SERIAL OPENED BIOPSIES APPLIED IN NONHUMAN PRIMATE MODEL TO STUDY THE PATHOGENESIS OF HEPATITIS A VIRUS.

ABSTRACT

Nonhuman primates are useful model to study the pathogenesis of viral hepatitis. In this paper we described a serial organs biopsies in HAV experimental infection carried out in *Callithrix jacchus* to determine morphologic and immunological changes using histological and immunohistochemical methods to detect HAV antigens in samples of liver, kidney, mesenteric lymph nodes and intestine, collected by biopsies using surgical techniques. The opened approach was used throughout a serial of experiments involving HAV infection. It was described and critically evaluated by us confirming that the biopsies procedures employed in experimental hepatitis virus infection in nonhuman primates are very useful for obtaining data on the pathogenesis of this infectious disease.

INTRODUCTION

The use of nonhuman primates as an experimental animal model has contributed to increasing of scientific understanding of many infectious diseases, mainly viral hepatitis as related (Lemon 1994). Numerous attempts have been made to transmit human hepatitis virus to nonhuman primates and several species of them have been showing susceptible to the human
hepatitis viruses (Smetana 1965, Beacroft 1969, Lorenz et al., 1970, Ebert et al., 1978, Trahan et al., 1987, Baptista et al., 1993). Because of host specificity of hepatitis A virus (hepatovirus), monkeys have been the only animal model to study the pathogenesis of HAV infection. However, other authors have been described HAV infection in guinea pigs (Hornei et al., 2001).

Experimental HAV infections have been reproduced in various nonhuman primate species which HAV antigens have been detected in the cytoplasm of hepatocytes before clinical illness, rise in hepatic enzyme activity in serum, and histological changes in liver (Vallbracht et al., 1993, Lemon, 1994, Pinto et al., 2000 and 2001). These features were accompanied by the release of the hepatitis A virions in feces (Mathiesen et al., 1980). However, extrahepatic sites of HAV replication as small-intestinal epithelium, lamina propria (Karayiannis et al., 1986, Asher et al., 1995), palatine tonsils and oropharynx (Cohen et al., 1989) have been investigated, but its relevance for viral spread and replication is not known for the human HAV.

The criteria to evaluate the infectivity and pathogenesis of experimental hepatitis virus infections include virus replication site, cellular changes and the pathophysiological alterations in liver and in various organs (Talcott & Dysko, 1991). Liver biopsy becoming an important diagnostic tools in the evaluation of hepatic morphology and assessment of biochemical properties of the liver to both clinical and research purposes (Bravo et al., 2001). In addition, tissue biopsies represent an invaluable source of material for the molecular characterization of RNAs, DNAs and viral proteins. The lack of clinical signs after infection with HAV in marmosets and in other nonhuman primate species has been reported (Dienstag et al., 1975, Lemon, 1994, Tabor, 1998, Chitambar et al., 2001, Pinto et al., 2001) contributing to increase the importance of tissue investigation in confirmation of virus-induced liver lesions.

Liver biopsy can be performed in several ways: open-wedge specimens, obtained from the free edge of the liver at laparotomy or semi-open under peritoneoscopic observation, whether with a forceps or, preferably, by a fine needle aspiration guided by ultrasonography or computed tomography (CT) and the “blind” liver biopsy, without visual control (Bravo et al., 2001). In the closed technique the biopsy was performed using transthoracic or transabdominal approach (Voss, 1970). Percutaneous liver biopsy is the quickest and the simplest method used to collected small biopsy samples. The risk of an aberrant puncture can be decreased combining the needle biopsy with a “keyhole” technique using when a small cranial ventral midline incision is made to ease localization and immobilization of the liver lobes by direct digital palpation purposes (Carter & Valli, 1990, Talcott & Dysko, 1991). Transjugular liver biopsy involves percutaneous puncturing of the right internal jugular vein, the introduction, with the use of fluoroscopy, of a catheter into the hepatic right vein, and a needle biopsy of the liver performed through the catheter (De Hoyos et al., 1999).
Surgical methods for liver wedge biopsy use methods such as finger fracture of the liver parenchyma, clamping or laparotomy followed by wedge biopsy using an automatic stapling device (Nolan & Conti, 1980). Open biopsy procedures have been previously described in dogs, cats, nonhuman primates and humans (Furneaux, 1975, Nolan & Conti, 1980, Breznock, 1983, Eichiberg, 1985). Laparotomy provided the adequate quantities of liver, kidney, mesenteric lymph node and small bowel tissues for light and electron microscopy observations.

In the present study, we describe the method of open biopsy used to collect tissue samples in a small nonhuman primates, *Callithrix jacchus*, that has served as animal models for hepatitis A virus (HAV) infection.

**MATERIALS AND METHODS**

**Nonhuman primates and inoculum:**

Four clinically healthy young-adult common marmosets, *Callithrix jacchus*, kindly provided by colony from São Bernardo do Campo Zoo (São Paulo, Brazil), were maintained in individual cages during quarantine and experiments. They were submitted to serological screening for HAV antibodies and liver enzyme (ALT). Environmental conditions included a temperature of 20-24°C, a relative humidity of 60%, and light for 12 hours per day. Laboratory standard diet was used with continuous free access to water. Animals were cared according to biosafety level 2 precautions and procedures outlined in the *Interagency Primate Steering Committee*. HAV strain HAF-203 (accession: GenBank AF268396) has been recovered from stools of a Brazilian child with sporadic hepatitis A infection (Gaspar et al., 1992). The stool samples were diluted 1% (w/v) in PBS (10 mM sodium phosphate, 0.15 M NaCl) with penicillin (100 IU/mL) and streptomycin (100 mg/mL), clarified by low-speed centrifugation and filtered through a 0.45 mm membrane. HAV was detected by a reverse transcription-polymerase chain reaction (RT-PCR) and quantified by an enzyme immunosorbent assay (ELISA) (1:320) (Gaspar et al., 1992). The amount of protein (15 ng/mL) was determined using the Lowry method modified (Peterson 1983). Each animal was given 0.5 mL of this suspension that was inoculated in the posterior pharynx with a blunt syringe under pharmacological sedation and immobility, one animal was orally inoculated with 0.5 mL of fecal suspension HAV free.

**General anesthesia and surgical procedure:**

The monkeys were sedated with 15mg/kg ketamine HCL (Vetalar, 100mg/ml, Parke-Davis, Detroit, Michigan) and with 0.1mg/kg midazolan (Roche, Brasil) administered intramuscularly. The intramuscular dose takes about 15 minutes to develop a rapid and excitement-free loss of consciousness. Additional doses were given for delayed effect. The surgical sites were clipped-free of hair and prepared for sterile surgery.

The biopsies were performed using standard median and paramedian abdominal incisions,
cranial to the level of the umbilicus. The liver was identified and randomized liver wedge samples were collected. When the liver has been incised attempts were made to stops the bleeding. The right kidney was exposed to collected the desired amount of kidney tissue by two longitudinal incisions. The wound wedges were coated with a flap using perirenal tissues and sutured with size 6-0 catgut in simple interrupted pattern. The loops of small bowel were with draw and covered with towels soaked with saline solution. Two Doyen intestinal clamps were inserted on each side of the section to be removed to prevent ingesta into the operative field. The sample of small intestine was resected through longitudinal incision made along the antimesenteric border. The intestine was sutured transversely in one layer. It is closed by a row of Schmieden suture that joins mucosal and serosal surface with 6-0 polypropylene stitches. The incision was adjusted by partial splectomy that included removal a third part of the organ. The mesenteric lymph nodes were collected with careful blunt dissection. Hemorrhage could be arrested by pressure or with ligatures. After the abdominal cavity was washed with warm physiological saline solution. Muscle and fascia were closed with size 5-0 polypropylene in a continuous suture. The subcutaneous pocket between skin and fascia were apposed with a continuous interrupted suture of size 6-0 catgut and skin was completely closed by simple isolated suture with size 5-0 mononylon. Each animal was biopsed twice during the 46 days of evaluation shortly monitored at least 48 hours after biopsies when bleeding or animal’s pain were evidenced.

**Evaluation of HAV infection**

**Stools:** Faeces were collected daily from the time of inoculation until histopathology liver changes return to normal morphology. The virus genome was detected by RT-PCR.

**Tissues/Immunofluorescent staining:** Liver, spleen, mesenteric lymph node, small intestine kidney Sections (4mm) fixed in formalin were twice washed xylene and alcohol (15min each, at 60°C) and PBS (3x 15min each) the slides were incubated at 37°C in humidified dark chamber with tripsin for 10min. They were washed and blocked. The first antibody (anti-HAV/ rabbit diluted in PBS 1:10) and the second antibody (anti-rabbit FITC, Sigma) were applied at 37°C for 30min. The slides were washed with water, dried and coverlips applied over a mounting of glycerol and anti-fade solution.

**Morphologic studies:** Sections of livers, spleen, small intestine and mesenteric lymph nodes, immediately after biopsy was divided into three sections. One sections was fixed in neutral 10% formalin solution for routine histological examination, a second section was immediately stored in nitrogen frozen for immunohistochemistry studies, and a third section with glutaraldehyde for standard thin-section electron microscopy.

**RESULTS**

**Post general anesthetic and surgical procedure:** Anesthetic procedures showed
depression of consciousness followed smoothly after administration with ketamine hydrochloride (15mg/kg) and midazolam (0,1mg/kg) intramuscularly. Hypothermia caused by anesthesia was rapidly reverted with warm blankets or monkeys usually warms at its own rate. All animals used in this study showed a safer induction of anesthesia and recovery was uniformly rapid and smooth.

Liver, small intestine, kidney and mesenteric lymph node biopsy samples were successfully collected in nonhuman primates to determine HAV presence. The peritoneal cavity was entered by a median or Para median approach with transverse incision through the rectus abdominal muscle. The incisions were chosen because they are advocated for repetitive laparotomy. The biopsies specimens were removed aseptically. Attachments were moderate between the omental fat and surgery areas. These were removed under traction always carefully to avoid the rupture of blood vessels. Hemorrhage from an injured lobe of liver was controlled by temporarily digital compression. Adhesions resulting from extensive use of suture were observed in all marmoset monkeys. This sometimes required blunt dissection to relieve the strictures. At 7 days after second laparotomy one animal showed a duodenal fistula and acute circumscribed peritonitis that was surgically repaired. It has been treated successfully after resection and duodenal anastomosis.

**Biological findings associated to hepatitis A virus infection**: Histological hepatocellular findings with biochemical changes associated with oral HAV infection were listed in table 1. Elevation of the serum alanine aminotransferase level occurred at the 3rd week post inoculation, fecal virus shedding was detected as early as 07 days after oral inoculation, seroconversion with appearance of anti-HAV IgG and IgM and morphological changes in the liver. All these findings suggested the prevalence of acute virus hepatitis. The presence of viral RNA in feces preceded development of liver enzyme abnormalities in all monkeys (Table 1) Seropositive monkeys were seen after day 30 postinoculation (IgG and IgM). Average baseline ALT (Alanine aminotransferase) values for *Callithrix sp.* have been previously reported by us and also morphological changes in the liver (Pinto et al., 2000).

<table>
<thead>
<tr>
<th>Days postinoculation</th>
<th>ALT (X*)</th>
<th>HAV RT-PCR in fecal samples. (positive animals/total animals)</th>
<th>Histopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4</td>
<td>45</td>
<td>(0/4)</td>
<td>No changes</td>
</tr>
<tr>
<td>7-15</td>
<td>40</td>
<td>(3/4)</td>
<td>No changes</td>
</tr>
<tr>
<td>15-20</td>
<td>50</td>
<td>(3/4)</td>
<td>Hepatocyte vacuolization</td>
</tr>
<tr>
<td>20-25</td>
<td>60</td>
<td>(3/4)</td>
<td>Focal inflammatory injury</td>
</tr>
<tr>
<td>45-46</td>
<td>60-70</td>
<td>(3/4)</td>
<td>Inflammatory injury, piecemeal necrosis and briding necrosis.</td>
</tr>
</tbody>
</table>

* media of values in infected animals

Table 1 – Summary of liver findings in *Callithrix sp.* orally inoculated with hepatitis A virus.
DISCUSSION

In this paper we described experimental procedures that has been used to assay of HAV infection in small specie of nonhuman primate, we have focused mainly on pathologic and immunological assessment of the disease process. By utilizing biopsies techniques we have successfully demonstrated pathologic changes of the liver and the degree of involvement of other organs. For collecting biopsies samples in *Callithrix jacchus* we have assayed by open technique. This procedure has shown to be most appropriated to small animals with reduced rate of complications when performed by experienced surgeons.

In our experimental routine procedures, closed suction biopsy has been performed with Menghini needle using a transabdominal approach, has been more appropriated to be used in *Macaca mulatta* or *Macaca fascicularis*. However this method has been critized because the small size sample obtained and artifacts causing difficulties in histological interpretation (Voss 1970; Purcell et al., 1975). We particularly emphasize this procedure as relatively inexpensive, simple, safe and very efficient for collecting repetitive liver samples in larger nonhuman primates (Dienstag et al., 1975, Nolan & Conti 1980).

Open biopsy procedure was performed for collection of liver, small intestine, kidney and lymph nodes samples with different size. Our results suggested that monkeys who underwent laparotomy by median or paramedian approaches could be a safe operation if performed well technically. Repeated biopsies were taken from areas not involved in the previous biopsy. This is particularly suitable for universal usage. Some disadvantages do exist with this procedure such as considerably longer sedation; require extensive manipulation of the abdominal organs, adhesions and strictures (Furneux, 1975; Negro, 1996), local peritoneal inflammation and peritonitis (Breznock, 1983, Bravo et al., 2001). Nevertheless, our data indicate that one animal shows three complications related to the procedure: adhesions, focal peritoneal inflammation and fistula. Fortunately, none of these episodes were serious adverse factors that contraindicate its use. Finally the liver disease associated with HAV infection was observed in all inoculated animals, and was compatible with acute hepatitis and the open biopsies procedure were appropriated to obtain kinetic information about HAV pathogenesis, that compensated the potential risk of the surgical intervention.
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