FIGURE 4. Activated CD4⁺ T cells after immunization with 17D YF vaccine and Ig or saline.

Adverse events. There were no serious adverse events. Mild arm swelling, redness, and other reactions (itching, warmth, or rash) at the vaccine injection site were less common in the vaccine plus Ig group. None of the solicited systemic signs and symptoms were different between the two groups (Supplemental Table 2).

DISCUSSION

This randomized trial clearly showed that post-vaccination viremia measured by RT-PCR or infectivity was the same in participants who received concomitant Ig or saline. Quantitative RT-PCR is more sensitive than infectivity assays for detection of viremia, and the former detects viral RNA in both infectious and non-infectious virus. A previous study of comparative viremia by quantitative RT-PCR and infectivity in 12 primary vaccinees showed that 12 of 12 were viremic by RT-PCR, but only 7 of 12 were positive by plaque assay.²³ Similarly, in our study, fewer subjects had post-vaccination viremia with the infectivity assay, and there was no difference in the two treatment groups.

Our result argues that Ig has no effect on YF virus viremia post-vaccination. Peak viremia occurred between days 5 and 7, which was also shown in previous studies.^{23–25} After intramuscular delivery of human Ig, peak serum levels are obtained approximately 2 days later, and the half-life of Ig is 23 days (per the manufacturer's package insert). Thus, a difference in the kinetics of circulating virus and antibody seems unlikely to explain the result. We documented PRNT₉₀ titers of 1:20 to 1:40 in our vials of human Ig. It has been suggested that a \log_{10} neutralization index (LNI) of > 0.7 or a PRNT titer ≥ 20 is protective.^{7,17}

Viremia was well-controlled by the vaccinees, and viral containment was temporally correlated with the rise of antibody and T-cell responses. There was no effect of Ig on antibody, T-cell, or cytokine responses. In our study, the resolution of viremia in nearly all participants by day 11 was concurrent with and likely because of the day 11 appearance in nearly all subjects of neutralizing antibodies and the day 14 peak magnitudes of activated CD4⁺ and CD8⁺ T cells. Previously, seroconversion rates of 86% and 100% were noted on days 14 and 28, respectively, in a study ($N = 93$) that used a new seed lot of the 17D-204 strain.²⁶ Interestingly, in the present study, two participants in the Ig group did not have detectable viremia but did develop neutralizing antibodies (with peak PRNT₉₀ titers of 1:80 and 1:2,560) and T-cell responses. Perhaps surprisingly, given the absence of an effect on circulating vaccine virus levels or immunity, we did observe less local reactogenicity at the vaccine injection site in the vaccine plus Ig group compared with the vaccine plus saline group.

We previously characterized both innate and adaptive immune responses to YF vaccine in both murine and human systems.^{27–30} In the present study, CD8⁺ and CD4⁺ T cell responses and the cytokine responses were similar in the Ig and saline recipients. Ki-67 is up-regulated in recently divided or cycling cells. Bcl-2 down-regulation indicates susceptibility to apoptosis, which is also a characteristic of T cells differentiating to become effectors.^{20,30} HLA-DR and CD38 are expressed by activated CD8⁺ and CD4⁺ T cells during acute viral infections in humans.^{20,29,30} In our study, 3–4% of total CD8⁺ T cells were activated (determined by Ki-67⁺/Bcl-2^{low} and HLA-DR⁺/CD38⁺ markers) at day 14, confirming our earlier work.²⁰ A single outlier value that we observed

on day 30 with high frequencies of activated CD8⁺ T cells (19%) in one participant likely represents an intercurrent infection unrelated to the study, because this activated phenotype may be seen with any acute infection (Figure 4).

Importantly, our results argue against one hypothesis for why an increase in reported serious YF vaccine-associated adverse events has been observed over the last 15 years concomitant with the advent of active hepatitis A vaccination in 1996 (i.e., the reduction in usage of coadministered Ig and therefore, the absence of the postulated lowering of post-vaccination viremia by Ig). Increased surveillance for YF vaccine-related adverse events, which was called for in 2002 by the CDC's Advisory Committee on Immunization Practices (ACIP),³¹ is another potential explanation for the increased numbers of reported adverse events.

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