Phenotypic and Molecular Analyses of Yellow Fever 17DD Vaccine Viruses Associated with Serious Adverse Events in Brazil

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The yellow fever (YF) 17D virus is one of the most successful vaccines developed to date. Its use has been estimated to be over 400 million doses with an excellent record of safety. In the past 3 years, yellow fever vaccination was intensified in Brazil in response to higher risk of urban outbreaks of the disease. Two fatal adverse events temporally associated with YF vaccination were reported. Both cases had features similar to yellow fever disease, including hepatitis and multiorgan failure. Two different lots of YF 17DD virus vaccine were administered to the affected patients and also to hundreds of thousands of other individuals without any other reported serious adverse events. The lots were prepared from the secondary seed, which has been in continuous use since 1984. Nucleotide sequencing revealed minor variations at some nucleotide positions between the secondary seed lot virus and the virus isolates from patients; these differences were not consistent across the isolates, represented differences in the relative amount of each nucleotide in a heterogeneous position, and did not result in amino acid substitutions. Inoculation of rhesus monkeys with the viruses isolated from the two patients by the intracerebral (ic) or intrahepatic (ih) route caused minimal viremia and no clinical signs of infection or alterations in laboratory markers. Central nervous system histological scores of rhesus monkeys inoculated ic were within the expected range, and there were no histopathological lesions in animals inoculated ih. Altogether, these results demonstrated the genetic stability and attenuated phenotype of the viruses that caused fatal illness in the two patients. Therefore, the fatal adverse events experienced by the vaccinees are related to individual, genetically determined host factors that regulate cellular susceptibility to yellow fever virus. Such increased susceptibility, resulting in clinically overt disease expression, appears to be extremely rare.

Key Words: YF vaccine; 17DD vaccine virus; adverse events; human vaccination.

INTRODUCTION

Brazil, like several other countries in South America, has an extensive Amazonian forest region in which jungle yellow fever (JF) is endemic and a coastal zone in which yellow fever does not occur (Robertson et al., 1996; Vasconcelos et al., 1997). The boundary between these zones may be affected by periodic expansions in epizootic activity. The densely populated coastal zone has become reinfested with the urban vector, Aedes aegypti, and is thus receptive to the introduction and spread of YF from the endemic area (Vasconcelos et al., 1997, 1999).

Between 1998 and 2000, yellow fever cases occurred over a very wide area in Brazil and expanded beyond traditional boundaries of the endemic zone, to involve the western regions of three states, Minas Gerais, São Paulo, and Bahia States. Due to the upsurge in yellow fever activity in the endemic area and the increasing mobility of the human population, there have been recent imported cases of yellow fever in the coastal zone, e.g., Rio de Janeiro and the interior of São Paulo State, renewing concerns about reurbanization (Vasconcelos et al., 2001a). The risk of urban yellow fever is enhanced by the expanding distribution and density of Aedes aegypti, which now include all 27 states of the country (Travassos da Rosa et al., 2000).

The YF 17D virus is one of the most successful vaccines developed to date. It has a well-defined and efficient production methodology, with strict quality control including monkey neurovirulence testing. It is inexpensive, and a single dose provides long-lasting immunity in ~100% of vaccinated individuals. Its use has been estimated to be over 400 million doses with an excellent record of safety. Only 21 cases of postvaccinal encephalitis have been recorded after seed lot system implementation in 1946, with incidence on very young infants.
In 1998 and 1999, yellow fever vaccination was intensified in Brazil in response to higher risk of urban outbreaks of the disease. Approximately 34 million doses have been applied since 1998. Increased surveillance for vaccine-associated adverse events was also implemented in Brazil during this interval. The vaccine, manufactured by BioManguinhos, Rio de Janeiro, contains the 17DD substrain and has been in continuous use for over 60 years. This substrain was derived from 17D virus by separate downstream passages starting with passage 195. It is used in Brazil, while the 17D-204 vaccine is employed in other countries (reviewed in Monath, 1999).

Vaccination with 17DD virus led to three reports of adverse reactions in Brazil, all of neurological origin as were the ones previously reported after vaccination with other 17D substrains mainly 17D-204. There were one case of encephalitis and two of paralysis, for an incidence of 0.09 per million (Ministry of Health, unpublished data), which is not different from the historical incidence of postvaccinal encephalitis—an expected, rare complication of yellow fever vaccination.

In October 1999 and February 2000 two fatal adverse events temporally associated with YF vaccination occurred in Goiânia (Goiás State) and Americana (São Paulo State). Details of these cases have been described elsewhere (Vasconcelos et al., 2001b). Both cases had features similar to yellow fever disease, including hepatitis and multiorgan failure. Two different lots of YF 17DD virus vaccine (lots 29Z and 88Z) were administered to the affected patients. The same lots were also administered to hundreds of thousands of other individuals without any other reported serious adverse events. The lots were prepared from the secondary seed (102/84, passage 285), which has been in continuous use since 1984. A third fatal case occurred in Santos, São Paulo State, in May 1999. The clinical details of this are not well documented, and the vaccine lot used is unknown.

Virus was recovered from all three cases from blood or postmortem tissues and identified by PCR amplification and immunostaining as YF virus. Partial nucleotide sequence analyses showed that in at least two cases, Goiânia and Americana, the viruses corresponded to the YF 17DD vaccine (Vasconcelos et al., 2001a).

The main objective of the molecular studies described here was to define whether any genetic changes occurred in the virus during its production and/or multiplication in the vaccinees that could explain the fatal adverse events. These studies were complemented by the analysis of the viral phenotype after inoculation of rhesus monkeys with viruses recovered from two of the cases.

**RESULTS**

**Nucleotide sequence determinations**

Figure 1 depicts the passage history of each virus used in the molecular analysis of YF 17DD strains associated with serious adverse reactions in humans.

The genomic sequence of the secondary seed lot virus, 102/84, was derived entirely from RNA extracted...
directly from the reconstituted vial. The seed is used to inoculate embryonated eggs for vaccine production in the YF vaccine manufacturer laboratory. One Vero cell passage was also carried out to examine the influence of this passage on the 3′/H11032 end sequence stability.

The intermediate viruses between the secondary seed lot and the viruses isolated from patients are the vaccine lots 29Z and 88Z. The vaccine lot used in the Santos case is unknown. The 29Z lot virus structural region (nt 1–2475) and the 3′/H11032 end (10,452–10,860) were sequenced directly from the reconstituted material of the original vaccine vial. For 88Z lot virus part of the structural region (nt 1659–2507) and its 3′ end (nt 10,450–10,862) were sequenced from virus present in the vaccine vial suspension, whereas most of the structural region (nt 1–2495) sequence was derived from virus of a Vero cell passage.

The complete nucleotide sequence of Goiânia virus genome was derived directly from liver suspension material. Approximately 75% (8130 nt) of the genome was sequenced on both strands. This sequencing was extended to a Vero cell-passaged virus genome to obtain complementary strand sequence and to analyze its extreme 3′ end (nt 14–2434, 2885–3330, 3627–4530, 5552–6275, 6830–8650, 8850–10,562; Table 1). Further analyses of the 3′ end sequence included viruses from different organs (heart, spleen) of the same patient. These viruses were available as tissue suspension and as C6/36 cell culture supernatants with one and two passages, respectively. For all of these viruses, the nucleotide sequences were determined at the 3′/H11032 end (nt 10,450–10,862; Fig. 1).

For the Americana case, the identity of the infecting virus was determined by sequencing the E gene of viral RNA present in serum. The complete genome sequence (except for a gap in the NS5 gene (nt 8188–8343) was determined for virus recovered from a liver suspension virus that had been passaged once in C6/36 cells. Approximately 56% (6047 nt) of the genome sequence was on both strands. To complement this sequence, the virus was first isolated from liver tissue by mouse brain inoculation followed by a single passage in Vero cells. The whole structural region (nt 1–2453) plus the 3′/H11032 end and the NS5 gap (nt 8034–8573) of this virus was sequenced. Virus from the Santos case was isolated from serum by intracerebral (ic) inoculation of suckling mice. The complete genomic sequence was determined from viral RNA extracted from Vero cells infected with the suckling mouse brain passage.

Considering all sequences available, the 102/84, Americana, Goiânia, and Santos viruses had their complete genome as cDNA sequenced on both strands.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Codon</th>
<th>Gene</th>
<th>Asibi</th>
<th>17DD original</th>
<th>Secondary seed 102/84</th>
<th>Lot 29Z</th>
<th>Lot 88Z</th>
<th>Americana</th>
<th>Goiânia</th>
<th>Santos</th>
<th>Amino acid change</th>
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<td>T</td>
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<td>T/C</td>
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<td>T</td>
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</tr>
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<td>1887</td>
<td>305</td>
<td>E</td>
<td>C</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>T, T/C</td>
<td>T</td>
<td>T</td>
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</tr>
<tr>
<td>2110</td>
<td>379</td>
<td>E</td>
<td>C</td>
<td>A</td>
<td>G</td>
<td>G</td>
<td>G</td>
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<td>G</td>
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<td>T</td>
<td>A</td>
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<td>T</td>
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<td>C/T</td>
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<td>C/T</td>
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</tr>
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<td>3294</td>
<td>NS5</td>
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<td>C/T</td>
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<td>nd</td>
<td>nd</td>
<td>T</td>
<td>T</td>
<td>T</td>
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<td>A/G</td>
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<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>None</td>
</tr>
</tbody>
</table>

*Rice et al., 1985.
Hahn et al., 1987.
GenBank Accession No. U17066.
T, unpassaged virus recovered from serum; T/C, liver virus passaged in mouse brain and Vero cells.
YF 17D 204 ATCC (X03700), 17D-204 France (X15062), and 17D-213 WHO (U17067) showed nucleotide G at this position.
YF 17D 204 viruses (ATCC, France, and WHO) showed T at this position.
Original sequence as reported by Post et al., 1992, and Duarte dos Santos et al., 1995, shown in bold. Reexamination of the sequence 2000 revealed heterogeneity as shown. nd, not determined.
YF 17D 204 ATCC showed C, 17D-204 France showed A, and 17D-213 WHO showed T at this position.
Liver virus, unpassaged, showed nucleotide G at these positions; however, Vero 1 passage of the liver isolate virus, as well as unpassaged spleen and heart virus, revealed the sequence to be G.

**YF 17DD VACCINE VIRUS IN ADVERSE EVENTS**
A summary of the comparative sequence analysis is shown in Table 1. Differences are shown between the viruses listed and the original published 17DD sequence (GenBank Accession No. U17066, originally described by Post et al., 1992, and Duarte dos Santos et al., 1995). The original 17DD sequence was performed on a vaccine lot at passage 286 equivalent to vaccine lots 29Z and 88Z. The secondary seed lot virus corresponds to passage 285. The initial sequencing of the Americana and Goiânia isolates had found a number of inconsistent differences with the original 17DD sequence (Table 1). Therefore, we first concentrated on determining the complete nucleotide sequence of the BioManguinhos 102/84 secondary seed lot virus genome and on the heretofore undefined Santos case virus. If no differences could be found between the secondary seed and the viruses isolated from the cases, there would be little value in sequencing the intermediate vaccine lot viruses. As described below this was indeed the case and this is the reason for the limited sequencing carried out on the vaccine lot viruses.

Comparison of BioManguinhos 102/84 secondary seed and the original 17DD sequence

The original sequence of 17DD was reexamined in its original files and displayed heterogeneity at nt 2677, 4523, 10,243, and 10,367 (Table 1). The original published sequence is shown in bold; the other nucleotides at these positions found as a minority population are also shown. The importance of this reexamination will become apparent when considering the sequence data for the cases with adverse events.

A comparison of the secondary seed and the original 17DD sequence revealed differences at 2110 and 2356 (E gene) and 5362 (NS3) none of which resulted in an amino acid change. Several other positions displayed heterogeneity, that is, two nucleotides were detected by sequencing. This suggests the coexistence of viral populations bearing one or the other nucleotide at that particular position. When a particular heterogeneity was not observed in one virus, one of the nucleotides of that heterogeneous position was always present (at positions 1003, 2677, 4523, 9988, 10,174, 10,243, 10,367, and 10,675). Therefore, these variations cannot be considered mutations. Moreover, neither nucleotide in the heterogeneous mixture at a given position resulted in an amino acid change.

The nucleotide (A) at position 2110 in the original sequence represents one of the differences originally defined between 17DD and 17D-204 substrain vaccines, which have a G at this position (Post et al., 1992). However, we found the BioManguinhos secondary seed to have a G at this position and thus to be identical to all 17D-204 substrain vaccines and wild-type parental Asibi virus (Table 1). Since the original 17DD sequence was performed with virus derived from the same secondary seed, it is possible that the original sequence may contain an error and that there is no difference between any yellow fever vaccine and wild-type virus at this position.

The nucleotide (T) at position 2356 in the original 17DD sequence represents one of the differences originally defined between all vaccine viruses and Asibi virus (Post et al., 1992; Galler et al., 1998). We found the BioManguinhos secondary seed sequence (as well as the vaccine lots and patient isolates) to have a change (T → C) at this nucleotide, representing a reversion to the wild-type Asibi sequence (Table 1). However, since the original 17DD sequence was performed with virus derived from the same secondary seed, it is possible that the original sequence is in error and that 17DD vaccine does in fact have the wild-type sequence at this position. Since both 17DD and 17D-204 vaccines are attenuated, and since the nucleotide difference occurs at the third position in the codon and does not result in an amino acid change, it is unlikely that nt 2356 is relevant to attenuation/virulence or that it plays a role in the adverse events.

The nucleotide (A) at position 5362 (NS3) in the original 17DD sequence was found to be C in the secondary seed, the vaccine lots, and the patients’ isolates. However, there is considerable variability across yellow fever vaccine strains at this nucleotide (Table 1). These changes do not result in an amino acid change and are not involved in virulence/attenuation.

Differences between the BioManguinhos secondary seed 102/84 and the viruses recovered from patients with adverse events

There were no consistent differences between the secondary seed and virus isolates from patients. The secondary seed and Santos isolate were identical. The secondary seed differed from either Americana or Goiânia strains but never from both strains at nucleotides 1003, 4523, 9988, and 10,174 (Table 1). At these positions, the seed and patient viruses displayed heterogeneity of the same nucleotides, and therefore these differences cannot be considered mutations. None of these changes encoded an amino acid substitution.

The only notable exception is a difference between the secondary seed and the Americana patient virus at nt 1887. The initial sequence of liver (C6/361) and unpassaged serum virus revealed no difference from the secondary seed at nt 1887 (T). However, the sequence of the Americana strain isolated from liver and passaged once in mouse brain and once in Vero cells revealed a minor heterogeneity (T/C) at this position. Based on the chromatogram, it was estimated that the C nucleotide at position 1887 represents only 10% of the total in the heterogeneous mixture. The C nucleotide was not seen in the secondary seed or in the vaccine lot 29Z used to
immunize the patient. Therefore, the heterogeneity detected probably resulted from selection or mutation during the additional mouse brain and cell culture passage of the virus rather than mutation during replication in the patient. Interestingly, virus containing the C nucleotide at position 1887 constitutes a reversion to the parental Asibi virus sequence and does result in an amino acid substitution (phenylalanine → serine) at residue 305 of the E protein. E305 has been suspected to be an important virulence factor (at least for neurotropism), since virus recovered from a fatal case of postvaccinal encephalitis contained a mutation at a juxtaposed amino acid (E303) (Jennings et al., 1994). Other studies have shown this region of the E protein to contain important neurovirulence determinants. For example, Japanese encephalitis virus selected for resistance to binding brain cell membranes contained a mutation at E306 and had an attenuated phenotype (Ni and Barrett, 1998).

It is unlikely that the mutation at nt 1887/E305 in the passaged virus from the American case is related to the adverse event in this case for the following reasons: (a) it is not seen in the Goiânia case (or Santos case); (b) it is not seen in the sequences from serum (unpassaged) or from liver (passaged in C6/36 cells); (c) it is present in the mouse brain + Vero passage material only as a minority subpopulation, i.e., approximately 10% of the total virus present; and (d) the virus containing the heterogeneity was inoculated into rhesus monkeys by either route, ic or intrahepatic (ih), and yet no clinical signs were observed (see below).

Differences between the BioManguinhos secondary seed 102/84 and the vaccine lots used to vaccinate the cases

No differences were found between the structural genes of the 102/84 seed and vaccine lots 29Z and 88Z (Table 1). Since the NS genes did not differ significantly across the secondary seed and the patient isolates, the complete sequences of the vaccine lots were not determined.

Observations on experimental infections of rhesus monkeys with YF 17DD 102/84, Goiânia, and Americana viruses

To further confirm that the viruses isolated from the Americana and Goiânia cases were in fact vaccine viruses without any altered phenotypic properties as predicted from the nucleotide sequence data we designed a protocol to inoculate a limited number of healthy rhesus monkeys by the ic or ih route and examined the outcome of the experimental infections. These results are shown in Tables 2 and 3.

**Febrile response.** All animals inoculated via ic developed fever equal or above 40.0°C (Table 2). The onset of fever in these animals was on day 8 in four of nine animals, increasing to six of nine animals on day 9; four of nine on day 10; three of nine on days 11, 12, and 13; and two of nine on days 14 and 15. During this interval in which animals more often developed fever, we observed 9 days of fever for animals inoculated with each virus, 102/84, Goiânia, and Americana. The onset of fever does
TABLE 3
Viremia in Rhesus Monkeys Inoculated by the Intracerebral (ic) or Intrahepatic (ih) Routes with Yellow Fever 17DD Isolates from Fatal Vaccine-Associated Cases and the 17DD Secondary Seed

<table>
<thead>
<tr>
<th>Virus/route</th>
<th>Monkey</th>
<th>Viremia (log_{10} PFU/ml) by postinoculation days</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Goiânia—ic</td>
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<td>&lt;0.9</td>
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<tr>
<td>17</td>
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<td>&lt;0.9</td>
<td>1.38</td>
</tr>
<tr>
<td>60</td>
<td>&lt;0.9</td>
<td>&lt;0.9</td>
<td>&lt;0.9</td>
</tr>
<tr>
<td>Goiânia—ih</td>
<td>05</td>
<td>&lt;0.9</td>
<td>&lt;0.9</td>
</tr>
<tr>
<td>15</td>
<td>&lt;0.9</td>
<td>&lt;0.9</td>
<td>&lt;0.9</td>
</tr>
<tr>
<td>20</td>
<td>&lt;0.9</td>
<td>&lt;0.9</td>
<td>1.38</td>
</tr>
<tr>
<td>Americana—ic</td>
<td>09</td>
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<td>&lt;0.9</td>
</tr>
<tr>
<td>11</td>
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<td>&lt;0.9</td>
<td>2.20</td>
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<td>&lt;0.9</td>
</tr>
<tr>
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<td>&lt;0.9</td>
<td>&lt;0.9</td>
<td>1.98</td>
</tr>
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</table>

a Log_{10} PFU/ml.

b Days.

not differ with regard to the viruses, as two animals inoculated with 102/84 were feverish on days 8, 9, and 10, and for Goiânia and Americana viruses, a single animal had fever on day 8 and two on day 9.

Fewer monkeys inoculated ih developed fever. From the animals inoculated ih, monkey 56 presented with fever on the 4th and 10th days but not on other time points. Other animals (1, 5, and 15) had fever exclusively on days 17, 18, and 27, respectively.

Viremia. The viremia detected in the sera of monkeys during the first 7 days postinoculation is shown in Table 3. Circulating virus could be detected in all animals inoculated ic and in seven of nine animals inoculated ih (exceptions being one monkey in the secondary seed lot 102/84 and one monkey in the Goiânia group). The total number of days of detectable viremia for animals inoculated ic was 21 in contrast to only 14 days for animals inoculated ih.

All viremias were brief and low level as indicated by mean peak titers and mean duration (Table 3). Goiânia and 102/84 viruses had similar magnitudes of viremia after ic inoculation (1.3 log_{10} PFU/ml), whereas a somewhat higher mean peak titer was observed for Americana virus (1.9 log_{10} PFU/ml). After ih inoculation the same profile was observed, with the values for Goiânia (1.5 log_{10} PFU/ml) and 102/84 (1.6 log_{10} PFU/ml) viruses being similar and a higher titer for Americana virus (2.0 log_{10} PFU/ml). The highest viremia noted was in monkey 35, inoculated ih with Americana virus, which reached 2.28 log_{10} PFU/ml on the fourth day pi. Of all animals inoculated ic another monkey (11) inoculated with Americana virus also had the highest viremia (2.2 log_{10} PFU/ml) on the third day pi.

The mean duration of viremia was the same for all animals inoculated ic regardless of the virus (2.3 days, Table 3). Upon ih inoculation with Goiânia and 102/84 viruses, duration of viremia averaged 1.5 days but it was longer in animals given Americana virus (2.7 days).

Antibody response. All animals developed neutralizing antibodies 30 days after YFvirus inoculation as indicated by the results of plaque reduction neutralization tests (Table 2). Titers varied from 1:3715 to more than 1:32,768. There is no evident difference in seroconversion titers to any virus irrespective of the dose and route.

Clinical findings. No clinical signs of any magnitude were noted along the 30 days of observation p.i. by either route with any of the viruses (Table 2). The facts that most animals displayed viremia and all specifically seroconverted to YF in neutralization tests confirm that the animals were indeed infected by the respective virus inoculated. Even those animals that had the highest viremia did not present any overt clinical signs.

Clinical chemistry analysis included the analysis of serum transaminases (AST/ALT), total bilirubin, alkaline phosphatase, lactate dehydrogenase, y-glutamyltransferase, creatinine, blood urea nitrogen, total protein, albumin, and globulin. Hematological analyses included total and differential blood cell counts (polysegmented neutrophils, band cells, lymphocytes, monocytes, eosinophils, and basophils), hemoglobin concentration, plate-
let counts, and hematocrit. Samples from days 1 (preinoculation), 2, 4, 6, and 31 were analyzed. None of these tests did suggest any alteration in the physiology of the inoculated animals (data not shown).

All animals inoculated ic, except monkey 9, lost weight at the end of the 30-day observation period. None of them had signs of diarrhea or anorexia. The loss averaged 506 g for animals inoculated with 102/84 virus, 320 g for animals with Americana virus, and 366 g for animals with Goiânia virus. Animals inoculated ih with 102/84 and Goiânia viruses gained weight (average of 40 and 120 g, respectively) but animals which received the Americana virus lost an average of 126 g (data not shown).

**Histological findings.** All nine rhesus monkeys inoculated ic with Goiânia, Americana, and 102/84 seed lot viruses developed histological lesions (Table 2). None of the animals inoculated ih developed any histological lesions in either the CNS (Table 2) or the extraneural organs, including liver, kidney, adrenals, heart, spleen, and lung (data not shown). No lesions in these visceral organs were observed for the animals inoculated ic. For all animals, there were no abnormalities in any organs that could suggest damage or impaired function. In this regard immunostaining analysis employing hyperimmune sera (ATCC) and a YF-specific monoclonal antibody did not reveal any YF virus antigen in any of the tissues above of animals infected with a virus as it did for the positive control tissue from another monkey which had died as a consequence of direct inoculation of an experimental YF 17D virus (Marchevsky et al., 1995). A second positive control for the immunostaining assays was liver histological sections from the Goiânia case (data not shown). In addition, liver biopsies of all 18 animals at the 10th day postinoculation, regardless of inoculation route, were analyzed for histological lesions, immunostaining for viral antigen, and viral isolation. All tests were negative as there were no signs of liver tissue damage, no staining for viral proteins, nor isolation of virus.

As proposed by Levenbook et al. (1987) the target area in the rhesus monkey CNS for several vaccine viruses is the substantia nigra. In this study the substantia nigra presented with the highest histological scores for the monkeys inoculated ic with all three viruses. Based on the individual values shown in Table 2, 102/84 virus had an average score in this area of 1.5, with 1.67 for Goiânia and for 116 Americana virus. In five complete neurovirulence tests for the 17DD 102/84 seed lot virus the average target area score was 1.49 (R. S. Marchevsky and R. Galler, in preparation).

Among the discriminator areas in the CNS, the putamen, globus pallidus, and nucleus caudatus were the areas more affected but the lesion scores were never above 2 with any of the viruses. The only exception was a grade 3 lesion on the left side of the nucleus caudatus of monkey 17 inoculated ic with Goiânia virus. This animal also had the highest combined target/discriminatory score (1.58; Table 2) but yet no clinical signs were noted.

The average discriminator area score for 102/84 virus was 0.45, for Goiânia 0.53, and for Americana 0.40, values which are very close to each other and to the average value observed for 102/84 across five other full neurovirulence tests (0.67; R. S. Marchevsky and R. Galler, in preparation).

The degree of neurovirulence of a given virus is the average of combined target/discriminator areas scores of all the monkeys. For 102/84 virus this combined score was 0.97, for Goiânia 1.09, and for Americana 0.78. The values for the combined neurovirulence scores in five complete tests with 102/84 virus varied between 0.96 and 1.37 with an average of 1.07.

**DISCUSSION**

It is important to underscore the limitations of genome analysis in this investigation. A limitation is that yellow fever vaccine is not biologically cloned and is known to be a mixture of multiple subpopulations of virions reflecting the quasispecies nature of flaviviruses. Molecular analysis results in a consensus sequence and is relatively insensitive for the demonstration of minority virus subpopulations. Only when present at a concentration of >10–20% will such subpopulations be detected. In fact, this was the case in the current analyses, with heterogeneity in the nucleotide sequence demonstrated at multiple positions. The underlying assumption is that selection of a mutation responsible for an increased virulence, leading to death of the infected host, would result in the altered genotype being the majority species in the consensus sequence. A second assumption is that one or more amino acid changes would be required for a significant change in virulence, although it is theoretically possible that silent changes could alter secondary structure and biological properties of the virus.

No consistent sequence changes were noted between the BioManguinhos secondary seed virus and the viruses isolated from the Americana, Goiânia, and Santos. Minor variations at nucleotides 1003, 4523, 9988, and 10,174 were found between the secondary seed and the patient isolates; these differences were not consistent across the patient isolates, represented differences in the relative amount of each nucleotide in a heterogeneous position, and did not result in amino acid substitutions. These variations are not related to the adverse events.

At one position (nt 1887/E306), a mutation was detected in the Americana virus, resulting in an amino acid change to the wild type (Asibi sequence). However, this change probably occurred during laboratory passage of the isolate; it was not detected in unpassaged virus and was a minority subpopulation in the passaged strain. It
was not observed in the Goiânia and Santos cases. It is not considered relevant to the adverse event in this case.

Virulence is defined by the ability of the virus to cause in an experimental animal model clinical illness, clinical laboratory abnormalities, histopathology of visceral and neural tissues, and higher viremia compared to a reference vaccine strain. Rhesus monkeys are exquisitely sensitive to infection with YF viruses and have been developed as a system for the analysis of YF 17D virus attenuation (Monath, 1999). The histopathologic scoring methodology for neurotropism (Levenbook et al., 1987) was incorporated into the biological standards for YF 17D vaccines (WHO, 1995).

In the experimental infection of rhesus monkeys by the intracerebral route with 102/84, Goiânia, and Americana viruses, monkey serum viremia does not appear to differ among the viruses. Americana virus apparently caused longer viremia than the other viruses by the ih route of inoculation. However, the serum viremia in monkeys inoculated ih or ic with all viruses are of the same magnitude (1–2 log10 PFU/ml). The definition put forward by the WHO (1995) of viscerotropism of 17D virus limits the amount of circulating virus to below 500 mouse LD50/0.03 ml for all (10 of 10) sera and therefore was well below the established limits. In addition, the range of titers observed here is similar to that observed for rhesus monkeys inoculated with attenuated 17D/JE SA-14-14-2 and 17D- den2 chimeric viruses (Monath et al., 2000; Guirakhoo et al., 2000).

The absence of clinical signs of infection, no alterations in laboratory markers, CNS histological scores of animals inoculated ic within the expected range, and limited viremia suggest an attenuated phenotype of the viruses recovered from the two Goiânia and Americana fatal cases similar to that of 17DD 102/84 virus.

It is concluded that the fatal adverse events experienced by the vaccines are not due to genetic variation in the YF 17DD vaccine virus. The adverse events may be related to individual, genetically determined host factors that regulate cellular susceptibility to yellow fever viruses. Such increased susceptibility resulting in clinically overt disease expression appears to be extremely rare. This conclusion is supported by the data that originated from experimental infection of rhesus monkeys with viruses derived from the human cases of adverse events for which the nucleotide sequences had been determined.

There are many genetically determined factors that can contribute to the control of virus infection and influence the outcome of infection that may have led to these adverse events, such as specific (antibody and T cell responses) and nonspecific (e.g., interferon system) immunity. It has been proposed for alphaviruses that the endocrine system also can play an important role in virus-induced pathology due to induction of toxic levels of stress hormones and proinflammatory cytokines that can cause excessive tissue damage directly or lead to immunosuppression facilitating virus replication (Trgovich et al., 1996, 1997). Abnormalities in any of these systems (that interact in a complex way) may have led to the fatal outcome in the adverse postvaccination events studied here. Other genetic determinants may be host cell factors which are hypothesized to participate in virus genome replication (Shi et al., 1986). Theoretically, these proteins, if mutated, could lead to abnormalities in the virus replication cycle, e.g., accumulation of higher levels of viral double-stranded RNA intermediates in infected cells that may result in more pronounced cell death (due to apoptosis) and tissue damage. Apoptosis itself is a complex multicomponent cascade and alterations in this cascade may result in higher than normal reactogenicity to infection with a certain virus and thus profound pathology. These possibilities require further investigation. If new adverse cases happen, tissue samples from victims should be preserved to undertake comprehensive genetic analysis (e.g., using microarray technology), which may open new possibilities for making vaccines safer (via genetic screening) and possibly discover new genes that play important roles in susceptibility to virus-induced pathology.

**MATERIALS AND METHODS**

**Cells**

Vero cells (ATCC, CCL 81) were maintained in Medium 199 with Earle’s salts, buffered with sodium bicarbonate and supplemented with 10% fetal bovine serum (FBS) and antibiotics. C6/36 cells were maintained in Leibovitz’s L-15 medium supplemented with 5% FBS, nonessential amino acids, and antibiotics.

**Viruses**

The virus strains available for study with their passage history are depicted in Fig. 1. The operating principle was to sequence viruses at the lowest passage level possible, in order to avoid mutations or selection of variants that could occur during laboratory passage. The 102/84 secondary seed and vaccine lots 29Z and 88Z viruses present in the original vials were reconstituted in 0.5 ml of sterile water as specified by the manufacturer (BioManguinhos, Rio de Janeiro, Brazil).

For the Americana case, viral RNA was extracted directly from patient serum or C6/36 or Vero cell culture supernatant. The Vero cell passage was carried out with virus resulting from one passage of patient serum virus in suckling mouse brain (Fig. 1). The C6/36 cell and mouse brain passages were made at the Adolpho Lutz Institute, São Paulo, Brazil.
The Goiânia case virus (GOI 4191) was derived from tissue suspensions (liver, spleen, and heart). Virus from liver tissue was passaged once in Vero cells at FioCruz. Heart and spleen tissue viruses were passaged once and twice in C6/36 cells, respectively, at the Evandro Chagas Institute, Belém, Brazil.

The Santos case virus, available only as a serum sample, was isolated by inoculation into suckling mouse brain (Adolpho Lutz Institute). The resulting virus suspension was used to inoculate Vero cells (single passage) at FioCruz (Rio de Janeiro, Brazil).

Viruses were passaged once in Vero cells at a multiplicity of infection (m.o.i.) of 0.1–1 PFU/cell and cell culture supernatant was harvested at 3–4 days p.i. There was extensive cytophatic effect on the cell monolayers. The m.o.i. for the infection of Vero cells with tissue suspension material was not known, as the suspension (10%) was diluted 1:10 and used for inoculation. The supernatants were frozen in the presence of 10% sorbitol as stabilizer. The Vero cell culture supernatants or the reconstituted material was used directly for RNA extraction. All Vero cell passages were carried out at FioCruz on different days so as to avoid any potential cross-contamination. The 102/84 virus was passaged in Vero cells at Acambis, Inc.

Complementary DNA synthesis, amplification, and sequencing

RT, PCR, and sequencing primers were designed using the YF 17D strain sequence (GenBank Accession No. K02749) as reference. A Goiânia liver sample was homogenized to yield a suspension (10% w/v in 0.75% bovine serum albumin (Sigma Co.) in phosphate-buffered saline with antibiotics) and mixed with Trizol LS (Life Technologies) and RNA was extracted as indicated by the manufacturer. Reverse transcription and amplification were carried out with the GeneAmp RNA/PCR Core kit (Perkin-Elmer). A total of 14 RT/PCR products were synthesized encompassing nucleotides 1–1064, 940–1798, 1130–1978, 1640–2638, 2361–3387, 3002–4285, 4181–5070, 4979–6324, 6100–7271, 7161–8419, 8300–9346, 9148–9826, 9423–10,345, and 10,166–10,862. PCR products were purified using QIA Quick PCR Purification kit (Qiagen). RNA from Americana virus samples was extracted from the serum or from C6/36 cell culture supernatants using the QiAmp Viral RNA Extraction kit (Qiagen) and RT/PCR performed with Superscript One-Step RT-PCR kit (Life Technologies). A total of 13 PCR products were produced, being 2 (625–1312 and 1220–2550) on RNA extracted directly from serum and 11 from C6/36 supernatant (1–1064, 940–1798, 1549–1823, 1823–3380, 3002–4285, 4181–5070, 4979–6324, 6100–7271, 7161–8419, 8300–9346, 9148–9826, and 9423–10,862). RNA from the secondary seed lot virus 102/84, the 29Z and 88Z vaccine lots 7201–9025, and 8801–10,862 were prepared. PCR products were purified from agarose gels (QIAquick Gel Extraction kit; Qiagen) and sequenced using CEQ dye termination cycle sequencing (Beckman) followed by reaction product purification with DyeEx Spin kit (Qiagen) or using dye-terminator rhodamine sequencing reaction mix (Perkin Elmer). Sequencing was carried out using a Beckman CEQ 2000 or ABI 377 (Perkin Elmer) sequenator directly on RT/PCR amplification products without any molecular cloning. Complementary DNA sequences were evaluated using Sequencher version 3.0 (GeneCodes) software. Nucleotide sequence heterogeneities were registered only when a heterogeneous signal was observed in all chromatograms representing both plus and minus cDNA strands.

Experimental inoculation of rhesus monkeys

Six groups of three captive-bred healthy rhesus monkeys (Macaca mulatta) (13 male and 7 female, weighing 3740 to 7480 g) were obtained from the Primatology Division (CECAL/FioCruz). Each animal was kept in a separate cage under controlled environmental conditions (temperature 20–22°C, relative humidity ~60%, and 12 h of artificial light and 12 h of darkness). Animals were fed twice daily with monkey chow supplemented with fresh fruits and allowed water ad libitum. All monkeys were shown to be free of YF neutralizing antibodies by plaque reduction neutralization assay.

Virus inoculation. All monkeys were anesthetized by intramuscular injection of ketamine hydrochloride (20 mg/kg body weight) and inoculated with 0.25 ml of viral suspension by the intracerebral route into the right frontal cortex or with 0.5 ml by intrahepatic injection. Intracerebral inoculation was carried out by making an incision through the skull about 2.5 cm above the middle of the right superior orbital ridge and a hole was drilled through the skull. Virus solution was inoculated into the frontal lobe using a 1-ml syringe fitted with a 25-gauge × 1/2 needle. For intrahepatic inoculation, the liver was located by palpation and percussion and the site of inoculation was the intercostal space between the fifth and the sixth ribs over the liver. The liver was inoculated using a 3-ml syringe fitted with a 1.5-in. 22-gauge needle. The inoculations were double blind. The investigator who prepared the virus coded the syringes and had no knowledge of what each monkey received and those
inoculating did not know what each syringe contained. The codes were broken when the histological analyses were completed.

The viral inocula were back-titrated by plaque assay on Vero cells. Yellow fever secondary seed lot (YFV 17DD 102/84) was prepared in November 1984 with a titer of 6.47 log$_{10}$ PFU/ml. Doses were administered with a titer of 5.49 log$_{10}$ PFU/0.25 ml (or 61,368 mouse LD$_{50}$), via ic, and 5.81 log$_{10}$ PFU/0.5 ml (128,520 mouse LD$_{50}$) via ih. For Goiânia virus the ic dose was 5.03 PFU/0.25 ml (21,278 mouse LD$_{50}$) and the ih dose was 5.42 log$_{10}$ PFU/0.5 ml (52,356 mouse LD$_{50}$). For Americana virus, the ic dose was 4.62 log$_{10}$ PFU/0.25 ml (8318 mouse LD$_{50}$) and the ih dose was 4.92 log$_{10}$ PFU/0.5 ml (16,596 mouse LD$_{50}$). The Goiânia and Americana viruses used to inoculate monkeys were those resulting from one passage in Vero cells (Fig. 1). The mouse LD$_{50}$ was established for YF 17DD virus and the values given for Goiânia and Americana viruses were inferred from their PFU values using a ratio established at Fiocruz with 17DD virus as reference.

Viremia. Blood samples for the determination of viremia were collected on days 1 through 9 after inoculation. Serial dilutions (1:3, 1:30, 1:300) of each monkey serum were titered by plaque assay on Vero cell monolayers ($10^5$ cells/cm$^2$) with 3.5% carboxymethyl cellulose as overlap in six-well plates. One hundred microliters of virus suspension was inoculated per well with four wells per dilution.

Seroconversion. Antibody titers were determined by a 50% plaque reduction test on Vero cells (Stefano et al., 1999).

Clinical observation. Monkeys were observed for 30 days and rectal temperatures recorded. Temperature equal to or grater than 40.0°C was considered elevated. Records of clinical observation were obtained using the following grading system: 1 (minimal), one to three small, focal inflammatory infiltrates, a few neurons may be changed or lost; 2 (moderate), more extensive focal inflammatory infiltrates, neuronal changes or loss that affects no more than one-third of neurons; 3 (severe), neuronal changes or loss of 33–90% of neurons, with moderate focal or diffuse inflammatory infiltration; 4 (overwhelming), more than 90% of neurons are changed or lost, with variable, but frequently severe, inflammatory infiltration.

Three separate scores were calculated for each monkey: discriminator areas only, target area only, and discriminator plus target areas (Levenbook et al., 1987). The target area is the substantia nigra, whereas the discriminator areas include the caudate nucleus, globus, pallidus, putamen, anterior and medial thalamic nucleus, lateral thalamic nucleus, and cervical and lumbar enlargements. A final neurovirulence score was given by the combination of the scores of both areas (combined score).

Overall mean scores were calculated for each group of monkeys as the arithmetic mean of individual monkey scores for discriminator areas only and for discriminator plus target areas. For the histological criterion of the neurotropism test to be satisfied both overall mean scores for the test monkeys could not be significantly greater (at the 5% significance level) than the overall mean scores for the monkeys injected with reference virus.

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Note added in proof. Recently, Martin and co-workers published an article (M. Martin, T. F. Tsai, B. Cropp, G. J. I. Chang, D. A. Holmes, I. Tseng, W. J. Shieh, S. R. Zaki, I. Al-Sanouri, A. F. Cutrona, G. Ray, L. H. Weld, and M. Cetrone, Lancet 358, 98–104, 201) describing four cases of multiorgan failure associated with YF 17D-204 vaccination and resulting in three deaths. Nucleotide sequence analysis suggested that in one patient, but not in the others, de-novo mutation and selective amplification of the mutated virus subpopulation was the major cause of illness.

REFERENCES


