Hepatocyte lesions and cellular immune response in yellow fever infection

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Summary The study of the in-situ cellular immune response is very important for the understanding of different liver infections. In the present study, 53 liver samples obtained by viscerotomy from patients who died during the course of jungle yellow fever were analyzed. The diagnosis was confirmed by serology, viral isolation and virus-specific immunohistochemistry. The specimens were analyzed by immunohistochemistry using specific antibodies for apoptosis, CD45RO, CD4, CD8, CD20, S100, CD57 and CD68. Quantitative analysis of the labeling pattern showed a clear predominance of the different phenotypes in the portal tract and mid zone region of the acini. There was a predominance of T CD4+ lymphocytes, accompanied by the presence of T CD8+ lymphocytes, natural killer cells (CD57), macrophages and antigen-presenting cells (S100). The disproportion between the intensity of inflammation and the degree of hepatic injury was probably due to the intense apoptotic component, which classically does not induce an inflammatory response. The present study demonstrates that, despite the disproportion between injury and inflammation, the cellular immune response plays an important role in the pathogenesis of the hepatocytic injury observed in yellow fever, probably as a result of cytolytic actions through mechanisms involving MHC II and the activation of Fas receptors and granzymes/perforins.

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1. Introduction

Yellow fever (YF) is caused by an arbovirus of the genus \textit{Flavivirus}, family Flaviviridae (Westaway et al., 1985). There are two different epidemiologic forms of YF, which differ in...
their transmission cycle. In urban YF, the virus is transmitted directly to humans by the mosquito *Aedes aegypti* in urban areas, while jungle YF is transmitted by various mosquitoes of the genus *Aedes* in Africa, and *Haemagogus* and *Sabethes* in the Americas. In jungle cycles, non-human primates are the primary vertebrate hosts on both continents and, as transmission is sylvatic, human infections are incidental when humans enter the forest while working, such as when opening roads and exploring the area, etc. (Dégalier et al., 1992). Urban transmission has not been reported in the Americas since 1959. The present level of *A. aegypti* infection in large urban centers near YF endemic areas, the frequent migration of populations between endemic and non-endemic areas, and a low YF vaccine coverage in many countries mean that re-urbanization of the disease in the Americas could happen (Vasconcelos, 2003; Vasconcelos et al., 2004). Consequently, YF once again represents a serious public health problem in the New World. The danger is particularly great in Brazil, the most populated country in the endemic area, and increasing figures of YF activity have been recorded over the last 5 years (Vasconcelos et al., 2001, 2004).

Since the classical studies of Councilman, the histopathologic presentation of the liver in YF has been characterized by the presence of hepatocytic lesions represented by steatosis, necrosis and apoptosis (Councilman, 1890; Klotz and Belt, 1930a, 1930b; Quaresma et al., 2005; Rocha-Lima, 1912). This intense picture of tissue injury is characteristically accompanied by an inflammation that is dispropor-
tional to the degree of liver tissue involvement and by the absence of viral particles in electron microscopy material (Quaresma et al., 2005; Vieira et al., 1983). Despite this characteristic picture, no studies are available in the literature regarding the local immune response and its relationship with hepatic lesions in YF. The objective of the present study was to analyze qualitative and quantitative aspects of the cellular immune response and its relationship with the occurrence and distribution of hepatic lesions in YF.

### 2. Materials and methods

#### 2.1. Histologic examination and immunohistochemical detection of YF virus antigen

Liver samples obtained from the archives of the Department of Pathology, Evandro Chagas Institute (Belém, Brazil), were fixed in 10% neutral-buffered formalin, paraffin embedded, micron-thick sectioned and stained with hematoxylin and eosin. All 53 patients, 46 males and 7 females, were from Brazil (Table 1) and ranged in age from 3 to 74 years. The diagnosis was made by serology, viral isolation and immunohistochemistry for the detection of specific YF antigens using monoclonal antibodies (Hall et al., 1991). Five-micrometer-thick sections were also stained with Masson’s trichrome, reticulin and Perl’s stain for examination by light microscopy. The sections were evaluated semiquantitatively using a subjective scale ranging from 0 to 3, where 0 = none, 1 = mild, 2 = moderate and 3 = severe damage, according to the degree of inflammatory infiltration, hepatic apoptosis and steatosis, as previously described (Xiao et al., 2001).

<table>
<thead>
<tr>
<th>State</th>
<th>No. (%)</th>
<th>Male</th>
<th>Female</th>
<th>Clinical form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goias</td>
<td>15 (28)</td>
<td>13</td>
<td>2</td>
<td>Hemorrhagic</td>
</tr>
<tr>
<td>Para</td>
<td>8 (15)</td>
<td>8</td>
<td>0</td>
<td>Hemorrhagic</td>
</tr>
<tr>
<td>Maranhao</td>
<td>7 (13)</td>
<td>4</td>
<td>3</td>
<td>Hemorrhagic</td>
</tr>
<tr>
<td>Roraima</td>
<td>6 (11)</td>
<td>5</td>
<td>1</td>
<td>Hemorrhagic</td>
</tr>
<tr>
<td>Minas Gerais</td>
<td>5 (9)</td>
<td>5</td>
<td>0</td>
<td>Hemorrhagic</td>
</tr>
<tr>
<td>Tocantins</td>
<td>4 (8)</td>
<td>4</td>
<td>0</td>
<td>Hemorrhagic</td>
</tr>
<tr>
<td>Amazonas</td>
<td>3 (6)</td>
<td>3</td>
<td>0</td>
<td>Hemorrhagic</td>
</tr>
<tr>
<td>Mato Grosso do Sul</td>
<td>3 (6)</td>
<td>2</td>
<td>1</td>
<td>Hemorrhagic</td>
</tr>
<tr>
<td>Mato Grosso</td>
<td>1 (2)</td>
<td>1</td>
<td>0</td>
<td>Hemorrhagic</td>
</tr>
<tr>
<td>Distrito Federal</td>
<td>1 (2)</td>
<td>1</td>
<td>0</td>
<td>Hemorrhagic</td>
</tr>
</tbody>
</table>

The project was approved by the Ethics and Research boards of the Evandro Chagas Institute.

#### 2.2. Immunologic markers

The immunohistochemical technique originally described by Hsu et al. (1981) was used to characterize the phenotype of the inflammatory cells. Immunostaining for the detection of apoptosis was carried out according to the manufacturer’s instructions, as previously described (Gold et al., 1994). The following antibodies were used: CD45RO, CD4, CD8, CD20, S100, CD57, CD68 and anti-apoptosis (ApopTag).

A grid-scale with 10 × 10 subdivisions in an area of 0.0625 mm² was used for quantitative analysis of cell phenotype and apoptosis. Ten fields were counted at large magnification (×400) in all three areas of the acini (I = periportal area; II = midzone; III = central vein area).

#### 2.3. Negative controls

The following liver samples obtained during routine autopsies were included as negative controls: five liver samples from patients with negative serology for the main hepatotropic viruses, who showed no morphologic alterations; and five liver specimens from cases diagnosed as leptospirosis based on clinical presentation, specific serology, histopathology and immunohistochemical analysis.

#### 2.4. Statistical analysis

All data are reported as mean ± SD and were analyzed statistically by one-way ANOVA followed by the Bonferroni test, with the level of significance set at *P* ≤ 0.05. Statistical analysis was performed using the GraphPad Prism 3.0 software for Windows (GraphPad Software, San Diego, CA, USA).

#### 2.5. Theory

The study of the immune response is of great importance for the understanding of the evolution of tissue lesions inherent in different infections (Bertolleti and Maini, 2000). In viral infections affecting the liver, such as hepatitis B and C, the cytotoxic cellular response and the action of cytokines are important factors involved in the control of viral replication.
and hepatocyte death (Willuweit et al., 2001). Indeed, the existence of clinically asymptomatic patients infected with hepatitis B virus indicates the importance of the immune response in the genesis of tissue lesions and, consequently, its determining role in the evolution of the disease (Ganem and Prince, 2004). Furthermore, over the last few years, the differentiated clinical evolution of individuals in response to the same etiologic agent has led to increased emphasis on the importance of the role of the immune system in the evolution of hepatic viral infections (Willuweit et al., 2001). In addition to these eminently hepatotropic agents, viral infections that progress with important vascular impairment have been the target of studies, because they cause important and characteristic hepatic lesions that are still not well understood (Zaki and Peters, 1997). Among these viral infections, dengue and YF raise attention due to their peculiar pathogenesis, which is characterized by intense hepatic involvement (Monath, 1995, 2001). Furthermore, the mechanisms that induce tissue lesions and the type of immune response in the human host are poorly understood, despite the classic description of the main histopathologic aspects of hepatic injury during YF infection more than a century ago (Councilman, 1890; Rocha-Lima, 1912). Hepatic damage caused by the YF virus is intense and is characterized by a preferential involvement of the midzone area, which results in necrosis and eosinophilic degeneration of the hepatocytes, associated with a discrete lymphomononuclear inflammatory response disproportionate to the degree of hepatocyte involvement (Vieira et al., 1983).

3. Results

Histologic analysis confirmed previous findings and demonstrated hepatocytic alterations characterized by lytic necrosis, steatosis and apoptosis, which showed a preferential midzone distribution and were accompanied by a mild to
moderate inflammatory infiltrate predominantly consisting of mononuclear cells. Semiquantitative evaluation of the morphologic events observed in the present material showed a clear predominance of hepatocyte apoptosis over lytic necrosis (Figures 1 and 2A, B).

Immunohistochemical analysis of the lesions demonstrated positivity for hepatocyte apoptosis (ApopTag) and for inflammatory cells of the CD45RO, CD4, CD8, CD20, S100, CD68 and CD57 type (Figures 2–4). The quantitative analysis of the inflammatory cells is shown in Table 2.

ApopTag-labeled cells were observed in all regions of the lobule, mainly including hepatocytes, but also Kupffer cells and some mononucleated inflammatory cells. More intense immunolabeling was observed in hepatocytes of midzone areas ($P \leq 0.001$). The generally frequent immunolabeled apoptotic hepatocytes corresponded to the morphologic aspect characteristic of Councilman bodies (Figure 5A). A smaller percentage of these bodies was not immunoreactive by this technique, and immunolabeling of apoptotic hepatocytes in normal controls (Area I = $0.05 \pm 0.00$; Area

Table 2 Immunohistochemical analysis of lesions from 53 Brazilian yellow fever patients

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Mean ± SD</th>
<th>Area I$^b$</th>
<th>Area II$^b$</th>
<th>Area III$^b$</th>
<th>Portal tract</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApopTag</td>
<td>$2.51 \pm 0.64$</td>
<td>$16.41 \pm 3.07^{**}$</td>
<td>$1.48 \pm 0.73$</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>CD45RO (Pan T)</td>
<td>$2.29 \pm 1.09$</td>
<td>$4.97 \pm 1.85^*$</td>
<td>$0.61 \pm 0.20$</td>
<td>$10.26 \pm 2.95^{**}$</td>
<td></td>
</tr>
<tr>
<td>CD20 (Pan B)</td>
<td>$0.52 \pm 0.24$</td>
<td>$1.03 \pm 0.28^*$</td>
<td>$0.28 \pm 0.14$</td>
<td>$3.19 \pm 1.89^{**}$</td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>$1.70 \pm 0.55$</td>
<td>$3.04 \pm 0.59^*$</td>
<td>$0.81 \pm 0.29$</td>
<td>$11.24 \pm 2.93^{**}$</td>
<td></td>
</tr>
<tr>
<td>CD8</td>
<td>$1.20 \pm 0.41$</td>
<td>$2.63 \pm 0.85^*$</td>
<td>$0.55 \pm 0.17$</td>
<td>$7.52 \pm 2.09^{**}$</td>
<td></td>
</tr>
<tr>
<td>CD57</td>
<td>$0.46 \pm 0.17$</td>
<td>$1.04 \pm 0.32^*$</td>
<td>$0.24 \pm 0.24$</td>
<td>$1.37 \pm 0.45^{**}$</td>
<td></td>
</tr>
<tr>
<td>S100</td>
<td>$0.11 \pm 0.08$</td>
<td>$0.29 \pm 0.14^*$</td>
<td>$0.08 \pm 0.10$</td>
<td>$1.15 \pm 0.49^{**}$</td>
<td></td>
</tr>
<tr>
<td>CD68</td>
<td>$2.66 \pm 1.09$</td>
<td>$5.54 \pm 1.33^*$</td>
<td>$1.24 \pm 0.59$</td>
<td>$1.30 \pm 0.45^{**}$</td>
<td></td>
</tr>
</tbody>
</table>

$^*P \leq 0.05$; $^{**}P \leq 0.001$; ANOVA followed by Bonferroni.

$^a$ Results per mm$^2$ of tissue of cellular phenotype in the portal tract and three acinar areas.

$^b$ Areas I, II and III: hepatic areas 1, 2 and 3, respectively.
II = 0.07 ± 0.01; Area III = 0.02 ± 0.00) and leptospirosis (Area I = 0.62 ± 0.05; Area II = 0.98 ± 0.45; Area III = 0.30 ± 0.00) was less and not significant.

Cells immunolabeled with anti-CD45RO antibodies were observed in both the acini and in the portal tract. In the acini, immunolabeling was more frequent in the midzone, followed by zones I and III, respectively (P ≤ 0.05). At times, aggregates of immunolabeled T lymphocytes and neutrophils were observed around areas of lytic hepatocyte necrosis (Figure 5B). In the portal tract, the immunohistochemical pattern demonstrated a higher density of immunolabeled T lymphocytes than in the acini (P ≤ 0.05) (Figure 5C, D).

CD4+ and CD8+ T cells were observed throughout the hepatic parenchyma, both in the portal tract (P < 0.001) and in the acini, with a higher concentration in zone II, followed by zones I and III (Figure 5C, D). CD4+ T cells were more frequent than CD8+ T cells, both in the acini and portal tract. Immunolabeled cells exceeding the limiting plaque and arranged in the periportal region were frequently observed. Anti-CD20-immunolabeled cells were observed in the acini and portal tract. In the acini, the frequency of these cells accompanied that observed for T cells, i.e. there was predominance in zone II (P < 0.05). A lower density of B lymphocytes than T lymphocytes was observed in the portal tract (Figure 5E).

Anti-CD68 positivity was detected in Kupffer cells in the acini and in macrophages in the portal tract. The frequency of immunolabeled cells in the areas of the hepatic lobules continued to show a midzone predominance (P ≤ 0.05), although the quantitative difference in the positivity pattern between zones I, II and III was less significant (Figure 5F).

Anti-CD57-immunolabeled cells were observed both in the acini and in the portal tract, with their distribution in the different zones of the lobule demonstrating a predominance only in zone II (P ≤ 0.05). Immunolabeled natural killer (NK) cells were frequent in the portal tract and showed a higher frequency per area than in the acini (P ≤ 0.05) (Figure 5G).

Antigen-presenting cells labeled with the anti-S100 antibody were present in both acini and portal tract, with their distribution in the acini demonstrating predominance in zone II (P < 0.05) (Figure 5H).

4. Discussion

A fact that calls attention to YF, in addition to the intense apoptotic component, is the disproportion between the degree of parenchymal involvement and the scarcity of an inflammatory infiltrate, a remarkable characteristic of the liver in this disease. This marked characteristic has been investigated in classical studies (Hudson, 1928; Klotz and Belt, 1930a, 1930b) and was later confirmed by Smetana (1962), Vieira et al. (1983), Branquet (1996) and Quaresma et al. (2005), both in human material and in experimentally infected monkeys. In the present samples, this characteristic poor inflammatory response was observed in the lobule and the portal tract.

Some data are important to an understanding of the causes of this discrete inflammatory component. First, there is the intense degree of apoptosis in hepatocytes that induces no important inflammatory response. In this process, the apoptotic bodies are phagocytosed by neighboring macrophages and thus do not elicit large regional inflammatory alterations (Kauffman and Hengartner, 2001; Kerr et al., 1972; Majno and Joris, 1995; Tran and Miller, 1999). However, recent studies have demonstrated neutrophil migration to the hepatic parenchyma in rats during the course of apoptosis induced by experimental infection with Listeria monocytogenes. Park et al. (2002) demonstrated that the activation of Fas receptors by the administration of moderate doses of anti-Fas antibodies, accompanied by the induction of hepatocyte apoptosis, was able to induce gene expression and the synthesis of proteins such as chemokines and macrophage inflammatory proteins, which led to the accumulation of neutrophils.

It should be emphasized that, in the present study, neutrophils, although rare, were mainly observed adjacent to
Figure 5  Quantitative analysis of immunostaining for yellow fever (YF), leptospirosis (LE) and negative controls (NC). In YF, comparison of the different acinar areas shows a predominance of immune response and apoptosis in the midzone area compared with other lobular areas. A strong immune response was also observed in the portal tract. (A) Apoptotic hepatocytes (△) and Councilman bodies (▼). (B) Neutrophils. (C) CD4+ lymphocytes. (D) CD8+ lymphocytes. (E) CD20+ cells. (F) CD68+ cells. (G) Natural killer cells (CD57). (H) S100 cells (antigen-presenting cells). I: hepatic area 1; II: hepatic area 2; III: hepatic area 3; PT: portal tract (*P ≤ 0.05; **P ≤ 0.001; ANOVA followed by Bonferroni).
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...areas of lytic hepatocyte necrosis (Figure 5B). In addition, the absence of neutrophils in areas of intense apoptosis may reflect, at least in part, the lack of induction of a significant inflammatory response. This is in agreement with reports in the literature showing an association between apoptosis and the absence of inflammation (Cotran et al., 1999).

The predominantly lymphomononuclear inflammatory infiltrate mainly consisted of T cells, as shown above. The predominance of T lymphocytes, mainly CD4+ and CD8+ T cells, is similar to findings obtained for other hepatic viral infections in which these cells, among other factors, are involved in the process of viral clearance during the course of the disease (Tseng et al., 2001; Valiante et al., 2000).

An important aspect of viral infections is that the immune response triggered by the presence of the infectious agent is aimed at neutralizing circulating viruses and at interfering with the intracellular viral cycle (Chisari and Ferrari, 1995), inducing a cellular immune response that is classically triggered to eliminate cells infected by the viral agents. More recently, it has been shown that this process does not always lead to destruction of the host cell but causes the release of cytokines with eminently antiviral functions (Samuel, 2001). Data obtained in studies involving other viruses have shown that the immunopathologic response to viral infections involves not only the participation of CD8+, or cytotoxic, T lymphocytes that necessarily contribute to tissue injury, but also the action of CD4+ T cells, macrophages, polymorphonuclear cells, leukocytes and NK cells, as well as cytokines, chemokines and components of the complement system (Chisari and Ferrari, 1995). In the present study, the cellular immune response associated with the evolution of YF virus infection was characterized by CD4+ T lymphocytes and, to a lesser extent, by CD8+ T lymphocytes.

The expression of antigen-presenting cells, macrophages and NK cells was also observed, and their concentrations were higher in the midzone region. Despite the discrete quantity of these cells, their participation in the determination of the evolutionary picture of the disease seems to be important. Some of the present data indicate a direct influence of this inflammatory response on the determination of YF lesions, especially the presence of the larger number of cytotoxic cells in the midzone region where the pattern of cellular injury is more intense. However, the exact role of each of these specific cell populations in the immunopathogenesis of YF remains a complete mystery. So far, reports regarding the evolution of these lesions are available in the literature but without emphasis on the role played by the immune system in this process in the liver.

Despite the poor local inflammatory infiltrate, it seems that during the course of infection and after the first replications in the lymph nodes, the virus reaches the liver and infects macrophages and antigen-presenting cells (S100), which promote the antigen processing and presentation to CD4+ T cells through the expression of MHC class II-related products. This was demonstrated by Hall et al. (1991) and confirmed in studies on viral antigen detection in Kupffer cells (Monath, 2001). During this initial phase, NK cells also play an important role. These cells are able to recognize virus-infected cells that possibly escaped the action of cytolytic T lymphocytes through mechanisms involving the downregulation of the expression of MHC class I-related molecules, leading to cell lysis induced by the release of granzymes and perforins (Zinkernagel, 1996). This mechanism is of great importance in the compartmentalization of immune response in the liver, mainly because of the large number of NK cells present in hepatic tissue (Biron et al., 1999). Natural killer cells also release IFN-γ, a cytokine with a confirmed antiviral effect that activates macrophages and potentiates the expression of MHC-related molecules. After recognition and presentation of the antigen, activated CD4+ T cells start to release cytokines such as IFN-γ and TNF-α, which are characteristic of cells of a Th1 type response. These cytokines act on the differentiation of naive CD8+ T lymphocytes into cytolytic T cells, on the induction of inflammation and on the activation of macrophages. CD4+ T cells seem to play an important role in the immunopathogenesis of hepatic lesions in YF, as indicated by the present quantitative data.

Once activated, macrophages release substantial amounts of oxygen-derived free radicals, nitric oxide and TNF-α. The latter cytokine, in turn, plays a highly important role in the pathogenesis of shock and tissue injury in YF (Monath, 2001). Cytolytic CD8+ T lymphocytes, activated either by immune processing of professional S100 cells related to the expression of MHC class I molecules or by the action of cytokines stimulating their maturation, interact with infected hepatocytes, inducing lysis and apoptotic cell death. It should be emphasized here that the induction of lysis of infected cells is an important mechanism of the immune response and viral clearance, and at times is able to completely eliminate virions present in hepatic cells. However, as described for hepatitis C and B, this process is not always able to completely eliminate the etiologic agent, a fact that frequently leads to the occurrence of hepatic lesions. In YF, the absence of virions in hepatocytes upon electron microscopy, mainly during the period of increased hepatic injury, is an indicative factor of the importance of the immune response, which also acts as a mechanism of tissue injury in the liver (Quaresma et al., 2005).

CD8+ T lymphocytes can lyse infected cells and thus release viral particles into the extracellular medium. These particles are submitted to the action of specific antibodies, mainly produced by B cells that undergo maturation induced by the release of cytokines from activated CD4+ T cells. In the present study, B cells were frequently present both in the acini and in the portal tract, and were probably also involved in antigen processing during the effector phase of the immune response.

Further studies using immunohistochemistry to cytokines, experimental models, especially non-human primates, and molecular biology techniques may help define the natural history of hepatic injury leading to cell death and provide therapeutic regimens aimed at reducing disease lethality.

Conflicts of interest statement
The authors have no conflicts of interest concerning the work reported in this paper.

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