

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 *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+ T lymphocytes, followed by CD8+ 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 *Flaviviridae*, genus *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 transmit-

ted from a sick individual to a non-immunized person through the bite of infected *Aedes aegypti*. In the jungle form in the South America, the virus is mainly transmitted accidentally to man by mosquitoes of the genus *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 characteristically more intense in the midzone region and are associated with a portal and

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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 not 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 Hsu 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. (1994). 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 we used in a grid-scale, with 10×10 subdivisions in an area of 0.0625 mm^2 , to count fields under high magnification ($\times 400$) 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

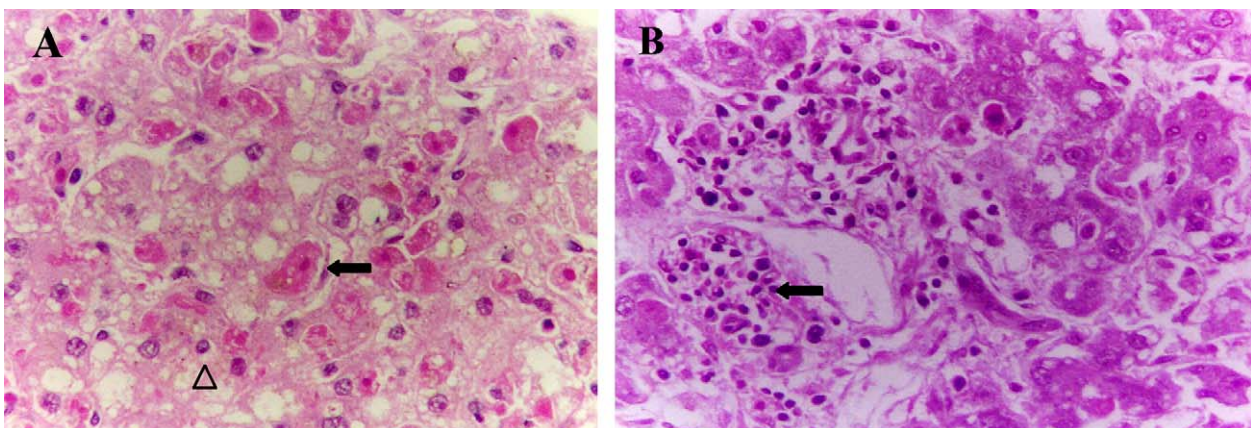


Fig. 1. Yellow fever liver: (A) histopathologic aspects of Councilman bodies (\leftarrow), macro and microvesicular steatosis (Δ) ($400\times$); (B) portal tract showing minimal inflammatory infiltrate of lymphocytes ($200\times$).

Table 1
Yellow fever—mean and standard deviation of apoptosis, lymphocytes and FasL expression in acini and portal tract

	Apoptosis			CD45RO			CD4			CD8			FasL		
	YF	LE	NC	YF	LE	NC	YF	LE	NC	YF	LE	NC	YF	LE	NC
	2.51 ± 0.64	0.50 ± 0.45	0.02 ± 0.00	2.29 ± 1.09	0.68 ± 0.07	0.06 ± 0.05	1.70 ± 0.55	0.22 ± 0.07	0.11 ± 0.05	1.20 ± 0.41	0.20 ± 0.05	0.12 ± 0.09	0.06 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
I	16.41 ± 3.07	0.10 ± 0.62	0.03 ± 0.00	4.97 ± 1.85	1.12 ± 0.10	0.25 ± 0.08	3.04 ± 0.59	0.57 ± 0.08	0.13 ± 0.05	2.63 ± 0.85	0.57 ± 0.08	0.12 ± 0.09	0.10 ± 0.04	0.02 ± 0.00	0.01 ± 0.00
II	1.48 ± 0.73	0.32 ± 0.09	0.00 ± 0.00	0.61 ± 0.20	0.25 ± 0.05	0.00 ± 0.10	0.81 ± 0.29	0.21 ± 0.08	0.07 ± 0.06	0.55 ± 0.17	0.17 ± 0.18	0.05 ± 0.08	0.06 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
PT	—	—	—	10.26 ± 2.95	6.87 ± 0.40	6.87 ± 0.40	11.2 ± 2.93	10.19 ± 5.34	1.02 ± 0.64	7.52 ± 2.09	11.5 ± 1.39	0.71 ± 0.39	—	—	—

YF: yellow fever, LE: leptospirosis, NC: normal control.

disproportional to the intense degree of involvement of the hepatic parenchyma (Fig. 1A and B). In addition, hemorrhagic foci and small neutrophil agglomerates were noted around the areas of lytic necrosis.

Immunohistochemical examination revealed apoptotic (ApopTag-labeled) cells in all regions of the lobule, mainly hepatocytes but also in Kupffer cells and in few mononucleated inflammatory cells (Table 1). Immunolabeling for apoptosis was more intense in hepatocytes of the midzone areas (Fig. 2A and B). The generally frequent immunolabeled apoptotic hepatocytes correspond to the morphological aspect characteristic of the Councilman bodies.

Activated T cells immunolabeled with the anti-CD45RO antibody were observed in both the acini and portal tract. In the acini, the immunolabeling intensity was higher in the midzone region, followed in decreasing order by zones 1 and 3. Immunolabeled T lymphocyte aggregates were observed around areas of lytic hepatocyte necrosis. In the portal tract, the immunohistochemical pattern demonstrated a higher density of immunolabeled T lymphocytes compared to the hepatic acini (Fig. 3A and B).

CD4+ and CD8+ T cells were observed throughout the hepatic parenchyma both in the portal tract and in the acini, with a higher concentration in zone 2, followed by zones 1 and 3. CD4+ T cells were more frequent than CD8+ T cells both in the acini and portal tract (Fig. 4A and B) (Fig. 5A and B).

Expression of the FasL protein was observed in all hepatic lobules and tended to be higher in the midzone region. In general, immunoexpression was noted especially in hepatocytes and was rare in cells lining the biliar ducts (Fig. 6A and B).

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).

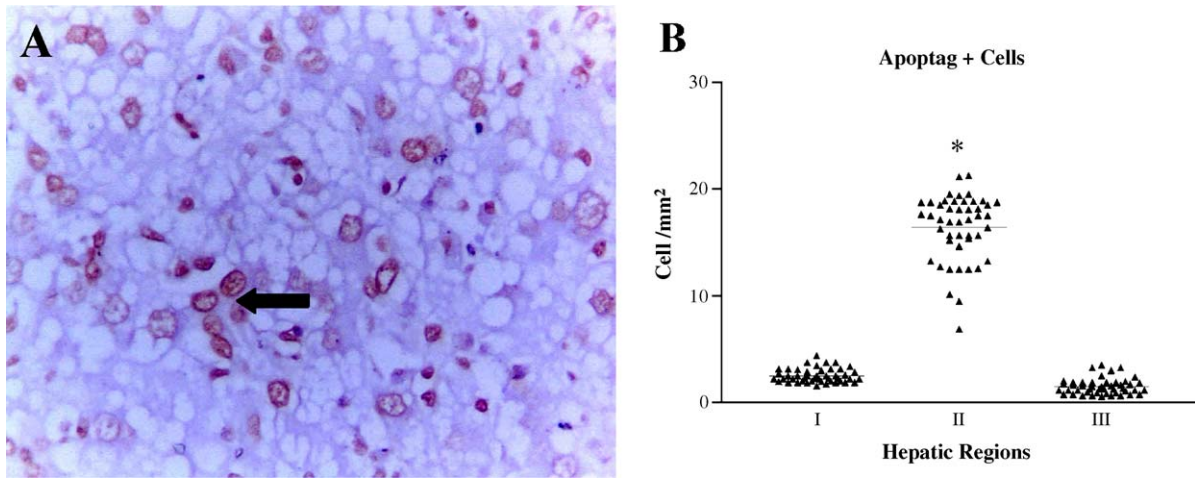


Fig. 2. Yellow fever liver: (A) immunohistochemistry for apoptosis showing hepatocytes in the acini (200×); (B) quantitative analysis of apoptotic hepatocytes in the areas I, II and III of the hepatic acini (* $p \leq 0.05$, ANOVA/Bonferroni).

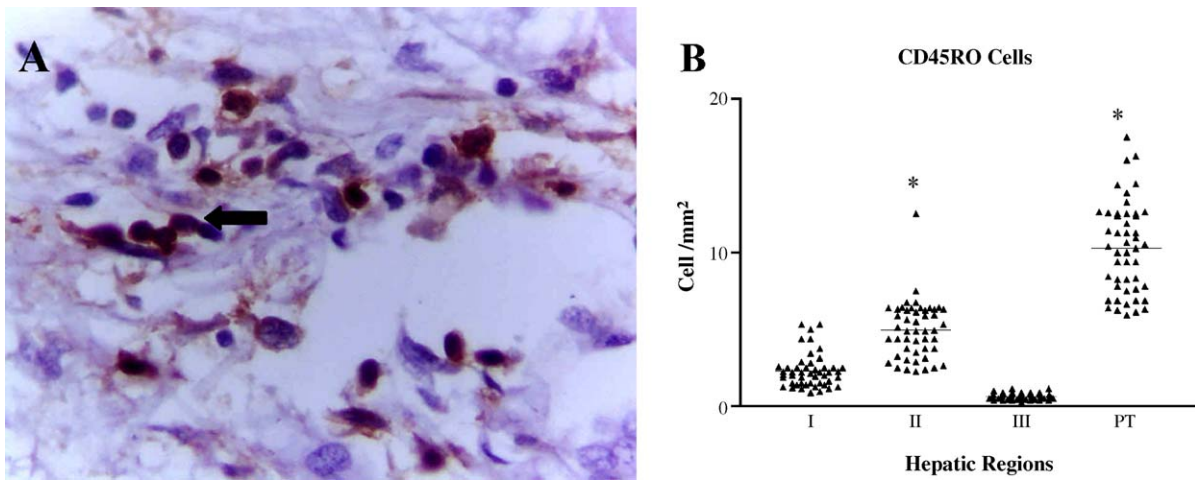


Fig. 3. Yellow fever liver: (A) immunohistochemistry of T lymphocytes (400×); (B) quantitative analysis of T lymphocytes with predominance of cells in the midzonal region and portal tract. (* $p \leq 0.05$, ANOVA/Bonferroni).

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 cyto-

pathic 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,

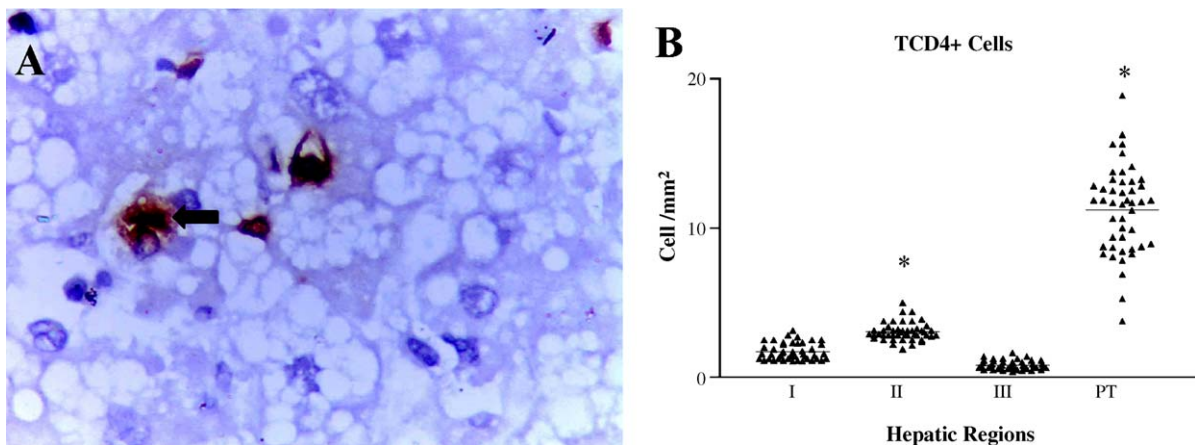


Fig. 4. Yellow fever liver: (A) immunohistochemistry of CD4 lymphocytes in the acini (400×); (B) quantitative analysis of distribution of CD4 positive cells showing predominance in the midzonal region. (* $p \leq 0.05$, ANOVA/Bonferroni).

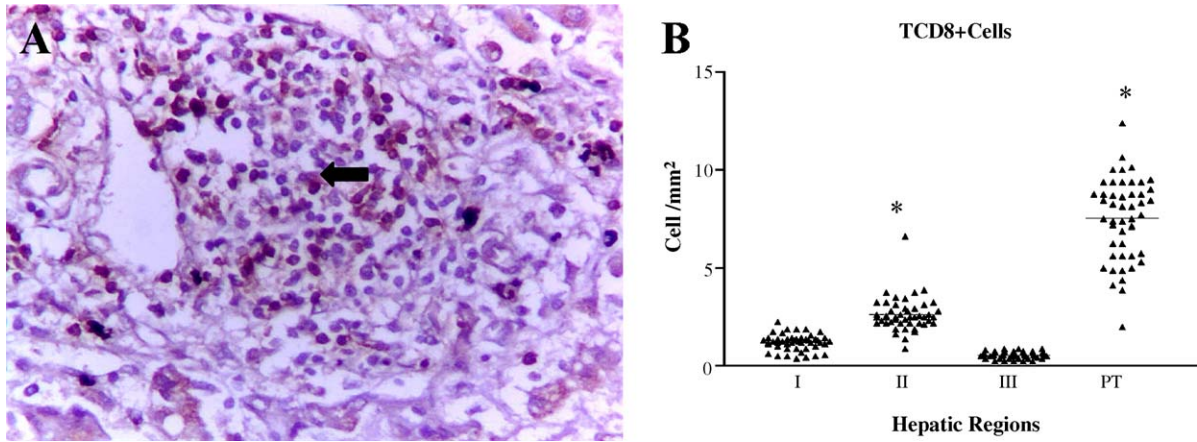


Fig. 5. Yellow fever liver: (A) immunohistochemistry of CD8 lymphocytes in the portal tract (200×); (B) quantitative analysis of distribution of CD8 positive cells showing predominance in the midzonal region. (* $p \leq 0.05$, ANOVA/Bonferroni).

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 immunorexpression 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).

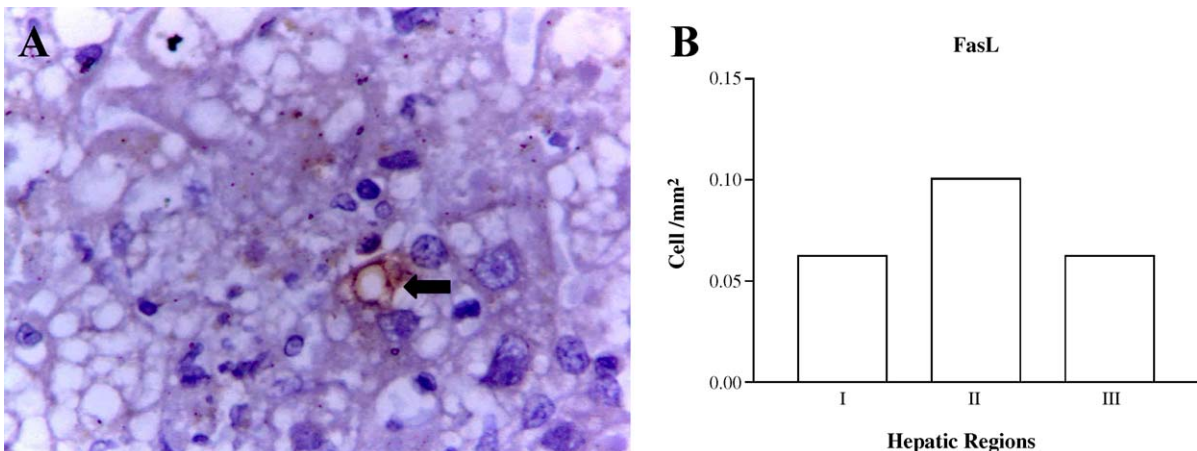


Fig. 6. Yellow fever liver: (A) immunohistochemistry of CD95 (FasL–Fas-ligant) in the acini (400×); (B) quantitative analysis of distribution of FasL positive cells showing predominance in the midzonal region. (* $p \leq 0.05$, ANOVA/Bonferroni).

It was interesting to observe in our samples the distribution of B cells, which are classically responsible for the production of antibodies, they were found in all acini areas and as observed for T lymphocytes were also predominantly found in the midzone.

More recently, it has been shown that the immune response to viral infections does not always lead to destruction of the host cell but causes the release of antibodies and cytokines with eminently antiviral functions (Samuel, 2001).

In conclusion, the present systematized quantitative study confirms that hepatocytes death in yellow fever is preferentially resulted from apoptotic phenomena and which several immune many factors are involved in induction of programmed cells death; the T lymphocytes probably induce apoptosis through its interaction with FasL receptors; because of the disproportion found between injury and inflammation, it is probably that other immune factors are involved in the pathogenesis of the liver in the yellow fever. Perhaps, the cytokines play an important role in this process; finally, other investigations are necessary to determine and quantify the role of lymphocytes and the cytokines in induction of necrosis and apoptosis observed during the course of severe yellow fever, especially in the liver failure.

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References

- Bertoletti, A., Maini, M.K., 2000. Protection or damage: a dual role for the virus-specific cytotoxic T lymphocyte response in hepatitis B and C infections? *Curr. Opin. Immunol.* 12, 403–408.
- Branquet, D., 1996. A yellow fever liver. *Med. Trop.* 56, 224.
- Chisari, F.V., Ferrari, C., 1995. Hepatitis B virus immunopathogenesis. *Annu. Rev. Immunol.* 13, 29–60.
- Councilman, W.T., 1990. Report on etiology and prevention of yellow fever. *Public Health Bull.* 2, 151–159.
- De Brito, T., Siqueira, S.A.C., Santos, R.T.M., Nassar, E.S., Coimbra, T.L., Alves, V.A.F., 1992. Human fatal yellow fever: immunohistochemical detection of viral antigens in the liver, kidneys and heart. *Pathol. Res. Pract.* 188, 177–181.
- De La Monte, S.M., Linhares, A.L., Travassos da Rosa, A.P.A., 1983. Immunoperoxidase detection of yellow fever virus after natural and experimental infection. *Trop. Geogr. Med.* 35, 235–241.
- Deubel, V., Huerre, M., Cathomas, G., Drouet, M.T., Wuscher, N., Le Guenno, B., Widmer, A.F., 1997. Molecular detection and characterization of yellow fever virus in blood and liver specimens of a non-vaccinated fatal human case. *J. Med. Virol.* 53, 212–217.
- Elton, N.W., Romero, A., 1955. Clinical pathology of yellow fever. *Am. J. Clin. Pathol.* 25, 135–137.
- Galle, R.P., Hofmann, W.J., Walczak, H., Schaller, H., Otto, G., Stremmel, W., Krammer, P.H., Runkel, L., 1995. Involvement of the CD95 (APO-1/Fas) receptor and ligand in liver damage. *J. Exp. Med.* 182, 1223–1230.
- Ganem, D., Prince, A.M., 2004. Hepatitis B virus infection—natural history and clinical consequences. *N. Eng. J. Med.* 350, 1118–1129.
- Gremion, C., Cerny, A., 2005. Hepatitis C virus and the immune system: a concise review. *Rev. Med. Virol.* 15, 235–268.
- Gold, R., Schmied, M., Giegerich, G., Breitschopf, H., Hartung, H.P., Toyka, K.V., Lassmann, H., 1994. Differentiation between cellular apoptosis and necrosis by combined use of in situ tailing and nick translation techniques. *Lab. Invest.* 71, 219–225.
- Hall, W.C., Crowell, T.P., Watts, D.M., 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.
- Harwick, J.M., 1998. Viral interference with apoptosis. *Sem. Cell Dev. Biol.* 9, 339–349.
- Hsu, S.M., Raine, L., Fanger, H., 1981. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase technique: a comparison between ABC and unlabeled antibody (PAP) procedure. *J. Histochem. Cytochem.* 29, 577–580.
- Ishak, K.G., Walker, D.H., Coetzer, J.A., Gardner, J.J., Gorelkin, L., 1982. Viral hemorrhagic fevers with hepatic involvement: pathologic aspects with clinical correlations. *Prog. Liver Dis.* 7, 495–515.
- Kaufmann, S., Hengartner, M.O., 2001. Programmed cell death: alive and well in the new millennium. *Trends Cell Biol.* 11, 526–534.
- Klotz, O., Belt, T.H., 1930a. The pathology of liver in yellow fever. *Am. J. Pathol.* 6, 663–687.
- Klotz, O., Belt, T.H., 1930b. Regeneration of liver and kidney following yellow fever. *Am. J. Pathol.* 6, 689–697.
- Kerr, J.F.R., Wyllie, A.H., Currie, A.R., 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* 26, 239–257.
- Kerr, J.A., 1973. Letter: liver pathology in yellow fever. *Trans. R. Soc. Trop. Med. Hyg.* 67, 882–883.
- Maini, M.K., Boni, C., Lee, C.K., Larrubia, J.R., Reignat, S., Ogg, G.S., King, A.S., Herberg, J., Gilson, R., Alisa, A., Williams, R., Vergani, D., Naoumov, N.V., Ferrari, C., Bertoletti, A., 2000. The role of virus-specific CD8+ cells in liver damage and viral control during persistent hepatitis B virus infection. *J. Exp. Med.* 191, 1269–1280.
- Majno, G., Joris, I., 1995. Apoptosis, oncosis, and necrosis. An overview of cell death. *Am. J. Pathol.* 146, 3–15.
- Makinen, J.K., 2004. Histopathologic diagnosis of hepatitis. *Duodecim* 120, 2433–2440.
- Marinneau, P., Steffan, A.M., Royer, C., Drouet, M.T., Kirn, A., Deubel, V., 1998. Differing infection patterns of dengue and yellow fever viruses in a human hepatoma cell line. *J. Infect. Dis.* 178, 1270–1278.
- Monath, T.P., Ballinger, M.E., Miller, B.R., Salaun, J.J., 1989. Detection of yellow fever viral RNA by nucleic acid hybridization and viral antigen by immunocytochemistry in fixed human liver. *Am. J. Trop. Med. Hyg.* 40, 663–668.
- Monath, T.P., 2001. Yellow fever: an update. *Lancet Infect. Dis.* 1, 11–20.
- Monath, T.P., Barrett, A.D., 2003. Pathogenesis and pathophysiology of yellow fever. *Adv. Virus Res.* 60, 343–395.
- Ogasawara, J., Watanabe-Fukunaga, R., Adachi, M., Matsuzawa, A., Kasugai, T., Kitamura, Y., Itoh, N., Suda, T., Nagata, S., 1993. Lethal effect of the anti-Fas antibody in mice. *Nature* 364, 806–809.
- Peters, C.J., Zaki, S.R., 2002. Role of the endothelium in viral hemorrhagic fevers. *Crit. Care Med.* 30, 268–273.
- Quaresma, J.A.S., Barros, V.L.R.S., Fernandes, E.R., Pagliari, C., Takakura, C.F.H., Vasconcelos, P.F.C., Andrade-Junior, H.F., Duarte, M.I.S., 2005. Reconsideration of histopathology and ultrastructural aspects of the human liver in yellow fever. *Ac. Trop.* 94, 116–127.
- Ryo, K., Kamogawa, Y., Ikeda, I., Yamauchi, K., Yoneraha, S., Nagata, S., Hayashi, N., 2000. Significance of Fas-antigen-mediated apoptosis in human fulminant hepatic failure. *Am. J. Gastroenterol.* 95, 2047–2055.
- Roulston, A., Marcellus, R.C., Branton, P.E., 1999. Viruses and apoptosis. *Annu. Rev. Microbiol.* 53, 577–628.
- Samuel, C.E., 2001. Antiviral actions of interferons. *Clin. Microbiol. Rev.* 14, 778–809.
- Thompson, C.B., 1995. Apoptosis in the pathogenesis and treatment of disease. *Science* 267, 1456–1492.
- Vasconcelos, P.F.C., Costa, Z.G., Rosa, E.S.T., Luna, E., Rodrigues, S.G., Barros, V.L.R.S., Dias, J.P., Oliva, O., Vasconcelos, H.B., Oliveira, R.C., Souza, M.R.S., Silva Jr., J.B., Martins, E.C., Rosa, J.F.S.T., 2001a. An epidemic of jungle yellow fever in Brazil, 2000. Implications of climatic alterations in disease spread. *J. Med. Virol.* 65, 598–604.

- Vasconcelos, P.F.C., Rosa, A.P.A., Rodrigues, S.G., Rosa, E.S.T., Monteiro, H.A.O., Cruz, A.C.R., Barros, V.L.R.S., Souza, M.R.S., Rosa, J.F.S.T., 2001b. Yellow fever in Para State, Amazon region of Brazil, 1998–1999: entomologic and epidemiologic findings. *Emerg. Infect. Dis.* 3, 565–569.
- Vasconcelos, P.F.C., Bryant, J., Rosa, A.P.A.T., Tesh, R.B., Rodrigues, S.G., Barrett, A.D.T., 2004. Genetic divergence and dispersal of yellow fever virus. Brazil. *Emerg. Infect. Dis.* 9, 1578–1584.
- Vieira, W.T., Gayotto, L.C., de Lima, C.P., 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.
- Xiao, S.Y., Zhang, H., Guzman, H., Tesh, R.B., 2001. Experimental yellow fever virus infection in the Golden Hamster (*Mesocricetus auratus*). 2. Pathology. *J. Infect. Dis.* 183, 1437–1444.