Failure of secondary infection with American genotype dengue 2 to cause dengue haemorrhagic fever

Douglas M Watts, Kevin R Porter, Pavithat Putvatana, Bruno Vasquez, Carlos Calampa, Curtis G Hayes, Scott B Halstead

Summary

Background Population-based epidemiological studies have shown that infection with dengue type 2 (DEN-2) virus in individuals previously infected with a different serotype of the virus is a major risk factor for dengue haemorrhagic fever and dengue shock syndrome. However, the western hemisphere was spared epidemics of these two syndromes, until the introduction of a southeast Asian DEN-2 genotype. Possibly American DEN-2 genotype strains lacked properties necessary to cause severe disease. We report on a major epidemic of DEN-2 in Peru in 1995, about 5 years after an epidemic of DEN-1 in the same population.

Methods In Iquitos, a city of 344 686 inhabitants in Peru, cases of dengue fever were studied prospectively from 1990. Acute phase of illness serum samples from patients were tested for virus in C6/36 cells, and virus isolates were identified by immunofluorescence. Isolates of dengue 2 virus obtained from patients during an outbreak of mild febrile illness in 1995 were sequenced to determine the genotype. Serological analysis of paired samples from the patients was done with an IgM capture ELISA and an indirect IgG ELISA. In addition, serum samples collected annually between 1993 and 1996 from a large cohort of students were tested for dengue IgG antibody by an ELISA. Serum samples from a random sample of 129 students from this cohort were tested for dengue neutralising antibodies to quantify the serotype specific infection rates.

Findings Among the 129 students (aged 7–20 years in 1993) who had serum samples available before and after the epidemic, 78 (60·5%) had a secondary DEN-2 virus infection. From previous studies, between 887 and 10 247 American genotype of DEN-2 viruses cocirculated. 1–8 Epidemics of dengue haemorrhagic fever and dengue shock syndrome appeared in the western hemisphere only after a southeast Asian DEN-2 genotype was introduced 1960s and 1970s when DEN-3, DEN-1, and the American genotype of DEN-2 viruses cocirculated. 9,10 These complications occur in only a small proportion of secondary dengue infections (18–125 per 1000). The frequency may be associated with the specific sequence of serotype infection. For example, a previous study found that infection with DEN-1 followed by infection with DEN-2 caused dengue shock syndrome to occur with a frequency of 208 per 1000 (48 children), whereas no cases of either complication were observed among 496, 58, and 142 children who had second infection with DEN-1, DEN-3, or DEN-4 serotypes, respectively. 4 Finally, only sporadic cases of dengue haemorrhagic fever were reported from the American tropics during the 1960s and 1970s when DEN-3, DEN-1, and the American genotype of DEN-2 viruses cocirculated. 11,12

We investigated the characteristics of American genotypes DEN-2 through continuous clinical and serological surveillance of a Peruvian population with high DEN-2 secondary infection rates.

Methods

Study site and participants

In anticipation of the spread of the dengue viruses into Peru in the late 1980s, the US Naval Medical Research Institute Detachment (NAMRID) established an infectious-disease field surveillance site in Iquitos, a city with a population of 344 686 located 120 m above sea level (73°29’W, 3°7’S). We identified newly introduced DEN-1 viruses there in 1990, after reintroduction of Aedes aegypti, which had been eradicated from the city earlier this century. 13-15 Subsequent seroepidemiological studies showed that DEN-1 continued to cause classic cases of dengue fever, particularly in adults. Because of the expected introduction of DEN-2 into Iquitos from neighbouring countries of South America, longitudinal prospective studies began in 1993. Blood samples were obtained between November and December, 1993, from 1336 students aged between 5 years and 22 years, who attended six schools in Iquitos. Subsequent samples were obtained from 653 of the same students between September and December, 1994, and from 691 between December, 1995, June, 1996. Of the students for whom three samples were available, serum samples from 129 (61 male, 68 female) were selected by a computer-generated list of random numbers to test DEN-1 and DEN-2 for specific neutralising antibodies. In 1993, the median age of the 129 students was 13 years (range 7–20).
Since 1993, hospital and outpatient-clinic surveillance has been done continuously in Iquitos by means of a standard questionnaire about demographic and clinical data for patients presenting with clinical signs that suggest dengue fever or dengue haemorrhagic fever. The case definition was framed so as to detect children and adult patients who had a temperature of 38°C or higher for 5 days or less, accompanied by at least headache, myalgia, and other non-specific signs and symptoms. All patients were closely monitored for signs of haemorrhage. NAMRID staff members and local physicians were familiar with the case definitions for dengue fever syndromes from published guidelines.11 Surveillance has been done since 1993 by attending physicians at five of the six hospitals in Iquitos. The number of beds ranged from 20 to 180 in three military hospitals, and there were 280 in the two Ministry of Health hospitals. NAMRID and Ministry of Health staff and attending physicians surveyed six of the 11 outpatient Ministry of Health clinics three times per week. When a suspected case was identified, homes were visited for identification of cases in the family and nearby households. Blood samples were obtained during the acute phase of illness for isolation of virus and, when possible, samples were taken during convalescence (14–20 days later) for serological testing. Enrollment in this study complied with a human use protocol approved by the Peruvian Ministry of Health, the Peruvian Navy, and the US Naval Medical Research Institute.

Virus isolation and serology
C6/36 mosquito cell lines were used to test for virus in acute-phase serum samples.14 Viruses were identified with an immunofluorescence assay with reference monoclonal antibodies.14 Serum samples were tested for IgM antibody to the dengue virus by a capture ELISA22 and for IgG antibody by an indirect ELISA by means of virus-infected vero-cell culture lysate antigens. The case definition required isolation of dengue virus, detection of IgM antibody, or evidence of seroconversion. Serotype-specific antibodies to dengue fever were identified by means of a plaque reduction neutralisation test with prototype DEN-1 and DEN-2 viruses in BHK-21 cell lines (clone-15).14 Serum samples were judged to contain neutralising antibody against a single virus if at a final dilution of 1 to 60 the plaque-forming units of only one serotype were decreased by 70% or more; infection with two viruses was recorded if the plaque-forming units of both serotypes were decreased by 70% or more.4

Virus genotype identification
Isolates of dengue virus were amplified by passage in C6/36 cells to prepare a working stock for the sequencing experiments. Viral RNA was extracted directly from tissue-culture supernatant with a commercially available total RNA isolation kit (Qiagen, Santa Clarita, CA, USA). Genotypic analysis was done with reverse-transcriptase PCR. Superscript reverse transcriptase (Gibco BRL, Gaitherburg, MD, USA) was used to reverse-transcribe complementary DNA (cDNA) from total RNA with the primer ENS1 R (5'-CATGATTCCCTTTRATGTCTCCTGTC-3'). cDNA was then used in a PCR reaction with the reverse primer ENS1 R and a forward primer ENS1 F (5'-CGGCCAAATGTTTGAGACAATG-3'). The amplification product from this reaction was 417 bp. This product was used to find out the nucleotide sequence of 240 bp spanning the junction of the envelope gene and the first non-structural gene. This sequence has been used to compare the phylogenetic relationship of 40 DEN-2 isolates,13 and is thought to show the long-term evolutionary trends among the serotypes of dengue virus because this sequence has not been affected by immune selection. Sequencing was done with an ABI 377 automated sequencer (Applied Biosystem, Foster City, CA, USA). The reverse and forward primers used for the sequencing were the ENS1 primer and the ENS1 Fseq primer (5'-GACACAGCTCGGGATT-3'). The DNA sequences of DEN-2 isolates from the samples from Iquitos were then aligned with the published sequences of 30 other isolates of DEN-2 with

Results
Between May and October, 1995, an epidemic of a mild dengue-like disease was detected in Iquitos. Although official reports are not yet available from the Ministry of Health, medical personnel in Iquitos reported that there were thousands of cases of this syndrome. During the epidemic we enrolled patients in their homes and in the hospitals and outpatient clinics in the surveillance system. Owing to the mild nature of the disease, we studied most cases at home or at outpatient clinics. We confirmed 165 cases of dengue fever by virus isolation, IgM capture ELISA, or both.15 The largest number of confirmed cases occurred in August, 1995. The DEN-1 serotype was isolated from two acute-phase samples, and the DEN-2 serotype from nine. After the epidemic, between December, 1995, and June, 1996, serum samples were obtained from the student cohort.

Neutralising antibodies to dengue virus were found in 101 (78·3%) of the 129 students in 1993 (table); this proportion reflects cumulative infections since 1990. Most of these individuals (85) had antibodies to DEN-1 virus, and only four had antibodies to DEN-2 virus alone (table).12 Had antibodies against both serotypes. The distribution of serotype-specific antibody in 1994 was nearly identical to that of 1993. There were six more antibody-negative students in 1994 than in 1993 because the antibody titre had waned below the positive cut-off value. In 1994, therefore, 115 individuals were susceptible to DEN-2 (81 positive for DEN-1 only; 34 negative). By 1995–96, 99 (86·1%) of these 115 individuals had seroconverted to DEN-2 (113 participants were positive for DEN-2; 14 had been positive in 1994).

Thus, 78 (60·5%) of the 129 had a secondary DEN-2 infection (91 had evidence of DEN-1 and DEN-2 infections in 1995, compared with 13 in 1994). By extrapolation from this frequency, we estimate that 49·266 of 81·479 children aged 5–14 years who were living in Iquitos according to the 1993 census may have had a secondary DEN-2 infection in 1995. Based on results of previous studies, between 887 and 10·247 cases of dengue haemorrhagic fever or dengue shock syndrome could have occurred in this age-group,14 and the expected case-fatality rates would be in the range 1–5%.25

Neutralising antibodies to dengue viruses in the student cohort

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Number (%) with neutralising antibodies (n=129)</th>
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<tr>
<td></td>
<td>1993</td>
</tr>
<tr>
<td>DEN-1</td>
<td></td>
</tr>
<tr>
<td>85 (65·9%)</td>
<td>81 (62·8%)*</td>
</tr>
<tr>
<td>DEN-2</td>
<td>4 (3·1%)</td>
</tr>
<tr>
<td>DEN-1 and 2</td>
<td>12 (9·3%)</td>
</tr>
<tr>
<td>No antibody</td>
<td>28 (21·7%)</td>
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* Susceptible to DEN-2.
† Seroconversion in 21 during 1995.
‡ Seroconversion in 78 during 1995.

Neutralising antibodies to dengue viruses in the student cohort

SeqApp software (version 1·9a196, D G Gilbert, University of Indiana, Bloomington, IN, USA), corrected manually, and subsequently analyzed for phylogenetic relatedness with the software Phylip (version 3·5c; J Felsenstein, University of Washington, Seattle, WA, USA).

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recorded during 1994, 1995, and 1996 among children under 10 years old (70, 66, and 91, respectively); no deaths were attributed to haemorrhage or shock.

Iquitos has a mixed ethnic population; white, Hispanic, Amerindians, and a few black people. The risk of dengue haemorrhage fever and dengue shock syndrome is significantly lower among black people than other ethnic groups. Genetic factors may be determinants of the clinical outcome of DEN-2 infections; there is no evidence of genetic susceptibility among the ethnic groups in Iquitos.

Nucleotide comparison analysis of 240 bp spanning the junction of the envelope gene and the first non-structural gene showed that the sequenced DEN-2 viral isolates in Iquitos were of American (group I) genotype, prototype Trinidad 1751. Lettmeyer and colleagues sequenced the full genome directly from a viraemic serum from two of the dengue cases in 1995 in Iquitos. These virus strains differed only slightly from several other American genotype DEN-2 strains, but had structural properties that distinguished them from southeast Asian DEN-2 strains. Viruses of the American genotype include those that have circulated in the Americas since the mid-1950s but few isolations have been made over the past decade.13

Discussion

Dengue haemorrhagic fever and dengue shock syndrome appeared in southeast Asia after the second world war and became established there as a stable feature of endemic dengue virus transmission. As populations of both human beings and the mosquito vector increase worldwide, so the complications of dengue fever spread to the American tropics and the Indian subcontinent.4,5 Extensive studies in southeast Asia have established that presence of antibody to dengue virus, actively or passively acquired, is a predominant risk factor for such complications.1,23,26,27 Although the virulence of the infecting strain has been postulated as a mechanism that regulates severe disease,28 no differences in virulence were observed between sequenced strains from mildly or severely ill patients in countries where dengue haemorrhagic fever and dengue shock syndrome are endemic.29,30

What mechanisms might explain the finding that viruses of the American DEN-2 genotype do not cause dengue haemorrhagic fever and dengue shock syndrome? One possibility is the sharing or non-sharing of similar envelope antigens. Further studies should investigate whether DEN-1 viruses share with those of the American DEN-2 genotype antigens that raise heterotypic protective antibodies, or whether they do not share infection-enhancing envelope antigens. Lettmeyer and colleagues29 postulated that viruses of the American DEN-2 genotype replicate poorly in vivo producing mild disease even in the presence of infection-enhancing antibodies. However, an infection rate of 86·1% for DEN-1 viruses of the American genotype replicate poorly in vivo producing mild disease for both primary infection and reinfection but the transmission rate was high. Because of these properties this virus is likely to be widely distributed in the American tropics, but seldom isolated. Nonetheless, infection with the American DEN-2 genotype should confer protection against severe disease after reinfection with the southeast Asian DEN-2 genotype. Although American DEN-2 genotype viruses have not been isolated in southeast Asia, members of the group have been reported from New Caledonia and Tonga.13 An origin from southeast Asia cannot, therefore, be excluded. The full-length sequences of both American and southeast Asian DEN-2 genotype viruses are known.24,25 These data, and the availability of biologically distinct DEN-2 strains, should facilitate studies that will provide a better understanding of the pathogenesis of dengue haemorrhagic fever and dengue shock syndrome.

Contributors

All investigators were involved in designing the study. Douglas M. Watts was responsible for the execution of the study, analysed the data and prepared the report. Kewin R. Porter characterised the DEN-2 viral isolates and did the nucleotide sequencing of the isolates. Ravithat Putvatana established and supervised the virological and serological tests. Bruno Vasquez coordinated the enrolment of the students and verified the clinical description of the patients. Carlos Calampa was responsible for the overall execution of the field component of the study. Curtis G. Hayes and Scott B. Halstead analysed the data and helped to write the report.

Acknowledgments

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References