Virchows Arch (2001) 438:107–115
DOI 10.1007/s004280000329

ORIGINAL ARTICLE

**Michel René Huerre · Nguyen Trong Lan
P. Marianneau · Nguyen Bac Hue · Huot Khun
Nguyen Thanh Hung · Nguyen Thi Khen
M.T. Drouet · Vu Thi Que Huong · Do Quang Ha
Y. Buisson · V. Deubel**

Liver histopathology and biological correlates in five cases of fatal dengue fever in Vietnamese children

Received: 24 March 2000 / Accepted: 14 September 2000 / Published online: 21 November 2000

Abstract We studied five fatal cases of dengue haemorrhagic fever (DHF), confirmed using the reverse transcriptase-polymerase chain reaction (RT-PCR) method, in Vietnamese children. The liver seems to be a target for dengue virus, so postmortem examinations were performed to investigate elementary lesions, local recruitment of inflammatory cells and whether the virus was present in target cells of the liver. We detected severe, diffuse hepatitis with midzonal necrosis and steatosis in two patients, focal areas of necrosis in two patients, and normal histology in one patient. Dengue virus antigen was detected using immunohistochemistry in hepatocytes from necrotic areas in four cases. There was no recruitment of polymorphonuclear cells, and no lymphocytes were detected in the liver lesions of patients who died from DHF. Lymphocytic infiltration occurred in only one hepatitis B virus-positive patient, with no signs of chronic hepatitis. Kupffer cells had mostly been destroyed in cases with focal or severe necrosis. TUNEL tests were positive in necrotic areas, with positive cells forming clusters, suggesting that an apoptotic mechanism was involved. Thus, we suggest that the hepatocyte and Kupffer cells may be target cells supporting virus replication and that the councilman body is an apoptotic cell, as in the pathogenesis of yellow fever.

Keywords Dengue virus · Hepatitis · Necrosis · Apoptosis.

M.R. Huerre (✉) · H. Khun
Unité d'Histopathologie, Institut Pasteur, 25 Rue du Dr Roux, 75724, Paris XV, France
e-mail: mhuerre@pasteur.fr
Tel.: +33 01 45688661, Fax: +33 01 40613155

N. Trong Lan · N.Bac Hue · N. Thanh Hung · N. Thi Khen
V.T. Que Huong
Hopital Pédiatrique No. 1, Ho Chi Minh City, Vietnam

P. Marianneau · M.T. Drouet · V. Deubel
Unité des Arbovirus et virus des Fièvres hémorragiques, Institut Pasteur, Paris

D. Quang Ha
Institut Pasteur, Ho Chi Minh City, Vietnam

Y. Buisson
Hopital du Val de Grace, Bd de Port Royal, Paris V

Introduction

Dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) are hyperendemic in Southeast Asia, endemic in Oceania and have extended to the tropical areas of the Americas since the early 1980s, causing tens of thousands of deaths in childhood annually [12]. The causative agents are four serotypes of mosquito-borne virus, dengue 1–4, of the genus flavivirus, family Flaviviridae. DF may involve high fever, headache, retro-orbital pain, myalgia and arthralgia, often accompanied by petechial rash. These symptoms resolve after 3–7 days. In some cases, the acute febrile phase may be followed by haemorrhagic manifestations, including petechiae, ecchymoses, epistaxis, gastro-intestinal bleeding and haematuria. Some individuals, mostly children under the age of 15 years, may suffer from a capillary leak syndrome, which may progress to hypovolemic shock with undetectable pulse and blood pressure, requiring transfer to an intensive care unit.

Dengue disease has a spectrum of clinical signs and symptoms, ranging from unapparent infection to severe and lethal manifestations. The World Health Organization (WHO) classification [1] distinguishes DHF from DF with haemorrhage and classifies it into four grades, depending on severity. Fatal cases of dengue are classed as grade IV, including shock. The pathobiology of DF, DHF and DSS is unclear. The development of primary DF in a small proportion of cases, into DHF with or without DSS, has been attributed to "facilitating antibodies" elicited in a former infection with a different virus serotype [14]. This concept, now revived, is interpreted as a cascade of

pathological events. The lesions have not been completely investigated and, because pathologists cannot obtain tissues from living patients with thrombocytopaenia and coagulative disorders, most of our knowledge is limited to what has been observed in fatal cases.

The virus is mostly isolated from the spleen and liver of fatal cases [3, 16,27]. Liver function tests show high levels of alanine aminotransferase (ALT) activity, indicating hepatic injury [8,23]. During the autopsy, the most frequent gross anatomical findings are haemorrhages (gastrointestinal tract and serous cavities), effusions and oedema [6, 11, 19,26]. Histologically, the most common features in the liver are small foci of necrosis (65%) and microvesicular steatosis (75%) [4, 6,11]. The foci of hepatocyte necrosis may coalesce, and acidophilic bodies with pycnotic nuclei corresponding to councilman bodies may form [3, 4, 6,9]. Dengue virus has frequently been isolated from the liver, and antigens have been detected in Kupffer cells and hepatocytes [3, 9,28]. These observations suggest that the liver is a main target in DF, DHF/DSS and that the virus may replicate in these cells, causing hepatic injury. To check for the presence of dengue virus in hepatocytes, we analysed five cases of fatal dengue associated with haemorrhagic manifestations in Vietnamese children. We observed focal necrosis of hepatocytes in two patients, more extensive necrosis in two patients and almost normal histology in the fifth, with no more than moderate microvesicular steatosis. We tested whether the hepatocyte was a site of viral replication leading to necrosis and whether an apoptotic mechanism was involved. Our results have important implications for the understanding of fatal dengue infection and the pathogenesis of the disease.

Materials and methods

Clinical, biological and virological studies

Between October 1996 and September 1997, dengue fever grade III/IV was the final diagnosis, confirmed using virological methods, in 31 children who died after admission to the paediatric hospital in Ho Chi Minh City, Vietnam. Clinical and biological results for five of these children, for whom a liver biopsy was possible and family consent was given, are presented in this study. Material was collected during the autopsy with the informed consent of the relatives of the deceased under the Vietnamese and French regulations. Biological tests, such as determination of haematocrit, platelet count, aspartate/alanine amino transferase (AST), ALT and bilirubin assays, coagulation tests, fibrinogen assays and ionograms were performed using standard methods.

An enzyme-linked immunosorbent assay (ELISA) was used for the rapid classification of serological responses in dengue infections, based on the ratio of immunoglobulin (Ig)M and IgG [18]. Other standard serological tests for hepatitis A virus (HAV), B (HBV), C (HCV) and E (HEV) were also performed. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on blood or liver to detect dengue virus RNA as previously described [28].

Microscopy

All of the patients studied died from DHF infection. Specimens were therefore obtained from percutaneous liver biopsy immediately after death. Formalin-fixed, paraffin-embedded tissues were sectioned and stained with haematoxylin and eosin, Gordon-Sweet and Perls stains. In a study on yellow fever hepatitis, elementary lesions, such as necrosis, steatosis and eosinophilic degeneration were classified by Viera et al. [29] using a semi-quantitative scale: 0, no lesion; grade 1, slight or moderate damage; grade 2, marked damage and grade 3, severe damage. The following criteria were studied here: (1) lobular damage: swelling or necrosis of hepatocytes; councilman bodies; microvesicular and macrovesicular steatosis. Grade 1: a few cells; grade 2: 10–100 cells for ten microscopic fields at a magnification of ×400, grade 3 (necrosis): more than 100 cells aggregated in the foci of necrosis; (2) portal inflammatory infiltrate (monocyte, lymphocytes and polymorphonuclear cells). Grade 1: a few cells; grade 2: 10–50 cells for ten microscopic fields at a magnification of ×400; and (3) reticulin framework: collapse +, restricted to small foci of necrosis and ++, restricted to large foci of necrosis or present throughout the lobule.

The distribution of lobular lesions over the centrilobular, midzonal and periportal areas was analysed for each case. Immunohistochemical analysis was performed on poly-L-lysine coated slides with dengue and yellow fever anti-envelope E protein monoclonal antibodies (mAb) [5,13]. Briefly, the sections were immersed in 200 ml citrate buffer and heated three times in a microwave oven for 5 min at 650 W. They were then incubated with the five mAbs, each directed against a different dengue virus serotype (DEN-1 mAb b1F1, DEN-2 mAb 3H5, DEN-3 mAb 5D4, DEN-4 mAb 1H10*)* and yellow fever virus (mAb 5E3) [15]. All of these antibodies were obtained as hybridoma cell supernatant fluids and were used at a dilution of 1:75. The alkaline phosphatase method was used (LSAB2 universal alkaline phosphatase kit; Dako, Copenhagen, Denmark) to detect the secondary antibodies, using fast red as a chromogen. Slides were counterstained with Mayer's haematoxylin. Negative controls for each sample included incubation of the sections with non-immune ascitic fluids from mice rather than with antibodies. Negative controls also included sections of five livers taken from patients who died from non-infectious diseases.

A panel of commercial antibodies (Dako) was used to analyse and to quantify lobular and portal infiltration by T lymphocytes (UCHL-1, CD3, Dako), B lymphocytes (CD 20, L 26 Dako) and Kupffer cells (CD 68, Dako). The 5-µm deparaffinised sections were heated in a microwave oven (three times, 5 min at 650 W) and then were incubated with anti-T/B lymphocytes and anti-Kupffer cell antibodies using the streptavidin–peroxidase method and amino-ethyl carbazole (AEC) as a chromogen. A quantitative analysis was carried out by comparing the five livers from dengue patients with livers from five other patients who died in Paris from non-infectious diseases.

Apoptosis

We investigated the nature of cell death in the livers of infected patients by performing TUNEL assays on tissue sections using the in situ cell death detection kit (Boehringer Mannheim). Briefly, liver sections were incubated with proteinase K (20 mg/ml in 10 mM Tris HCl pH 7.4) for 15 min at 25°C. They were then permeabilised with 0.1% TritonX100 in 0.1% sodium citrate and incubated in the TUNEL reaction mixture for 60 min at 37°C. Sections were mounted and observed under a fluorescence microscope.

Results

Pathological findings

Clinical and biological findings

Table 1 shows the clinical manifestations and laboratory findings for five cases of DHF. All patients presented with severe forms on admission, with clinical features of

Table 1 Clinical manifestations and laboratory findings for liver biopsies from five fatal cases of dengue haemorrhagic fever with liver biopsy. *ND* not determined, *GI bleeding* gastrointestinal bleeding, *L/R* lactated/ringer, *AST/ALT* aspartate/alanine aminotransferase, *Ig* immunoglobulin, *RT-PCR* reverse transcriptase-ploymerase chain reaction, *HBV* hepatitis B virus, *HAV* hepatitis A virus, *HCV* hepatitis C virus, *HEV* hepatitis E virus

Patient	1 96/1709	2 97/1565	3 97/1622	4 97/1709	5 97/1710
Age (months)/ gender/weight (kg)	10/Male/8	48/Female/14	60/Female/16	10/Female/19	72/Male/17
Admission	16/10/96	10/07/97	2/09/97	30/09/97	30/09/97
Death	21/10/96	15/07/97	4/09/97	1/10/97	1/10/97
Grade	III	III	IV	III	II
Clinical manifestations					
Consciousness	Coma	Lethargic	Coma	Lethargic	Lethargic
Convulsion	–	+	–	–	+
Petechiae	–	+	–	+	–
GI bleeding	+	+	–	+	
Jaundice	+	–	–	–	+
Shock	+	+	+	–	+
Respiratory failure	+	+	+	+	+
Hematocrit	39	30	33	29	26
Platelet count	32,000	30,000	30,000	40,000	60,000
AST/ALT (*N*≤40 U/l)	18.500/3.450	1.050/106	1.563/23	9.9/650	108/3.072
Bilirubin (mg/dl)					
Total (*N*≤12.8)	4	0.84	0.64	1.47	1.87
Conjugated (*N*≤2.9)	3.6	0.44	0.35	0.55	0.90
Coagulation					
Quick time	ND	ND	19%	16.5%	≤13%
Cephaline K (s)			≥120	≥120	≥120
Fibrinogen 2<*N*<4 (g)	ND	ND	≤0.6	≤0.6	≤0.6
Ionogram (mmoles/l)					
Na+ (135–145)	117	ND	130	124	128
Ca++ (2.2–2.5)	0.93		1.07	1.03	0.95
K+ (3.5–50)	4.8		4.1	3.7	6.2
Cl (100–110	108		ND	ND	ND
IgM/IgG (Ig)	0.9/0.4	0.2/2.8	1.1/3	1/2.7	0.1/1.4
Interpretation	Primary	Secondary	Secondary	Secondary	Secondary
RT-PCR:					
Blood	+	+	+	ND	ND
Liver	+	ND	ND	+	+
HBV	–	–	–	–	+
HAV	–	–	–	–	–
HCV	–	–	–	–	–
HEV	–	–	–	–	–
Treatment	Infusion with L/R, gelatin, blood	Infusion with L/R, dextran 40, 70 blood	Infusion with L/R, dextran 70, blood	Infusion with L/R, blood	Treated as malaria

dengue grade III in four cases, grade IV in one case and gastro-intestinal bleeding in two cases. These patients had low platelet counts, under 60,000/mm^3. In each case, the disease was fatal, with shock occurring 1 day (two cases), 2 days (1 case), and 5 days (two cases) after admission. Four children showed clinical features of hepatitis; transaminase activity was high in each case. The AST/ALT ratio was greater than one in three cases and ALT levels were 10–100 times normal values in three cases (cases 1, 4 and 5). Bilirubin levels were normal except in case 1. Fibrinogen concentration was below 0.6 g/l in the three cases in which it was determined, with a pathological quick time and a cephalin kaolin time of over 120 s.

Other aetiologies of acute and severe infectious diseases, endemic in Vietnam, such as malaria and salmonellosis, were investigated. All tests were negative, although patient 5 was initially considered to be a case of cerebral malaria. Serological tests for dengue virus using IgM- and IgG-ELISA were positive in all cases (Table 1). The IgM/IgG ratio calculated for sera diluted 1 in 100 showed that patient 1 presented with a case of primary and the other patients secondary disease. One patient tested positive for HBV antigen in serological

Table 2 Liver histological changes in dengue fever. *PP* periportal, *MZ* midzonal, *CL* centrilobular, *M* monocytes, *Ly* lymphocytes, *IHC* immunohistochemistry for DEN-3 virus, – normal reticulin framework, –/+ subnormal or very moderate collapse, + limited destruction, ++ large foci of destruction

Patient	Lobular damage										Reticulin			Final diagnosis
	Swelling/necrosis of hepatocyte			Councilman bodies				Steatosis		Portal infiltration	Reticulin frame work collapse		IHC DEN virus	
	PP	MZ	CL	PP	MZ	CL	PP	MZ	CL		MZ	PP		
1	0/1	2	1	1	2	1	0/1	2	0	0/1, Ly	++	–	+	Diffuse
2	0	0	0	0	0	0	0	1	0	0	+	–	–	Moderate fatty
3	0	1	0	0	1	0	0	1	1	0	+	–	+	Focal
4	0/1	2	1	0/1	1	0/1	0	1	0	0	+/–	–	+	Diffuse
5	0	2	0/1	0	3	1	1	2	0	0/1, Ly	++	–	+	Focal

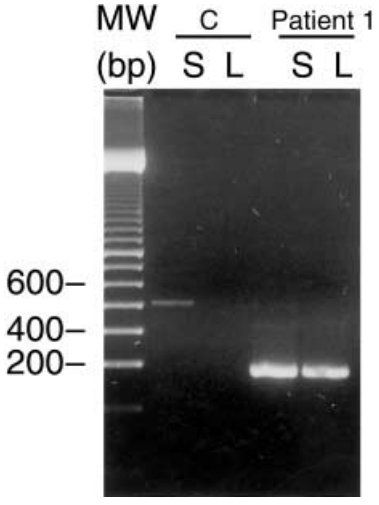


Fig. 1 Ethidium bromide-stained agarose gels showing the products of semi-nested polymerase chain reaction amplification of DEN-3 virus in the serum (*S*) and liver (*L*) of dengue negative control (*C*) and dengue-positive (patient 1) patients. *MW* Molecular weight, (*bp*) base pairs

tests but had no sign of active hepatitis. Tests for antibodies against HAV, HCV, HEV and HBV antigens were negative in all cases. Dengue 3 virus was detected in all patients by means of RT-PCR, whether blood or liver samples were tested (Fig. 1).

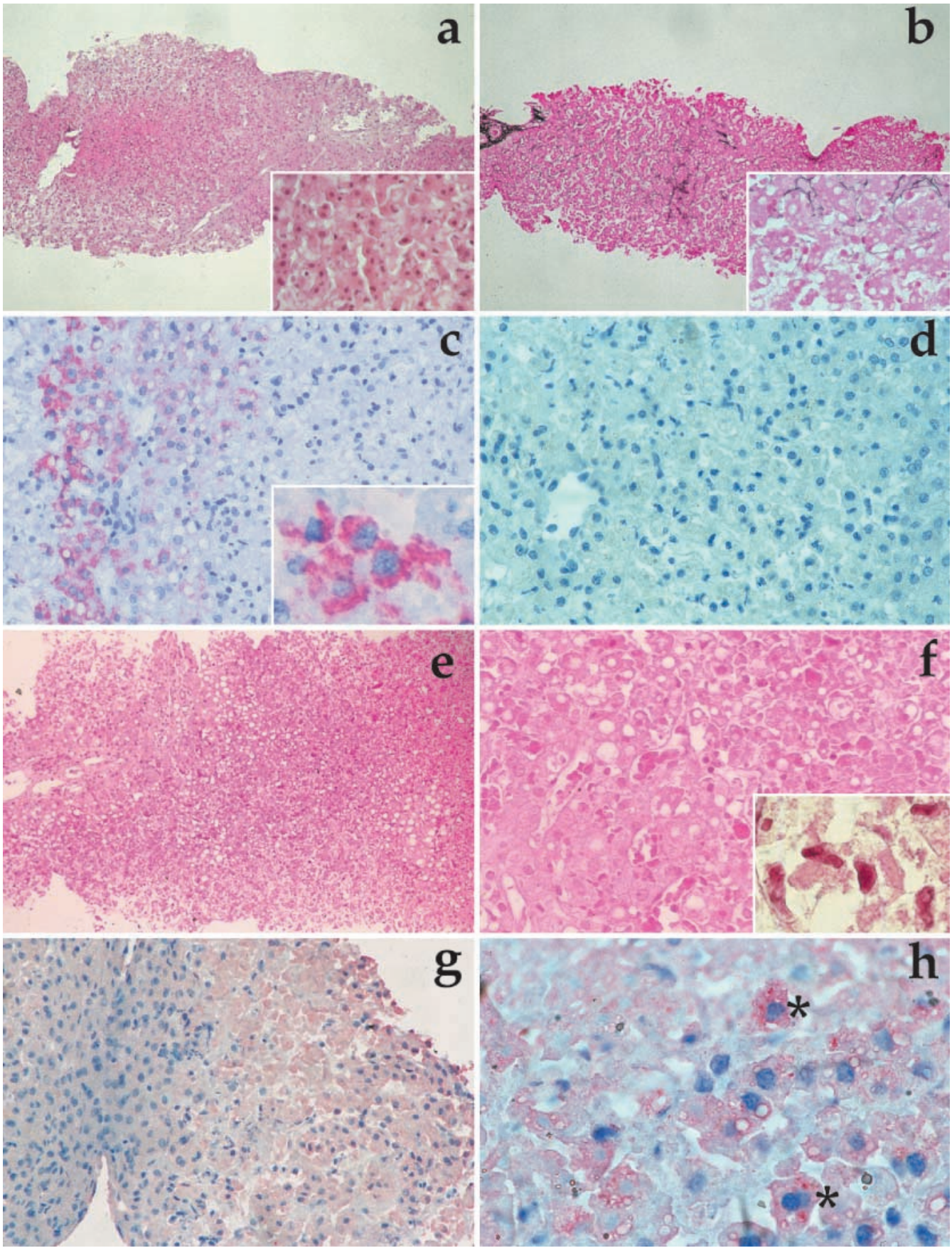
Histopathology

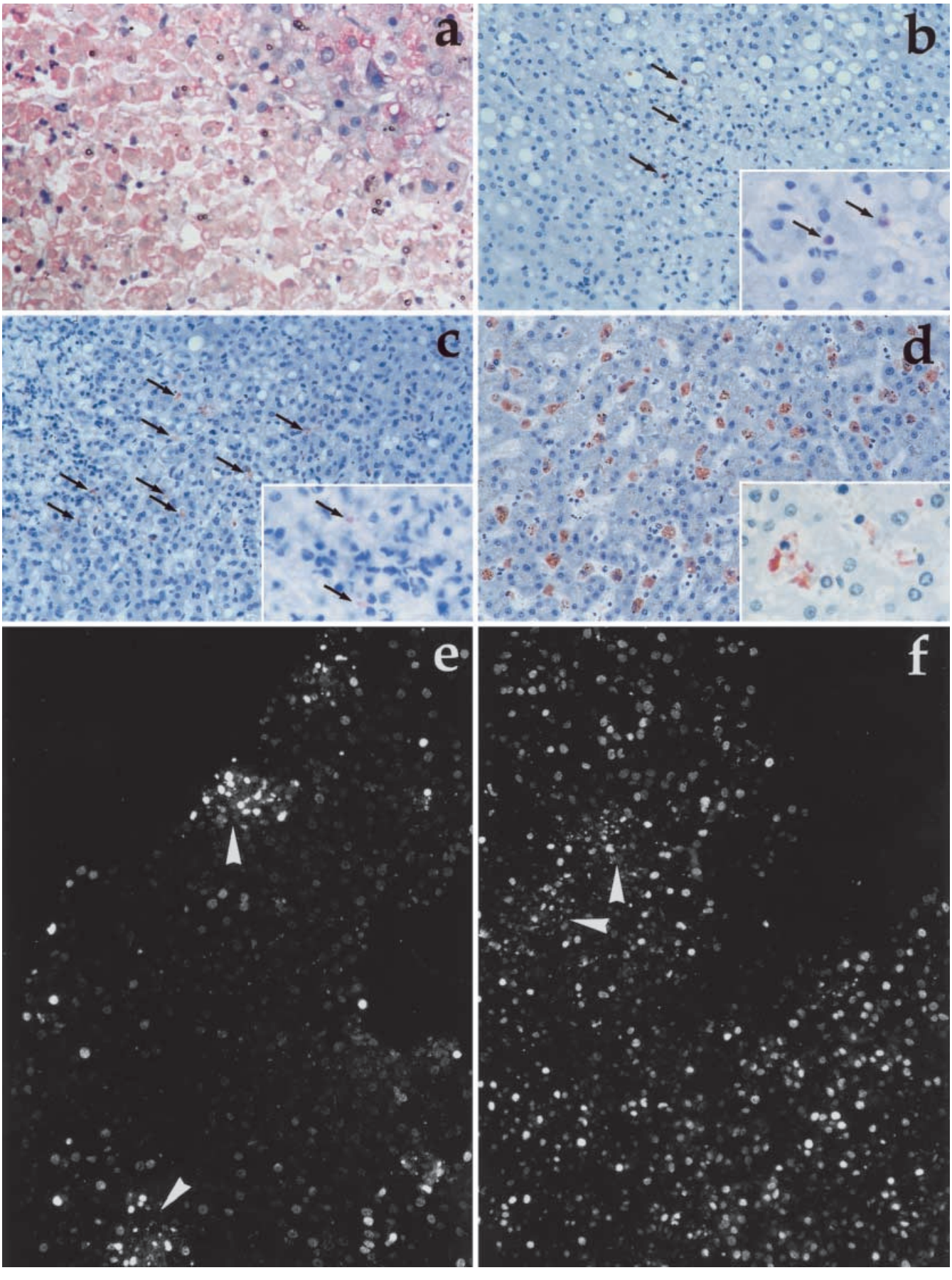
Table 2 shows the liver histological changes in DF. Lobular architecture was normal in one patient (patient 2) and very disturbed in four patients, with several foci of hepatocellular necrosis, mostly midzonal (patient 3 and patient 5; Fig. 2a–c) and sometimes present throughout the lobule (patient 1 and patient 4; Fig. 2e–h and Fig. 3a). In all cases, the periportal areas were intact, limited by a layer of hepatocytes and with no sign of necrosis (Fig. 2g and Fig. 3a). The reticulin network collapsed in the areas of necrosis or increased around the necrotic foci (Fig. 2b). Councilman bodies were clearly observed in four cases, generally midzonal in distribution, in and around the foci of necrosis (Fig. 2f). Microvesicular steatosis was a frequent feature (Fig. 2e, f and Fig. 3a–c), observed in four cases, extensive only in cases 1 and 5 and generally midzonal or centrilobular in distribution.

Immunohistochemical staining for DEN-3 virus was positive in the necrotic areas (Fig. 2c, g, h and Fig. 3a). The target cells were mostly hepatocytes (Fig. 2c) and especially councilman bodies (Fig. 2h and Fig. 3a). The staining was particularly strong in the cytoplasm around the nucleus, and hepatocytes in the areas surrounding the necrosis were also strongly stained (Fig. 2c and Fig. 3a). We were unable to identify the location of virus antigens in Kupffer cells, because these cells were necrotic. Dengue antigens were not significantly detected in endothelial cells. No liver cells were stained if dengue 1, 2 or 4 serotype-specific mAbs or yellow fever polyclonal antibodies were used.

There was little or no inflammatory response, with no polymorphonuclear cell, no B lymphocytes (data not shown) and few T lymphocytes: 4.85±1.55 for ten fields examined at ×400 (Fig. 3b). For the controls this was: 4.35±1.35,, $P<0.01$ (in using the non-parametric Mann Whitney U-test). In case 1, moderate lymphocyte infil-

Fig. 2 **a** Foci of necrosis in midzonal areas of the lobule. Haematoxylin and eosin staining (H&E; ×25; case 5). *Inset*: H&E; ×400. **b** Destruction of reticulum network in necrosis. Gordon-Sweet staining (×25). *Inset*: H&E; ×400. **c** Immunohistochemistry with DEN-3 antibody, alkaline phosphatase–antialkaline phosphatase (APAAP) and fast red. Detection of DEN virus antigen in and around areas of necrosis (×250). *Inset*: ×1000. **d** Immunohistochemistry with DEN-3 antibody, APAAP and fast red; negative control. Liver from a patient who died in the hospital in Paris with acute myocardial infarction. **e** Large areas of necrosis extending into the lobule with steatosis (H&E; ×65; case 1). **f** Numerous councilman bodies in midzonal necrosis combined with steatosis (H&E; ×250). *Inset*: H&E; ×1000. **g** Immunohistochemistry: foci of coagulative necrosis staining for DEN virus antigens alkaline phosphatase–antialkaline phosphatase (APAAP) and fast red (×100; case 3). **h** Immunohistochemistry: councilman bodies (*asterisk*) containing dengue virus antigens APAAP and fast red (×400; case 1) ▶


a
b
c
d
e
f
g
h
*
*


a
b
c
d
e
f

tration was observed, mainly in the periportal area (data not shown). A few rare Kupffer cells were observed in the liver of dengue patients, and they were mostly necrotic (Fig. 3c), whereas those of the controls were not (Fig. 3d).

Apoptosis

We have previously demonstrated that dengue virus replication triggers an apoptotic mechanism in human hepatoma Hep G2 cells [21] and in human Kupffer cells [22] but also in the liver cells of a human fatal case [9]. The five liver specimens were studied by means of TUNEL to assess whether TUNEL-positive nuclei were a specific feature of liver injury in dengue hepatitis. TUNEL-positive nuclei and small aggregates of fragmented nuclei, suggestive of apoptosis, were observed in hepatocytes located in the areas morphologically identified as foci with a hematoxylin staining. TUNEL-positive nuclei were observed in all sections studied, but differences were observed in their distribution. In patient 4, TUNEL-positive cells were distributed throughout the entire section (Fig. 3f), whereas positive cells were primarily restricted to foci in patient 3 (Fig. 3e) and in patients 1, 2 and 5 (data not shown). These results are consistent with those for overall histological examination, resulting in a classification of subfulminant hepatitis or mild hepatitis with well delimited paracentral necrotic foci.

Discussion

Several viruses that cause haemorrhagic diseases may also cause hepatic injury [7,31], and dengue virus is not generally considered to be an aetiological agent of hepatitis. Dengue virus antigens and RNA have been detected in many organs in DHF/DSS patients, including the spleen (cells lining the Billroth cords), lymphoid tissues, bone marrow, thymus, lung (alveolar macrophages), skin and liver. Only a few cases of hepatitis have been reported [4, 6, 7, 19,26] in dengue fever, although it has been demonstrated in recent years that the liver is an important site of dengue replication and the source of some of the patho-physiologic aberrations [8, 23, 27,28]. In addition, the combination of neurological signs and liver dysfunction in patients with DHF/DSS is considered by many investigators to indicate a poor prognosis.

S. Nimmannitya et al. [24] reported 18 cases of DHF with jaundice and encephalopathy. Ten had a fatal outcome. In Malaysia, eight cases of liver failure were reported [20] in 20 cases of grade III/IV DHF, generally associated with severe deterioration of mental status requiring cerebral protection. There was a fatal outcome in only one case, and most of the survivors (18 of 20) completely recovered hepatic and neurological function. Liver failure in DHF, as indicated by high levels of ALT activity, occurred in 82% of cases [16]. It has been suggested that the long time required for recovery in patients with severe circulatory failure in intensive care units may be due to the unusual liver tropism of some strains, but our patients died in 1–5 days.

The course of dengue fever does not seem to be influenced by concomitant hepatitis virus infection, although one case of HBV was observed in our series. It is well known that HBV is hyperendemic in Vietnam, South America and Oceania, but there is no evidence that HBV infection acts as a co-factor in dengue infections. Livers from patients who died of dengue fever with haemorrhage [14, 16,28] and of those who died of yellow fever [10,31] are similar in appearance under the microscope, and histopathological diagnosis relies on geographical and epidemiological data, clinical features and virological studies.

In dengue infections, three basic types of lesion are observed: microvesicular steatosis, hepatocellular necrosis and councilman bodies. Steatosis frequently occurs in hepatitis of viral origin, and no specific significance can be attributed to this process in DF. Hepatocellular necrosis in dengue fever generally occurs in midzonal and sometimes centrolobular areas, whereas in yellow fever, necrosis is more severe and midzonal in distribution. In severe cases of dengue, hepatocellular necrosis may extend beyond the midzone into the lobule, causing the complete destruction of the lobule although a layer of intact hepatocytes persists around the portal tracts. There are many analogies between yellow fever and DHF, so it has been suggested that the pathogenesis of the two diseases may be similar [27]. Councilman bodies are thought to be important in yellow fever but have also been observed in viral hepatitis and in haemorrhagic fevers (HF), such as Lassa fever, Argentine HF, Bolivian HF, Venezuelan HF and Rift Valley fever [7,31].

Councilman-like bodies have also been described as apoptotic cells [17], and our results show that apoptotic cells were observed in and around the areas of necrosis. We have previously demonstrated that dengue virus and yellow fever infection of hepatocytes induces cell apoptosis in vitro [21]. In a previous study, we detected apoptotic nuclei aggregated in clusters in the liver of an adult who died from a dengue 2 virus infection; this patient was not from Vietnam but was instead from a Caribbean island [9]. The presence of apoptotic cells in the livers of DHF/DSS patients suggests that this feature also occurs in vivo. However, we cannot determine

◀ **Fig. 3** **a** Immunohistochemistry with DEN-3 virus antibody, alkaline phosphatase–antialkaline phosphatase (APAAP) and fast red. Large area of necrosis extending to the portal area (×250; case 4). **b** A few T lymphocytes (CD3; *arrow*) detected by means of immunostaining, streptavidin peroxidase method (×250). *Inset*: ×400 **c** A few Kupffer cells, mostly necrotic, (*arrow*) from case 4; streptavidin peroxidase method (×250). *Inset*: ×400 **d** Kupffer cells from control; streptavidin peroxidase method (×250). *Inset* ×400. **e** Identification of apoptotic cells in liver necropsies from DHF/DSS children. Slides of liver samples from case 3; foci of fluorescent apoptotic cells detected using TUNEL as pycnotic nuclei (*arrowheads*). **f** Apoptosis in case 4; diffuse distribution of apoptotic cells

whether this apoptotic mechanism precedes or is associated simultaneously with necrosis, because the two mechanisms are probably combined and may account for the lesions observed. Indeed, hepatocyte lesions occur late in the natural history of DF.

Another question concerns the infiltrates observed in the livers of patients infected with dengue virus. Kupffer cells were destroyed in patients, and this finding is not consistent with autolysis because liver samples were taken a few minutes after the patient's death. The liver biopsies used as controls were performed 24, 48 and 72 h after death. However, we cannot exclude the possibility that long periods of fixation in formalin (usually over 2 weeks) may have caused a loss of epitopes from Kupffer CD-68 positive cells despite the technical procedure used, involving a microwave oven.

The lack of antigenic reactivity of most of the Kupffer cells in the liver section is consistent with the simultaneous lysis of these cells during the course of infection. We have observed that the infection of Kupffer cells with dengue virus in vitro is an abortive process, leading to apoptosis [22]. However, we could not demonstrate in this study that the shrunken Kupffer cells and fragmented nuclei observed in these fatal cases corresponded to apoptotic cells.

Infiltrates [lymphocytes, polymorphonuclear neutrophils (PMNs)] were absent or mild in the livers of these patients, and this finding is more frequent in arbovirus-induced liver injury, especially due to yellow fever and DF, than in liver damage caused by other viruses, such as the hepatitis B and hepatitis C viruses. The presence of dengue envelope proteins in hepatocytes mostly around necrotic foci when viraemia is no longer detected [28] suggests that dengue virus replication may occur late in these cells. However, attempts to detect dengue RNA using hybridisation in situ in liver biopsy tissue from DSS cases has not been successful in our experience and in the previous studies [3,9]. Dengue virus replication in hepatocytes may be a slow process, or it may induce the rapid destruction of these host cells, as observed in experimentally infected human hepatoma cells [21]. Thus, hepatocytes in infected humans and in vitro may be considered to be major targets of dengue virus, at least in the terminal stages.

Vascular injury (bleeding and plasma leakage) may result from direct endothelial cell injury by dengue virus or immunologically mediated injury, probably combined with the local production of pro-inflammatory cytokines. Viral antigens may be detected in vessels as has been shown in vitro in endothelial cells, the infection of which leads to chemokine production and apoptosis [2]. However, in our study, we observed no specific immunostaining of endothelial cells, showing that endothelial cells are permissive for the virus in vivo.

The prognosis does not seem to be correlated with the grade of histological lesion, and death may occur with only minor hepatic injury (patient 2). However, our results are consistent with hepatic injury, being common in cases of DHF/DSS. However, the replication of DEN in other cells, both within the liver, especially in Kuppfer cells, and in other organs, particularly at early stages of the disease, may account for clinical and biological disturbances not being correlated with the restricted damage to the liver. Recent results have demonstrated that dendritic cells, especially monocyte-derived dendritic cells (DC) and human skin Langerhans cells are targets for dengue virus infection at the early stages [25,30]. However, as immature DC cells are more permissive than macrophages, this result suggests that the differentiation of DC-derived cells plays a critical role in the intracellular cycle of the virus.

All patients studied were infected with DEN-3 virus. We do not know whether there are genetic and phenotypic differences between strains, but differences in the clinical patterns of hepatic injury suggest that host factors determine the outcome of the disease, at least in part [2]. The patient with the most severe hepatic injury in these five cases was the patient with the primary infection. Further histopathological studies are required in primary and secondary dengue-infected patients to assess the role of a primed minimal defence in limiting liver injury.

Acknowledgements The authors would like to thank Dr. Nick Karabatsos for providing the dengue antibodies, Dr. J.J. Sclesinger for providing the yellow fever mAb, Pr Callard (Tenon hospital, Paris) for the autopsy liver biopsies used as controls, Ms. Sabine Maurin for technical assistance and Ms. Julie Sappa for correcting the English of the manuscript.

References

1. Anonymous WHO (1986) Dengue haemorrhagic fever, diagnosis, treatment and control. WHO
2. Avirutnam P, Malasit P, Seligar B, Bahkdi S, Hussman M (1998) Dengue virus infection of human endothelial cells leads to chemokine production, complement activation and apoptosis. Immunol 161:6338–6346
3. Bahmarapravati N (1997) Pathology of dengue infections. In: Gubler DJ, Kuno G (eds) Dengue and dengue hemorrhagic fever. Cab International. Oxon, UK pp 115–132
4. Bhamarapravati N, Tuchinda P, Boonyapaknavik V (1967) Pathology of Thailand haemorrhagic fever: a study of 100 autopsy cases. Ann Trop Med Parasitol 61:500–510
5. Bhoopat L, Bhamarapravati N, Attasiri C, Yoksarn S, Chaiwun B, Khunamornpong S, Sirisanthana V (1996) Immunohistochemical characterization of a new monoclonal antibody reactive with dengue virus infected cells in frozen tissue using immunoperoxidase technique. Asian Pac J Aller Immunol 14:107–113
6. Burke T (1968) Dengue hemorrhagic fever, a pathological study. Trans R Soc Trop Med Hyg 62:682–691
7. Burt FJ, Swanepoel R, Shieh WJ, Sniith JF, Leman PA, Greer PW, Coffeld LM, Rollin PE, Ksiazek TQ, Peters CJ, Zaki SR (1997) Immunohistochemical and in situ localization of Crimean-Congo hemorrhagic fever (CCHF) virus in human tissues and implications for CCHF pathogenesis. Arch Pathol Lab Med 121:839–846
8. Chung HK, Dar IT, Chi SCC, Chi KL, Shue SC, Yun FL (1992) Liver biochemical test and dengue fever. Am J Trop Med Hyg 47:265–270
9. Couvelard A, Marianneau P, Bedel C, Drouet MT, Vachon F, Henin D, Deubel V (1999) Report of a fatal case of dengue infection with hepatitis. Demonstration of dengue antigens in hepatocytes and liver apoptosis. Hum Pathol 30:1106–1110

10. Deubel V, Huerre M, Cathomas G, Drouet MT, Wuscher N, Le Guenno B, Widmer A (1997) Molecular detection and characterization of yellow fever virus in blood and liver specimens of a non vaccinated fatal human case. Med Virol 53:212–217
11. Fresh JW, Reyes W, Clarke EJ, Uylangeo CV (1969) Philippine hemorrhagic fever: a clinical, laboratory and necropsy study. Lab Clin Med 73:451–458
12. Gubler DJ (1998) Dengue and dengue haemorrhagic fever. Clin Microbiol Rev 11: 480–496
13. Hall WC, Crowell TP, Watt DM, Baoors VLR, Kruger H, Pinheiro F, Peters CJ (1991) Demonstration of yellow fever and dengue antigens in formalin-fixed paraffin-embedded human liver by immunohistochemical analysis. Am J Trop Med Hyg 45:408–417
14. Halstead SB (1989) Antibody, macrophages, dengue virus isolation, shock and haemorrhages: a pathogenic cascade. Rev Infect Dis 11 [Suppl]:830–839
15. Henchal EA, Gentry MK, McCown JM, Brandt WE (1982) Dengue virus specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. Am J Trop Med Hyg 31:830–836
16. Innis B (1995) Dengue and dengue hemorrhagic fever. In: Porterfield JS (ed) Kass handbook of infectious diseases; exotic viral infections. Chapman Hall, London pp 103–146
17. Kerr JF, Cooksley WG, Searle J, Halliday JW, Halliday WJ, Holder L, Roberts I, Burnett W, Powell LW (1979) The nature of piecemeal necrosis in chronic active hepatitis. Lancet 2:827–828
18. Kuno G, Gomez I, Gubler DJ (1991) An ELISA procedure for the diagnosis of dengue infections. Virol Methods 33:101–113
19. Lukes RJ (1954) The pathology of thirty-nine fatal cases of epidemic hemorragic fever. Am J Med 16:639–650
20. Lum LCS, Lam SK, George S, Devi S (1993) Fulminant hepatitis in dengue infection. Southeast Asian J Trop Med Public Health 24:467–470
21. Marianneau P, Steffan AM, Royer C, Drouet MT, Kirn A, Deubel V (1998) Differing infection patterns of dengue fever and yellow fever viruses in a human hepatoma cell line. Infect Dis 178:1270–1278
22. Marianneau P, Steffon AM, Roger C, Drouet MT, Jaeck D, Kirn A, Deubel V (1999) Infection of primary cultures of human Kuppfer cells by dengue virus: no viral progeny synthesis but cytokine production is evident. J Virol 73:5201–5206
23. Nguyen TL, Nguyen TH, Tieu NT (1997) The impact of dengue haemorrhagic fever on liver function Res. Virol 148:273–277
24. Nimmannitya S, Thisyakorn U, Hemsrichart V (1987) Dengue haemorrhagic fever with unusual manifestations. Southeast Asian J Trop Med Public Health 18:398–406
25. Palucka AK (2000) Dengue virus and dendritic cells. Nat Med 6:748–749
26. Piyaratn P (1961) Pathology of Thailand epidemic hemorrhagic fever. Am J Trop Med Hyg 10:767–772
27. Rosen L, Khin MM, Tin U (1989) Recovery of virus from the liver of children with fatal dengue. Reflections on the pathogenesis of the disease and its possible analogy with that of yellow fever. Res Virol 140:351–360
28. Rosen L, Drouet MT, Deubel V (1999) Detection of dengue virus RNA by reverse transcription-polymerase chain reaction in the liver and lymphoid organs but not in the brain in fatal human infection. Am J Trop Med Hyg 61:720–724
29. Viera WT, Hayotto LC, de Lima CT, de Brito T (1983) Histopathology of the human liver in yellow fever with special emphasis on the diagnostic role of the councilman body. Histopathology 7:195–208
30. Wu SJL, Grouard-Vogel G, Sun W, Mascola JR, Brachtel E, Putvatana R, Louder MK, Filgueira L, Marovitch MA, Wong HK, Blauvelt A, Murphy GS, Robb ML, Innis B, Birx DL, Hayes CG, Frankel SS (2000) Human skin Langerhans cells are targets of dengue virus infection. Nat Med 6:816–820
31. Zaki S, Peters CJ (1997) Viral hemorrhagic fevers. In: Connor DH (ed) Pathology of infectious diseases, vol 1. Appleton and Lange, Stamford, pp 347–364