Immunohistochemical examination of the role of Fas ligand and lymphocytes in the pathogenesis of human liver yellow fever

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Abstract

Yellow fever is an infectious, non-contagious disease caused by an RNA virus of the family \textit{Flaviviridae}, which is transmitted to man by the bite of hematophagous mosquitoes. Infection with the yellow fever virus can progress with lesions in the heart, kidneys, central nervous system, and liver. In the liver, the histopathological picture is characterized by necrosis, steatosis and hepatocyte apoptosis, with a preferential midzone distribution. In the present study, liver samples from fatal patients with yellow fever were analyzed. The histopathological pattern was characterized by steatosis, lytic necrosis and hepatocyte apoptosis associated with a moderate mononuclear inflammatory infiltrate. The inflammatory component mainly consisted of CD4\textsuperscript{+} T lymphocytes, followed by CD8\textsuperscript{+} T lymphocytes, which showed a preferential portal and midzone distribution. Immunoreactivity to Fas ligand was mainly observed in hepatocytes of the midzone region. Based on these findings, we conclude that lymphocytes play an important role in the genesis of hepatic lesions in severe yellow fever, inducing hepatocyte apoptosis through the binding to Fas receptors. However, further studies are necessary to investigate the participation of other immune factors and to quantify the role of the cytotoxic cellular response in the lesion evolution during the course of disease in the liver.

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1. Introduction

Yellow fever is an acute infection disease whose symptoms vary within a broad spectrum of manifestations ranging from mild or subclinical cases to severe forms with intense vasculopathy and characterized by a clinical triad of jaundice, hemorrhage and acute renal failure (Ishak et al., 1982; Monath and Barrett, 2003; Peters and Zaki, 2002). Yellow fever is caused by an arbovirus of the family \textit{Flaviviridae}, genus \textit{Flavivirus}, and its case-fatality rate has ranged from 20 to 50% (Vasconcelos et al., 2004). Epidemiologically, this disease can occur in two forms, an urban and a jungle form. The urban form is transmitted from a sick individual to a non-immunized person through the bite of infected \textit{Aedes aegypti}. In the jungle form in the South America, the virus is mainly transmitted accidentally to man by mosquitoes of the genus \textit{Haemagogus}, whose natural habitat are the forests and which become infected through contact with viremic animals, especially monkeys (Monath, 2001; Vasconcelos et al., 2001a,b).

Clinically, after an asymptomatic period the patient may present fever, headache, generalized muscle pain, photophobia, shivering and jaundice, and can progress to hemorrhagic manifestations and acute renal failure (Elton and Romero, 1955; Monath, 2001).

In hepatic tissue, the virus may induce lesions such as macro- and microvesicular steatosis, eosinophilic degeneration and hepatocyte necrosis, which are characterized by more intense in the midzone region and are associated with a portal and...
acinar mononuclear infiltrate of mild intensity (Klotz and Belt, 1930a,b; Kerr, 1973; Branquet, 1996). Quantitative analysis observation had shown that no substantial alterations in the reticular network were found in liver and the hepatic damage resulted mainly from massive apoptotic death of hepatocyte and lesser extent due to lytic necrosis. This histopathologic picture was associated by an inflammatory infiltrate consisted of mononuclear cells with intensity disproportionate to intense death of hepatocytes, probably due to the apoptotic component that predominates in these cases with no activation of inflammatory cascade (Vieira et al., 1983; Quaresma et al., 2005).

Little is known about the role of the virus-host interaction, the cellular immune response and its role in the genesis of the histopathological alterations observed in yellow fever liver as done for hepatitis B and C (Ganem and Prince, 2004; Gremion and Cerny, 2005). We believe that such study would provide a better understanding of the physiopathological aspects involved in the genesis of hepatic lesions and the interaction with the host immune response in an attempt to fill the gaps in the knowledge of the pathogenesis and clinical evolution of yellow fever.

2. Materials and methods

2.1. Diagnostic procedures and histological examination

Samples of liver from Department of Pathology of Evandro Chagas Institute (Belem, Brazil) were obtained by post-mortem biopsy specimen of 53 fatal patients from Brazil, with age between 03 and 74 years, 13.20% female and 86.79% male. The diagnosis was made by serology, viral isolation, and immunohistochemistry. Samples were fixed in 10% neutral-buffered formalin, followed by paraffin embedding, micron-thick sectioned and stained by hematoxylin and eosin method. Immunohistochemical method for the detection of specific YF antigens using polyclonal antibodies and light microscopy was carried out as described elsewhere (Hall et al., 1991). Sections were also evaluated qualitatively according to the histological characteristics previously described (Quaresma et al., 2005).

2.2. Immunologic markers

An immunohistochemical technique to characterize the phenotype of the inflammatory cells followed the protocol originally described by Hou et al. (1981). The immunologic staining techniques for the detection of apoptosis were carried out as per the manufacturer’s instructions, as previously described by Gold et al. (1984). The following antibodies were used: CD45R0, CD4, CD8, CD95 and anti-apoptosis (APOPTAG plus peroxidase kit, Chemicon®, USA).

For quantitative analysis of the phenotype of the cells, cellular expression of Fas ligand (FasL) and ApopTag positive cells were used in a grid-scale, with 10 × 10 subdivisions in an area of 0.0625 mm², to count fields under high magnification (×400) in all three areas of the hepatic lobule (I: peri-portal area; II: midzonal area, and III: central vein area).

2.3. Negative controls and statistical analysis

For negative and positive controls we included 10 liver samples from patients with negative serology for the main hepatotropic viruses (viruses of A, B, C and D hepatitis) and which showed no morphological alterations in the liver architecture, and 10 liver specimens from cases diagnosed as leptospirosis by clinical presentation, specific serology, histopathology and immunohistochemical analysis. Statistical analysis was made using analysis of variance (ANOVA one-way) followed by the Bonferroni test. The level of significance for these analyses was established when \( p \leq 0.05 \). The analysis was performed using the GraphPad Prism 3.0 software for Windows (GraphPad Software, San Diego, CA).

3. Results

The pattern of histopathological alterations was more intense in the midzone region and was mainly characterized by hepatocytic lesions such as macro- and microvesicular steatosis, focal lytic necrosis and frequent eosinophilic bodies corresponding to apoptotic hepatocytes. A lymphomononuclear inflammatory infiltrate was observed which was minimal or moderate, and
Table 1

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<th>YF</th>
<th>LE</th>
<th>NC</th>
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<tr>
<td>Apoptosis</td>
<td>2.51 ± 0.64</td>
<td>0.50 ± 0.45</td>
<td>0.02 ± 0.00</td>
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<td>CD45RO</td>
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<td>FasL</td>
<td>2.29 ± 1.09</td>
<td>0.68 ± 0.07</td>
<td>0.06 ± 0.05</td>
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4. Discussion

The study of the local immune response is of great importance for the understanding and determination of yellow fever evolution, especially in the liver, the target organ of disease (Maini et al., 2000; Ryo et al., 2000; Chisari and Ferrari, 1995).

Monath (2001) mentioned the occurrence of apoptosis during severe yellow fever. Moreover, in an experimental study in hamsters it was demonstrated by Xiao et al. (2001) that yellow fever virus induced apoptosis in the infected animals. The histopathological analysis of the present tissue specimens, as described in the classical studies of Councilman (1890) and Vieira et al. (1983), had demonstrated which Councilman/apoptotic bodies were one of the marked findings in our sample. Indeed, the apoptotic cells showed a clear predominance in the midzone area and were widely predominating over hepatocyte lytic necrosis.

The relationship between apoptosis and infectious agents has been the subject of numerous studies (Thompson, 1995; Harwick, 1998). In some cases, probably it would represent a type of immune escape since the viral particles persist inside blebs lined with an intact membrane. However, it should be emphasized that the molecular mechanisms of virus-cell interaction involved in the induction of cell death are extremely complex and varies according to the specific molecular interactions between the etiological agent and host cell (Roulston et al., 1999).
Many factors are involved in the induction of programmed cell death during the course of C hepatitis, a Flaviviridae agent, with emphasis on the role of cytotoxic T lymphocytes through their FasL, TNF-α, and IFN-γ, as well as a direct viral cytopathic effect. The direct viral cytopathic effect and its potential in inducing apoptosis have been described for other mosquito-borne flaviviruses such as dengue, yellow fever, and West Nile (Marinneau et al., 1998; Monath, 2001). In the yellow fever liver,
contrary of is observed in the B and C hepatitis, the presence of an inflammatory infiltrate consisting mainly of helper and cytotoxic T lymphocytes in a preferential midzone distribution, in addition to the characteristic immunolabeling for FasL in zone 2 hepatocytes, is an indication of the role of T lymphocytes in the genesis of yellow fever lesions, since previous studies had shown preferential distribution of yellow fever antigens in midzonal region (Monath et al., 1989; De La Monte et al., 1983; De Brito et al., 1992; Deubel et al., 1997). Probably, viral interaction with MHC-related molecules leads to the activation of cytolytic CD8+ T lymphocytes that bind to T lymphocyte receptors or through FasL, inducing apoptosis through the interaction with specific death domains (FADD or TRADD) or through other mechanisms involving the release of granzymes/perforins. These mechanisms have been characterized as being involved in the pathogenesis of other chronic, acute, and fulminant forms of hepatitis (Galle et al., 1995; Ryo et al., 2000). In those studies, the authors called attention to the role of cytotoxic CD8+ T lymphocytes as one of the mechanisms underlying the genesis of hepatic parenchymal lesions in patients with B and C hepatitis (Bertoletti and Maini, 2000). Ryo et al. (2000), studying patients with signs and symptoms of acute hepatitis of viral etiology, detected an increase of FasL immunoreexpression in the hepatic tissue of the patients, which was accompanied by an increase in the expression of specific ligands on tissue and circulating lymphocytes associated with an intense apoptotic component. In addition, experimental studies using murine models of fulminant liver failure, have demonstrated that the administration of anti-Fas antibodies induces intense hepatocyte destruction (Ogasawara et al., 1993). In the present study, despite the observation of an increase in FasL expression compared to normal controls, the massive apoptosis detected during this process was not accompanied by the intense lymphocytic infiltration observed by other in acute and fulminant viral hepatitis (Makinen, 2004).

The causes of this discrete inflammatory component accompanied by an intense degree of apoptosis which has been quantified and interpreted as the main causal factor of hepatocyte death, remain unknown but it is known that apoptosis does not induce an important inflammatory response, and the apoptotic bodies are phagocytosed by neighboring macrophages, and thus do not activate large regional inflammatory response (Kerr et al., 1972; Majno and Joris, 1995; Kaufmann and Hengartner, 2001).

Fig. 5. Yellow fever liver: (A) immunohistochemistry of CD8 lymphocytes in the portal tract (200×); (B) quantitative analysis of distribution of CD8 positive cells showing predominance in the midzonal region. (*p ≤ 0.05, ANOVA/Bonferroni).
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