Revisiting the liver in human yellow fever: Virus-induced apoptosis in hepatocytes associated with TGF-β, TNF-α and NK cells activity

Juarez A.S. Quaresma a,b,*, Vera L.R.S. Barros c, Carla Pagliari d, Elaine R. Fernandes d, Fernanda Guedes d, Cleusa F.H. Takakura d, Heitor F. Andrade Jr. d, Pedro F.C. Vasconcelos c, Maria I.S. Duarte d

* Tropical Medical Center, Federal do Para University, Av. Generalissimo Deodoro 92, 66055-420, Belem, Para, Brazil
b Department of Pathology, State University of Para, Belem, Para, Brazil
c Evandro Chagas Institute, Ministry of Health, Belem, Para, Brazil
d Department of Pathology, Faculty of Medicine, Sao Paulo University, Sao Paulo, Brazil

Received 12 April 2005; returned to author for revision 22 August 2005; accepted 21 September 2005
Available online 8 November 2005

Abstract

Flavivirus infection as dengue and yellow fever persists as a terrible menace to pandemics, due to Aedes prevalence in the Americas. Yellow fever is characterized by hepatocyte damage, with steatosis, apoptosis and necrosis, mainly in the midzonal region of the liver, but the injury mechanism has not been studied at the light of recent knowledge, such as the advances in cell death mechanisms, inflammatory response and cytokine cell expression tools. We studied 53 human liver paraffin embedded blocks from patients who died with yellow fever, all with histological demonstration of higher prevalence of apoptosis over necrosis and mild disproportionate inflammatory response. Viral antigens were found most frequently in hepatocytes from the midzonal area than other lobule areas, as detected by specific immunohistochemistry. Infiltrating cell subpopulations showed mainly CD4+ T lymphocytes, with small numbers of CD8+ cytotoxic lymphocytes, CD20+ B lymphocytes, NKT+ cells and S100+ dendritic cells in the sites of inflammation, as compared to normal and leptospirosis liver blocks. Some cells expressed TNF-α and IFN-γ, but a much more intense proportion of TGF-β expressing cells were found, suggesting both a Th1 and Th3 patterns of immune response in yellow fever. Most affected hepatocyte presented apoptosis markers that appear at the cell death main pathway in this infection. Viral antigens, which production could interfere in hepatocyte biology, could induce the activation of apoptosis cascade, but TGF-β was also an apoptosis promoter.

Our finding supports the key effect of the yellow fever virus in hepatocyte injury, resulting in prevalence of apoptosis over necrosis, aside from a TGF-β action induced by the inflammatory response.

Keywords: Yellow fever; Apoptosis; Immune markers; Cytokine; Pathogenesis

Introduction

Yellow fever (YF) is a viral infectious disease transmitted by arthropod vectors, characterized by an acute and lethal systemic involvement, specially affecting the liver (Monath and Barret, 2003; Vasconcelos, 2003). Their symptoms vary from mild non-specific to hemorrhagic diathesis, jaundice and acute renal failure, with a high case-fatality rate (Monath, 2001). There are two transmission cycles for this arbovirus infection, with urban YF transmitted man to man by the mosquito Aedes aegypti, while jungle YF is transmitted by several mosquitoes, Aedes in Africa and Haemagogus and Sabethes in the Americas, with non-human primates as vertebrate hosts. Human beings become infected when they intrude in the forest and are bitten by infected mosquitoes (Degallier et al., 1992). Despite absence of urban transmission in the Americas since 1959, the present infestation of Aedes aegypti in large urban centers near YF endemic areas, population migration and a low YF vaccine coverage in many countries predispose to the possibility of re-urbanization of the...
disease in the Americas (Massad et al., 2001; Robertson et al., 1996; Vasconcelos et al., 2004). The danger is particularly great in Brazil, the most populated country in the area, and increased figures for YF activity have been recorded in the last 5 years (Vasconcelos et al., 2001a, 2001b, 2003).

The classic description of yellow fever hepatic injury established more than a century ago is characterized by involvement of the midzonal area of the hepatic lobule, with cell death and steatosis, with a disproportionate discrete inflammatory infiltrate (Vieira et al., 1983). The cell death in YF is usually attributed to the virus direct effect, without studies on their mechanism, especially without the recent available tools in immunology and cell biology (Monath, 2001). Most of the few reports ascribes the hepatic lesion to circulatory changes, as low blood flow resulting in midzonal distribution, associated to direct cytopathic viral effect on liver cells (De Brito et al., 1992; De La Monte et al., 1984; Monath et al., 1989). Recently, an increasing importance has been attributed to immune mechanisms of cell death induction, as the effects of certain cytokines, as IFN-γ and TNF-α, or other products of immune cells activation, as free radicals (Delves and Roitt, 2000a, 2000b; Liu et al., 1996).

We were unable to find any reports dealing with the phenotype of the constituent cells of the portal or lobular inflammatory infiltrate, the expression of cytokines, or the type of compartmentalized immune response of the liver during the course of severe YF infection. Here, we revisited files and viscerotomy livers paraffin blocks of fatal Brazilian YF patients, allowing systematic analysis of the hepatic histology, associated to immunohistochemistry for viral antigens, cell death pathways, immune cell phenotypes, and cytokine production.

**Results**

**Patient characteristics**

We found blocks from 53 patients whose liver fragments were studied, and whose infections were previously diagnosed between 1973 through 2001. The age of patients ranged from 3 to 74 years old; 86.7% were male. Viral infection was acquired in Brazilian States, with Goiás (28.3%) and Pará (15.9%) providing most cases. No differences in the severity of tissue injury in liver samples were detected in patients of different age or sex. For negative controls, we included blocks from ten patients with negative serology for the main hepatotropic viruses and which showed no morphological alterations, obtained during routine necropsies and blocks from ten cases diagnosed as leptospirosis by clinical presentation, specific serology, histopathologic and immunohistochemical analysis. All liver fragments showed YF antigens, which were more frequent in the midzonal area. No YF antigens were found in the two control groups.

**Qualitative histology**

The quantitative analysis of the histopathologic aspects was made in previous studies (Quaresma et al., 2005). Steatosis was one of the main liver alterations observed and was present in all three lobule areas but with a higher prevalence in the midzonal area (Fig. 1A). The lesion was characterized by both macro- and micro-vesicular steatosis. Micro-vesicular fatty changes in the cytoplasm of hepatocytes were also observed, showing a typical (morula-like) aspect for the hepatocytes with this kind of steatosis (Fig. 1B).

![Fig. 1. Histopathology and immunohistochemistry of human liver in the yellow fever. (A) Councilman bodies and midzonal necrosis (hematoxylin–eosin, original magnification ×200); (B) high magnification (×400) showing in details steatosis (arrow), apoptosis (arrowhead) and lytic necrosis (circle); (C) portal area showing mononuclear inflammatory infiltrate (×400); (D) immunohistochemical staining showing large amounts of YF antigens (in red) (×200).](image-url)
Councilman bodies were frequently observed in all three lobule areas, but they were more frequently found in the midzonal area. They were also recognized in the central vein and portal tracts. It is noteworthy that between the midzonal region and the other regions, there was an overlap in the different aspect of the Councilman bodies. These represent areas of focal eosinophilic condensation in the cytoplasm, showing a condensed and retracted cytoplasm accompanied by pyknotic nuclei. Sometimes classic corpuscles with or without nuclear debris were also observed (Fig. 1B).

Small focal points of lytic necrosis of the hepatocytes were observed mainly in the midzonal area, and less frequently in the central lobular and periportal areas (Fig. 1A). These focal points of necrosis were visualized as areas of parenchyma showing complete absence of cells that were in general substituted by an amorphous and slightly eosinophilic mass containing cellular debris accompanied by a consistent lymphocyte infiltrate and, occasionally, also by neutrophils. The degree of inflammatory cell infiltrate in the hepatic lobule was markedly disproportionate to the intensity of the parenchyma damage. The infiltrate comprised predominantly of mononuclear cells, with scant neutrophils, occasionally observed close to areas of lytic necrosis of the hepatocytes (Fig. 1B). Distribution of inflammation in the lobule maintained a pattern similar to that observed in steatosis, i.e., with a clear preference for the midzonal area. In the portal tract the infiltration of lymphomononuclear cells was mild-to-moderate (Fig. 1C), maintaining the same disproportion to the degree of tissue damage, with limited areas preserving the lobular limiting plaque. The ultrastructural aspects had confirmed the findings of hepatocytes apoptosis with Councilman bodies presented as dense round or elliptical cytoplasmic masses, partially delimited by a membrane.

Detection of specific YF antigens was easily observed in all three areas, but much more frequent in the midzonal area (Fig. 1D). Cells stained mostly by the APOPTAG procedure were observed in all lobular areas but mainly in the hepatocytes of the midzonal region (Fig. 2A). There was a more intense stain in the midzonal area, where frequent apoptotic hepatocytes were seen and, in general, their presence coincided with the characteristic aspect of Councilman bodies (Fig. 1B).

Quantitative immunohistochemistry

Quantitative data on specific cell populations in the liver performed both in YF blocks, as compared to normal or leptospirosis controls are shown in Table 1 and Fig. 4.

The main lymphocyte populations, CD4+ (Figs. 2B and 4A) and CD8+ (Figs. 2C and 4B) T lymphocytes, and B lymphocytes (Figs. 2D and 4C) were more intensely presented in portal areas, with a smaller numbers in the lobular areas, when they present a preferential occurrence in the midzonal area of the acinus. The quantitative data appear to show a

![Fig. 2. Immunohistochemical staining of YF in human liver using specific antibodies (×400). (A) Hepatocytes showing apoptosis (arrows); (B – D) portal tract shows intense immune-staining for CD4+ lymphocytes (B); CD8+ lymphocytes (C); B lymphocytes (D).](image)
pattern of traffic of those cells, from the zone I to zone III, for all three subsets. The presence of TCD4+ cells was slightly more intense than the TCD8+ and B lymphocytes. All of those cells were present in higher numbers than in normal and leptospirosis livers in all lobular areas, but leptospirosis presented the same values as YF in portal areas for T lymphocytes but not for B lymphocytes.

When the cells of the innate immune response in YF livers were analyzed, as CD68+ macrophages (Figs. 3A and 4D), NK cells (Figs. 3B and 4E) and S100+ antigen presenting cells (Figs. 3C and 4F), a different pattern was observed. There is similar involvement of the portal area as compared to the midzonal area of the lobule for most cells, without the preferential location of the cells as seen for T lymphocytes. CD68 was found in Kupffer cells, with a greater density in the midzonal area than in portal area. Despite lower intensity, this pattern was also found in leptospirosis livers (Fig. 4).

NK cells were present also in higher numbers in both portal and midzonal areas of YF livers, with some patients presenting extremely high values in midzonal area. This increment of NK cells was only seen in YF livers, as compared to the other controls. The distribution of S100+ antigen-presenting cells were quite similar to NK cells but with small numbers of positive cells in each area (Table 1).

Several cytokines showed a greater expression in the midzonal area and in the portal tract, occurring to a lesser extent in the other lobule areas (Table 2) (Figs. 5A, B and C). A marked expression of TNF-α and IFN-γ was observed. Intense cellular immunolabeling was observed for TGF-β. There was a mild immunoexpression of IL1-α and β, IL4, IL8 and IL10 in the lobule areas and also in the portal tract (Table 2). This immune-expression was not marked with any preference for a specific area of the hepatic lobule. The expression of Fas protein was observed in the cytoplasm of hepatocytes, and was detected at significantly higher levels in the midzonal area, when compared with areas I and III (Fig. 5D).

**Discussion**

The reemergence of YF in many areas of Africa and South America, and the possibility of its re-urbanization in those countries showing a wide distribution and high infestation-index of *Aedes aegypti* indicate this arboviral disease as a potential serious public health threat, especially in countries where transmission of dengue is common (Robertson et al., 1996; Vasconcelos, 2003; Vasconcelos et al., 2004). Although many studies have described and focused on the clinical manifestations and histological alterations of the liver, little is known about the mechanisms of cell damage or the type of local immune response by the host cells to the YF virus infection in humans. The knowledge of these mechanisms would be a very useful step in providing a better treatment of patients with severe YF infection in whom the case fatality rate is high.

What are the real dimensions of the hepatic role and the extension of cell damage during YF infection? Previous reports on the hepatic involvement give a description of midzonal necrosis, presence of Councilman bodies, and of a discrete inflammatory infiltrate (Councilman, 1890; Klotz and Belt, 1930; Rocha-Lima, 1912; Vieira et al., 1983). Similar observations have been seen in experimental YF of rhesus monkeys with total effacement of hepatic architecture due to necrosis of hepatocytes and Kupffer cells. At lobule level, no portions were spared and necrosis was accompanied with little or no inflammation (Monath et al., 1981). The same observations were noted in the hamster model (Tesh et al., 2001; Xiao et al., 2001).

In our systematized study of 53 liver fragments of patients with fatal YF, quantification of the histological abnormalities showed intense involvement of the hepatic parenchyma with a clear predominance of cell damage on an YF antigens expression in the midzonal area. We noted that cell lesion is much more intense and widely distributed in this area than in areas I and III (Fig. 1).

We also confirmed the existence of a consistent and intriguing disproportion between the intensity of tissue injury in the lobular and portal areas and the discrete or very poor...
inflammatory response, already recorded in previous literature (Klotz and Belt, 1930; Monath, 2001; Xiao et al., 2001). Indeed, the degree of inflammatory infiltration observed in our samples, which comprised almost exclusively of mononuclear cells, was estimated in our scale as mild-to-moderate. This contrasts with the exuberance of lesions observed in the hepatocytes.

Our findings of a clear predominance of apoptosis over lytic necrosis as the principal mechanism responsible for cellular death in the liver of fatal YF cases contrasts with data available in the literature (Bugher, 1951; Hall et al., 1991; Klotz and Belt, 1930; Monath et al., 1981; Xiao et al., 2001). Previous works had demonstrated that the coagulative and lytic necrosis represent the main mechanism of hepatocytes death in yellow fever both in human and animal models (Monath et al., 1981; Smetana, 1962; Vieira et al., 1983). On the other hand, Vieira et al. (1983) have described hepatocytes with morphologic aspects of apoptosis in human cases, but correlations concerning the mechanism of cell death and the immune response, evolution, and general aspects of histopathologic alterations of liver in the human infection had not been considered in these papers. It is important to emphasize that the semi-quantitative evaluation of the two events showed that apoptosis was much more important than lytic necrosis in the pathogenesis of hepatocyte death. We may state, therefore, that in fatal human YF, the death of hepatic cells is principally

Fig. 4. Quantitative analysis of specific immune cell frequency in hepatic areas during YF, as compared to controls and leptospirosis patients. (A) CD4+ lymphocytes; (B) CD8+ lymphocytes; (C) CD20+ cells; (D) CD68+ cells; (E) natural killers cells; (F) S100 cells (antigen-presenting cells). Closed dots (■) represent YF patients, open dots (○): normal controls and stars (*): leptospirosis patients. A bar represents mean values. P values are related to YF patients and controls.
Table 2

<table>
<thead>
<tr>
<th>Immunochemistry in yellow fever</th>
<th>TGF-α</th>
<th>IFN-α</th>
<th>IL4</th>
<th>IL8</th>
<th>IL10</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>YF</td>
<td>LE</td>
<td>NC</td>
<td>YF</td>
<td>LE</td>
</tr>
<tr>
<td>II</td>
<td>YF</td>
<td>LE</td>
<td>NC</td>
<td>YF</td>
<td>LE</td>
</tr>
<tr>
<td>III</td>
<td>YF</td>
<td>LE</td>
<td>NC</td>
<td>YF</td>
<td>LE</td>
</tr>
<tr>
<td>PT</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Results (Mean ± Standard Deviation) for cellular expression of cytokines and CD95L (FasL) per mm² of tissue in portal tract (PT) and the three acinus areas.


caused by apoptosis. This apparent paradox, found in comparison with it is described in the literature, is probably due the recent improvement of immunologic and molecular biology tools.

Although well recognized in other pathologic conditions, we could find no explanation in the literature as to the cause and effect of the frequent midzonal lesion observed by other authors and ourselves in the present study. Several authors have previously demonstrated midzonal lesions in the presence of hypoxia and in syndrome of hepatic injury caused by drugs (De La Monte et al., 1984; Jungermann and Katz, 1989; Suematsu et al., 1992).

Marked vascular damage in the liver was noted in almost all the severe cases of YF cases we examined, but no similar lesions were observed in the two sets of controls studied. These vascular lesions are associated with biochemical changes in the hepatocytes in all three regions of the hepatic lobule (Rappaport, 1976), resulting in hypoxic damage that would explain the preferential pattern of midzonal damage in YF infection, since this area of the liver has the poorest blood supply. The role of the other factors that might contribute to the genesis of this characteristic pattern of hepatic tissue damage must, however, be considered. Indeed, our findings support at least an important role for direct virus action due the presence of large amounts of YF antigens in the hepatocytes in the midzonal area.

The pathogenesis of hepatocyte injury in the course of hepatitis induced by flaviviruses is complex and remains to be defined. Cell-mediated immune regulation by the host is thought to attenuate the genesis and evolution of liver cell injury occurring during the course of hepatitis C virus infection (Marianneau et al., 1998). Other authors have mainly investigated the role of cytotoxic T lymphocytes and cytokines such as TNF-α and IFN-γ in hepatitis C (Bertoletti and Maini, 2000). During acute infection, Kupffer cells and S100+ cells rapidly take up the YF virus in the liver and play an important role not only in the liberation of cytokines such as TNF-α, but also during the process of production and presentation of the specific YF antigen to helper T lymphocytes (Monath, 2001).

In the hepatic tissue, the preferential place for expression of Th1 profile cytokines, associated with a lack of viral particles observed by electron microscopy of material from human cases (as previously described by several authors, and in agreement with our observations), indicates lesion-inducing mechanisms somewhat different from those observed for other forms of viral hepatitis. The discrete inflammatory infiltration with scarce participation of B lymphocytes, and the high TCD4⁺:TCD8⁺ ratio (especially in the midzonal area that coincides with the most intense immunolabeling by Fas), all point to an important role of cytokotic mechanisms mediated by cells with induction of apoptotic cell death of hepatocytes. At ultra-structural level, we have observed significant changes of mitochondria of the hepatocyte that may be due the hypoxia of low-flow and direct viral action.

The participation of cytokines in the process of viral clearance has already been described in hepatitis B and C (Bertoletti and Maini, 2000). In these infections, the cytokines
TNF-α and IFN-γ are of importance since both can also induce apoptosis in hepatocytes by binding to their specific receptors. The antiviral effect of IFN-γ is well established but its also plays a role in activation of macrophages, as well as in the increase of the expression of molecules related to the Major Histocompatibility Complex (MHC) types I and II, thus maximizing the presentation of antigens to the lymphocytes and increasing the binding of TCD8+ cells to the surface of the hepatocytes: this is an important mechanism that leads to cell death. While TNF-α has been implicated as a cytokine with strong antiviral action during the course of viral hepatitis B and C, its role during disease is not fully understood. Mechanisms of interaction of components of C virus with domains related to the induction of apoptotic death by TNF-α possibly represent factors associated with persistence of the infection in this disease (Bertoletti and Maini, 2000; Willuweit et al., 2001).

Regarding YF virus infection, the roles of IFN-γ and TNF-α are not completely understood (Monath et al., 1981; Monath, 2001). In our study, the immunolabeling of cells with these cytokines characterizing an immune response Th1 was more intense in the midzonal area, and probably, they were directly or indirectly implicated in both viral clearance and in the induction of cell injury. Due to the significant immunoexpression of TNF-α in the midzonal area, a region in which hepatocytes injury is marked by exuberant lesions, and where apoptosis is more intense, these cytokines may play a role during the induction of the hepatocytes apoptosis. Receptors such as Fas, which also showed an important expression in this area, may act as possible mediators (Figs. 3A and B).

Corroborating this hypothesis, it is important to emphasize the very intense TGF-β immunoexpression, especially in the midzonal area (Fig. 5C). Two important aspects should be mentioned: firstly, the high capacity of this cytokine to induce apoptotic cell death in hepatocytes by interacting with specific receptors; and, secondly, that TGF-β is a strong inhibitor of the cell immune response. In view of our findings, we believe that TGF-β plays a very important role during fatal human YF virus infection by inducing tissue damage. This finding may explain the intensity of apoptosis in association with the very poor inflammatory response observed in our patients.

Previous information in the literature has shown that the effects caused by TGF-β are inhibited by TNF-α (Tartaglia et al., 1993). Schuster and Kriegistein (2002) have suggested that the effects of these cytokines on tissue are closely associated with their interaction with the targeted cells. In our patients, both cytokines were prominent and concomitantly expressed in all lobule areas, but again were more intensively expressed in the midzonal area. Indeed, the expressive immunolabeling of these two cytokines in the midzonal area, where the lesions of the YF virus preferentially occurs, led us to suggest that there is a convergence of their action towards the development of cellular death by apoptosis. The low expression of the Th2 cytokine IL-10 in the samples was consisted with a weak local inflammatory response.

The role of NK cells in the pathogenesis of other viral hepatitis is based on the fact that they are able to induce lesions in cells infected by hepatotropic viruses through independent mechanisms of the MHC, and also by cytokines with a potential antiviral effect, such as TNF-α and IFN-γ that are also secreted by NK cells (Delves and Roitt, 2000a, 2000b). The considerable expression of NK cells in our study is interesting, being more intensively observed in the midzonal area and portal tract (Fig. 4E), and points to the role of these cells in the clearance process of the YF virus. These cells also contribute to the genesis of the intense apoptotic component.
observed during acute YF virus infection, through mechanisms that involve the release of granzymes and perforin.

In summary, our findings support the hypothesis that hepatic injury is due by a combined factors i.e. direct viral action, low-flow hypoxia, and by immune response. The more important mechanism of hepatocytes death is apoptosis that it is mainly flow hypoxia, and by immune response. The more important that involve the release of granzymes and perforin.

Further studies using experimental models, especially in non-human primates could help to define the natural history of hepatic injury leading to cell death, the role of cytokines and other factors in this evolution, and how inappropriate or inadequate immune responses might culminate in fatal outcome. If these experiments can be carried out, much more information will be acquired on the pathogenesis of YF virus infection in a model that completely reproduces the human disease, and this may lead to therapeutic schemes aimed at diminishing disease lethality.

Material and methods

Histological examination and immunohistochemical detection of YF virus antigen

Paraffin embedded blocks from formaldehyde fixed liver samples were obtained from files of the Department of Pathology, Evandro Chagas Institute (Ananindeua, Brazil), and resectioned, with sections stained by hematoxylin and eosin method and placed in sylane-treated slides. Liver blocks from 10 patients with negative serology for the main hepatotropic viruses and which showed no morphological alterations, obtained during routine necropsies and liver blocks from cases diagnosed as leptospirosis, were used as controls and submitted as the same histopathologic and immunohistochemical analysis.

YF infection was proven by serologic methods, viral isolation, and immunohistochemical detection of YF antigens using monoclonal antibodies and light microscopy examination as described elsewhere (Hall et al., 1991). In this report, 5 μm sections were also stained with Masson’s trichrome, reticulin and Perls stain and examined by light microscopy by two independent observers, using a semi-quantitative scale ranging from 0 to 3, for none, mild, moderate and severe or intense phenomenon, for inflammatory infiltrates, hepatic apoptosis, and steatosis, similarly to reported elsewhere in experimental YF infections (Xiao et al., 2001). Paraffin-embedded tissue was also reprocessed for electron microscopy studies in accordance with the described protocol for Duarte et al. (1992).

Immunohistochemistry cell surface, apoptosis and cytokine expression markers

All sections were analyzed by immunohistochemistry of several cell phenotype or intracellular cytokine production, according to minor modification of reported protocols, including the detection of cell apoptosis were carried out as previously described (Amato et al., 2003; Gold et al., 1994; Hsu et al., 1981). The following antibodies were used: CD20, CD45R0, CD4, CD8, S100, CD57, CD68, CD95, TNF-α, IFN-γ, IL-1α, IL1-β, IL4, IL8, IL10, TGF-β and anti-apoptosis (APOPTAG). All sections were analyzed for density of the labeled cells for each stain, using a grid-scale, with 10 × 10 subdivisions in an area of 10 mm², to count fields under high magnification (×400) in at least 10 fields of all three areas of the hepatic lobule, defined as peri-portal area (I); midzonal area (II); and near central vein area (III), aside to the portal tract.

Statistic analysis

All data obtained were reported as mean ± standard deviation (SD) and studied statistically by analysis of variance (ANOVA one-way) followed by the Bonferroni test. The level of significance for these analyses was established when P ≤ 0.05. The analysis was performed using the GraphPad Prism 3.0 software for Windows (GraphPad Software, San Diego, CA).

Acknowledgments

This work was supported by FAPESP (grant 00/09745-9) and CNPq (grants 302770/02-0 and 401349/2004-7). We are grateful to Dr. Samuel Allen, Chelsea and Westminster Hospital, London, for comments and suggestions. The authors wish to thank Rosana Cardoso and Marliva Araujo for technical support.

References
