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Isolation and characterization of dengue viruses serotype 1 from an epidemic in northern Queensland, Australia

J. Blok*, **B. H. Kay**, **R. A. Hall**, and **B. M. Gorman**

Queensland Institute of Medical Research, Herston, Brisbane, Queensland, Australia

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Summary. Thirteen strains of dengue type 1 were isolated from the lymphocyte fractions of 69 acute phase blood samples collected at Thursday Island Hospital during 1981 and 1982. One further strain of type 1 was isolated from 7 blood samples despatched by air from Cairns Base Hospital during 1982. Four of these Australian isolates representing the beginning, middle, and end of the epidemic were examined by restriction enzyme mapping and were found to be identical for the nine restriction enzymes used. The maps differed from those derived from two Malaysian dengue type 1 strains isolated during the epidemic of 1981–82 in that country. This suggests reliance on serological typing to establish global circulation patterns of epidemic dengue is insufficient and that more specific methods such as genome mapping are useful.

Introduction

There have been periodic outbreaks of dengue fever in northern Australia since 1879. From the 1897 epidemic, Hare [1] provided clinical descriptions of dengue, some of which are recognized as being the earliest recorded cases of dengue haemorrhagic fever. Between 1955 and 1981 the only cases recorded in Australia were from patients infected elsewhere. During this time, it was generally believed that dengue would not be a significant problem for Australia since populations of *Aedes aegypti* were thought to be restricted to parts of northern Queensland. In March 1981, cases of dengue fever were diagnosed in the resident population of northern Queensland and in late October, the Medical Superintendent at the Thursday Island Hospital notified 30 suspected cases. A team of workers was sent from Brisbane to investigate the outbreak, details of which have been published elsewhere [2].

In this paper, we describe the methods used to isolate dengue viruses and their characterization as type 1. The isolates were also compared with dengue

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* Present address: Sir Albert Sakzewski Virus Research Laboratory, Royal Children's Hospital, Herston Rd, Brisbane, Queensland 4029, Australia.

type 1 viruses isolated in Malaysia by restriction enzyme mapping in an attempt to trace the origin of the Australian strains.

Materials and methods

Virus isolation

Blood samples were collected from patients on presentation at the pathology departments of the Cairns and Thursday Island Base Hospitals. At Thursday Island Hospital, 69 samples were collected in two visits during November 1981 and April 1982, and these were inoculated into tissue cultures on site prior to despatch by air to the Queensland Institute of Medical Research (QIMR), Brisbane. Seven blood samples from Cairns were mixed with RPMI 1640 tissue culture medium by Dr. R. W. Guard and despatched by air to Brisbane during June 1982.

Sterile tubes, Pasteur pipettes, Ficoll-paque (Pharmacia), 20 ml roller tubes containing *Ae albopictus* C6/36 cells and RPMI 1640 tissue culture medium were taken to Thursday Island. A bench centrifuge and a Bunsen burner were the only items of laboratory equipment required.

The processing and inoculation procedures were as follows: (1) 10 ml of heparinized blood was drawn intravenously using Vacutainers; (2) immediately, an equal volume of RPMI 1640 medium was added to the sample; (3) 4 ml of Ficoll-paque was then drawn by syringe from a stock bottle and added to a 10 ml capped centrifuge tube; (4) using sterile techniques, 6 ml of the blood-1640 mixture was drawn from the original tube and carefully layered on top of the Ficoll-paque; (5) after centrifugation at 2,000 rpm for 20–30 min, the mixture separated into four layers consisting of a plasma-RPMI 1640 mixture which lay above the lymphocyte layer, followed by the Ficoll-paque layer and the red blood cells settled at the bottom of the tube. The lymphocyte layer was carefully removed from directly above the Ficoll-paque layer using a Pasteur pipette; (6) 0.2 ml of the lymphocytes were then inoculated into duplicate roller tubes containing *Ae albopictus* C6/36 cells and left for 1 h at room temperature; (7) the inoculum was removed from the tubes, fresh RPMI 1640 medium containing 0.2% bovine serum albumin was added and the tubes were sealed with tape and left to rest obliquely until the next air service to Brisbane was available, sometimes 3 days later; (8) serum was stored in screw capped vials at 4 °C and the remaining lymphocyte fraction placed in ampoules and stored in liquid nitrogen at −179 °C.

On delivery at QIMR, from 12 to 48 h after leaving Thursday Island, the culture medium in the inoculated tubes was changed and the cultures incubated at 28 °C. The tissue culture fluids were tested daily for presence of haemagglutinin using fresh gander cells at a range of pH values. The identity of each virus isolate was established by indirect immunofluorescence tests [3] using type-specific monoclonal antibodies to the four dengue serotypes 15F3-1-15 (type 1, Hawaii), 3H5-1-21 (type 2, New Guinea C), 5D4-11-24 (type 3, H-87) and 1H10-6-7 (type 4, H-241), provided by Drs. T. Monath and N. Karabatsos, CDC, Fort Collins, U.S.A.

Isolates of dengue type 1 from Malaysia were obtained from Dr. T. Pang, Department of Medical Microbiology, University of Malaya, Kuala Lumpur. These viruses were isolated and passaged twice in mosquito cells and were identified as dengue-1 by immunofluorescence in Malaysia. The identity of the Australian isolates was also confirmed in Dr. Pang's laboratory.

Serology

Antibody levels to dengue viruses in patients' sera were determined by haemagglutination-inhibition tests based on the methods of Clarke and Casals [4].

Synthesis of double-stranded DNA from viral RNA and mapping by restriction enzyme analysis

Selected virus isolates were grown in C6/36 cells in Roux flasks. Virus was precipitated from 1.5 litres tissue culture medium by polyethylene glycol and the methods used to extract viral RNA from these precipitates and to prepare cDNA from it have been described [5, 6]. Briefly, viral RNA and a primer AGATCCTGTGT (synthesized by BRESATEC, Adelaide, Australia), which is complementary to the 3′ sequence of the West Nile virus RNA genome [7] were heated together at 90 °C for 1 min, quickly chilled in ice-water and used as a template for cDNA synthesis. The cDNA was synthesized in the presence of ^{32}P dATP (3,000 Ci/mmol, Amersham) and avian myeloblastosis virus reverse transcriptase (Life Sciences) at 38 °C for 40 min. The resulting cDNA-RNA hybrids were used for dsDNA synthesis using RNase H and DNA polymerase I (Boehringer Mannheim) in the presence of ^{32}P dATP as described [8]. All dsDNA syntheses were performed simultaneously to minimise the differences in lengths which might occur under different reaction conditions.

The dsDNA was digested with *Bam HI, Bgl II, Eco RI, Hae III, Hinc II, Hinf I, Pst I, and Sau 3A at 37 °C,* and *Taq I* at 65 °C for 3 h, and the resulting fragments were separated on 5% polyacrylamide gels containing 7 M urea as described [6].

Dot blot hybridization

Viral RNA extracted from purified dengue virus (10 ng in 1 µl) and nucleic acid extracted from C6/36 cells (100 ng in 1 µl) were spotted onto nylon membranes (Hybond N, Amersham) and then bound to the membranes by UV irradiation for 5 min. No pretreatment or denaturation of the RNA or cellular nucleic acid was carried out. The dot blots were then hybridized with ^{32}P-labelled cDNA probes synthesized from the prototype dengue-1 and dengue-2 RNA genomes as described [5]. Hybridization was carried out in aqueous solutions containing 0.45 M NaCl at 65 °C for 16 h and the filters were washed in 0.3 M NaCl at room temperature (four 15 min washes). Autoradiography was performed at − 70 °C for 16 h.

Results

Virus isolation

Thirteen virus isolates were obtained from acute phase blood of 69 patients at Thursday Island and another isolate from one sample of seven collected in Cairns (Table 1). The ages of patients from which these viruses were isolated ranged from nine months to 51 years. All patients presented with classical symptoms of dengue as described previously [2], except for DM (TI 6) who had a sub-clinical infection. The preparation of lymphocytes to be used as an inoculum for virus isolation from C6/36 cells was from one to four days after the reported day of onset of illness. No antibodies detectable by haemagglutinin-inhibition (HI) assays were present in any acute phase sera except for patient BG. In this case HI titres of 1 : 160 to Murray Valley encephalitis virus and 1 : 80 to dengue type 1 virus suggest that the infant's mother did not estimate accurately the date of onset of illness.

The methods used to isolate viruses were based on growth of dengue viruses in mosquito cell lines which could be maintained for reasonably long periods at room temperature. Since dengue viruses grow in macrophages and monocytes [for review, see 9], a lymphocyte fraction rather than whole blood or serum was used as an inoculum to increase the chances of isolating virus.

Table 1. Dengue serotype 1 strains isolated from lymphocytes of patients from Thursday Island (TI) and Cairns (CS), 1981–1982

Strain designation		Patient	Age/Sex (yrs)	Date of onset	inoculation
TI	1	DM	29 M	30 Oct	3 Nov
	2	BC	24 M	2 Nov	3 Nov
	3	MM	33 M	30 Oct	3 Nov
	4	SO	28 M	31 Oct	3 Nov
	5	MW	32 F	3 Nov	4 Nov
	6	DM	23 M	4 Nov	4 Nov
	7	FI	9 F	5 Nov	9 Nov
	8	BO'B	43 M	5 Nov	6 Nov
	9	PA	31 M	8 Nov	9 Nov
	10	ZK	51 M	31 Oct	2 Nov
	11	TH	10 F	18 April	20 April
	12	BG	<1 F	1 Nov	2 Nov
	13	LD	18 M	25 April	27 April
CS	1	DH	— M	—	1 June

Virus characterization

All of the dengue virus isolates from Thursday Island and Cairns were identified as serotype 1 by an indirect immunofluorescence test using monoclonal antibodies specific for the four serotypes of dengue. Four isolates (TI 2, TI 5, TI 6, CS 1) were confirmed as serotype 1 by dot blot hybridization. The RNA from these four isolates as well as a Malaysian virus (32507) isolated during the same period and RNA from the prototype viruses of the four dengue serotypes were hybridized with cDNA probes specific for the dengue-1 and dengue-2 serotypes (Fig. 1). The dot blots in Figure 1 show that the Malaysian and all Australian virus isolates hybridized very strongly with the dengue-1 cDNA probe but only minor hybridization was detected with the dengue-2 cDNA probe. The dengue-1 and -2 cDNA probes hybridized strongly with their homologous RNA and some cross hybridization was also detected for serotypes 3 and 4. The minor cross-reactions of the probes with RNA from the prototype viruses of the four dengue serotypes is to be expected since there is a certain amount of sequence similarity which has previously been detected by cDNA-RNA hybridization [10].

Comparative one-dimensional restriction enzyme mapping studies of the four Australian isolates (TI 2, TI 5, TI 6, CS 1) revealed that, despite the differences in location of the isolates and the seven month difference in time of isolation, the maps were identical for the nine restriction enzymes mentioned in materials and methods. By contrast, the restriction enzyme maps of two Malaysian isolates of dengue type 1 were distinct from those of the Australian isolates. The Malaysian virus 32507 was isolated in November 1981 which was

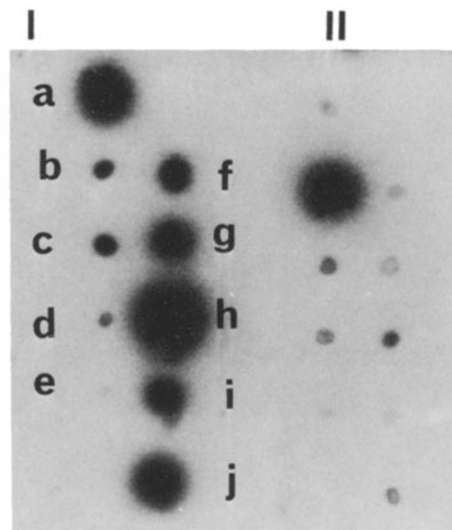

Fig. 1. Autoradiographs of dot blots of 10 ng RNA from dengue virus isolates of *a* dengue-1 Hawaii, *b* dengue-2 New Guinea C, *c* dengue-3 H-87, *d* dengue-4 H-241, *e* nucleic acid from the C6/36 mosquito cell line (100 ng), *f* Malaysian 32507, *g* TI2, *h* TI6, *i* TI5, and *j* CS1. The cDNA probe used in **I** was synthesized to dengue-1 Hawaii viral RNA while the cDNA probe used in **II** was synthesized from dengue-2 NGC RNA. Hybridization of the nylon membrane was carried out at 65 °C for 16 hr and washing was performed at room temperature

at about the same time as those from Thursday Island (except TI11 and TI13) were isolated. The other isolate from Malaysia was obtained in June 1982 which was similar to the isolation time of CS1. An example of the *Hae III* restriction enzyme profiles for the two Malaysian and four Australian isolates examined are shown in Fig. 2.

In a genetic analysis of dengue strains isolated in different geographic regions, Trent et al., 1983 [11] defined five sets or "topotypes" based on their oligonucleotide maps. The similarity of the four Australian isolates by restriction enzyme mapping and their differences from the two similar Malaysian isolates indicates that two distinct topotypes of dengue virus serotype 1 were circulating in Australia and Malaysia during 1981/1982 outbreaks. By contrast, the maps of isolates obtained over seven months within each country, were identical except for the difference in intensity of some of the restriction enzyme fragment bands. These one-dimensional maps obtained using nine restriction enzymes represent about 4% of the viral RNA genome.

Discussion

The origin of the viruses in the 1981–82 epidemic of dengue in northern Australia is not known but these are the first indigenous dengue virus isolates from either man or mosquitoes. Using newborn mice, Marshall and Hawkes [12] previously isolated dengue-2 virus from a patient who had just returned from Papua

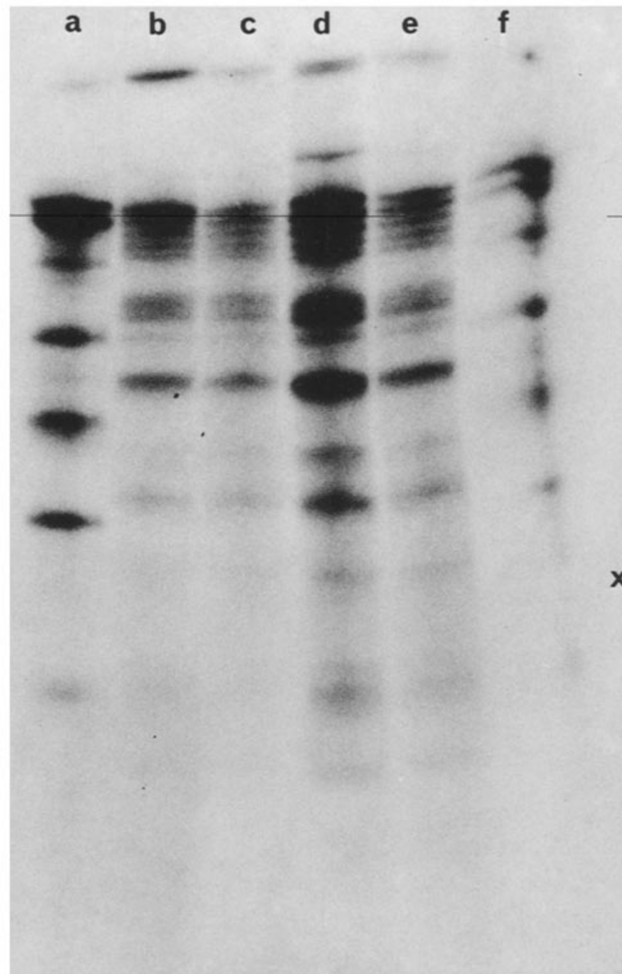

Fig. 2. Autoradiograph of a denaturing 5% polyacrylamide gel in which *Hae III* restriction enzyme fragments of ^{32}P-labelled cDNA transcribed from genomic RNA of different dengue-1 isolates have been fractionated. The viral RNAs were from *a* Malaysian 32507, *b* TI2, *c* TI6, *d* TI5, *e* CS1, and *f* Malaysian 35350. X Position of the xylene cyanole dye

Niugini-New Britain. An epidemic of dengue in Fiji in 1981 was identified as being caused by dengue-4 viruses [13]; and a few dengue cases from Papua Niugini and the Solomon Islands, tested by the Queensland Health Department, were identified as serotypes 2 and 3, respectively (N. F. Stallman, pers. commun.) However, the apparent source of one early clinical case (a mariner) on Thursday Island was traced to Port Moresby, where clinical dengue was occurring. In view of the early concentration of cases in the vicinity of the marine pilot station, it seems that the virus may have been introduced from Papua Niugini.

The methods for dengue virus isolation presented in this paper were devised to take into account the distance of ca. 2,200 kilometres between the site of collection of samples and the laboratory, and the irregularity of air services between them. Only the minimum of equipment was used in the field and the

samples had to remain viable for virus isolation at room temperature. The isolation rate of 13 from 69 samples (from Thursday Island) suggests that this method can be used for dengue virus isolation from clinical samples but no comparison with previously reported isolation methods was carried out. Subsequently, other workers isolated strains of Japanese encephalitis virus (a related flavivirus) from clinical specimens collected in northern Thailand by weekly transport of culture flasks of *Ae pseudoscutellaris* mosquito cells by road to and from Bangkok [14].

Reliance on serology alone could have led to the conclusion that the same virus was involved in concurrent epidemics of dengue in Malaysia and Australia. The one-dimensional mapping of the dengue virus genomes from four Australian dengue-1 isolates from the 1981–82 epidemic has shown that these viruses were identical with respect to the enzymes used to analyse them. This is in contrast to a series of dengue-2 isolates from the 1980 epidemic in Bangkok where restriction enzyme differences ranging from 4% to 39% were detected among three of the viruses, and oligonucleotide fingerprint differences of 9% to 42% among 10 of the dengue-2 viruses were found [6]. The one-dimensional maps of the Malaysian and Australian viruses are very different, thereby suggesting that the dengue-1 viruses in each country belong to different topotypes. Methods such as one-dimensional restriction enzyme mapping or two-dimensional fingerprinting of viral genomes [6, 11, 15, 16] or signature analysis using a bank of monoclonal antibodies [17] are therefore useful in tracing possible patterns of circulation of dengue viruses.

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Authors' address: Dr. B. H. Kay, Queensland Institute of Medical Research, Bramston Terrace, Herston, Brisbane, Queensland 4006, Australia.

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