An ex Vivo Assay for Estimating the Antiviral State of Hepatocytes

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An ex vivo antiviral assay was established which uses hepatocytes from mice given recombinant mouse interferon-β (rmlIFN-β). Assay results were compared with results obtained with a 2',5'-oligoadenylylate synthetase (2-5AS) assay. rmlIFN-β was intraperitoneally administered to C3H mice and the antiviral state of their liver parenchymal cells was evaluated in an in vitro cytopathic effect assay. In this assay, cells are infected with vesicular stomatitis virus (VSV) and surviving cells are determined colorimetrically. The antiviral state was measured as the resistance of hepatocytes to VSV infection with increasing doses of rmlIFN-β. The antiviral state correlated well with the dose-dependent increase in hepatic 2-5AS activity. This good correlation suggests that induction of 2-5AS mediates the antiviral action of interferon in liver tissue. This ex vivo assay could be a useful tool for estimating the ability of hepatocytes to resist hepatitis virus infection.

Keywords ex vivo antiviral assay; recombinant mouse interferon-β (rmlIFN-β); hepatocyte; vesicular stomatitis virus (VSV); 2',5'-oligoadenylylate synthetase (2-5AS)

Interferon (IFN) therapy has been widely used to treat patients with chronic hepatitis B and C, and some patients show a good response to this therapy.1-4 The antiviral action was reported to involve the expression of antiviral proteins such as 2',5'-oligoadenylylate synthetase (2-5AS),5 protein kinase6 and phosphodiesterase7 which destroy viral mRNA and inhibit the synthesis of viral proteins. Experimental animal models of hepatitis have been established in chimpanzees,8 woodchucks,9,10 ducks,11 mice12,13 and rats.14 These models, however, are not necessarily appropriate for the routine laboratory investigation of IFNs because of their high cost, the species specificity of IFNs, and lack of organ-specific infection or inflammation in the liver.

Kramer et al. reported in vitro and in vivo the antiviral effects of recombinant human IFN-α (rhIFN-α) including subtypes A and D and the hybrid A/D.15 Witter et al. investigated antiviral responses, including both 2-5AS activity and resistance to vesicular stomatitis virus (VSV) infection, of peripheral blood mononuclear cells (PBMCs) isolated from healthy volunteers treated with rhIFN-α.16 Here, we have established an ex vivo assay for estimating the antiviral state of hepatocytes, isolated from mice given mouse IFN-β, against VSV, which has been widely used for measuring the titer of IFNs. In addition, the relationship between antiviral state and hepatic 2-5AS activity was investigated.

MATERIALS AND METHODS

Preparation of Rat Hepatocytes Recombinant mouse IFN-β (rmlIFN-β; 3.4 x 10^7 IU/mg), purchased from Funakoshi (Japan), was administered intraperitoneally at doses of 1.2 and 5 μg/mouse in 0.2 ml phosphate buffered saline (PBS) once daily for 3 d. Each group consisted of four male C3H/He mice aged 7 weeks. For a control, 0.2 ml PBS was injected into eight mice in a similar fashion. Hepatocytes were isolated 24 h after the third injection according to the method described by Seglen et al.17 Briefly, each mouse was anesthetized by intraperitoneal injection of sodium pentobarbital and its liver was exposed by a midline abdominal incision. The portal vein was catheterized with a polyethylene cannula (O.D. = 0.8 mm) attached to the perfusion system. The thoracic inferior vena cava was catheterized through the right atrium with another polyethylene tube, and the subhepatic inferior vena cava was ligated just above the renal vein. The liver was perfused at 37°C for 5 min with Ca^2+-Mg^2+-free Hanks solution (pH 7.2) containing 0.019% EGTA, followed by 15-min perfusion with Hanks solution (pH 7.5) containing 0.05% collagenase (Wako, Japan) and 0.005% trypsin inhibitor (Sigma Chem. Co., MO). The liver was then taken out, cut into small pieces and suspended gently in 10 ml Eagle's minimum essential medium (E-MEM). After filtering through gauze, parenchymal cells were separated by repeated centrifugation (50 × 1 min, 3 times) and resuspended in E-MEM containing 10% fetal calf serum. All procedures were carried out under sterile conditions.

Antiviral State Mouse parenchymal cells were seeded at a concentration of 1.5 x 10^6 cells/100 μl well in 96-well cell culture plates (Primaria®, Falcon, NJ), followed by cultivation at 37°C in a 5% CO2 incubation for 3 h. VSV suspension (donated by the Ministry of Agriculture, Forestry and Fisheries) in E-MEM containing 10 mM HEPEs was then added to each well at concentrations ranging from 8 x 10^3 to 2 x 10^5 PFU/50 μl/well and incubated at 37°C in a 5% CO2 incubator for 3 d. The viral cytopathic effect (CPE) was determined by colorimetric measurement of surviving cells using 3-(4,5-dimethylthiazo-l-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chem. Co., MO). Briefly, 20 μl MTT solution (5 mg/ml in PBS) was added to each well, followed by incubation at 37°C in a 5% CO2 incubator for 4 h. One hundred μl 10% sodium dodecyl sulfate were then added and incubated overnight at 37°C. The absorbance at 590 nm was measured by a multiwell scanning spectrophotometer (Titertek multitiskan®, ICN Biomedicals, CA), and plotted against the logarithm of the viral concentration. The VSV concentration needed to produce a 50% CPE was used as an index of the antiviral response of parenchymal cells to interferon treatment.
2-5AS Activity: Mouse parenchymal cells were also subjected to 2-5AS assay using a 2-5A kit (Eiken, Japan). After washing 3 times with PBS, the cells were resuspended in the same buffer, frozen and thawed once, and then sonicated for 15 s on ice. After centrifugation (7200 g × 30 min) at 4 °C, the supernatant was filtered through a 0.2 μm filter and the 2-5AS activity measured by radioimmunoassay using 125I-labeled 2',5'-oligoadenylate (2-5A). The enzyme activity was expressed as the amount (pmol/dl) of 2-5A synthesized from adenosine-5'-triphosphate. The protein concentration of each filtrate was determined by a protein assay kit (Bio-Rad, CA) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

The viral CPE on hepatocytes was evident, with increasing concentrations of VSV, 3d after infection, when the antiviral state of the cells was measured. The hepatocytes from mice given rmIFN-β exhibited more resistance to VSV infection than those from control mice. Approximately 2.0- and 5.7-fold higher concentrations of VSV were needed to achieve a 50% CPE on the hepatocytes from mice given 1.2 and 5 μg/mouse of rmIFN-β, respectively, when compared with control mice given PBS alone (Fig. 1). Similar dose-dependent resistance to VSV was also observed in hepatocytes 6 d after infection (data not shown).

2-5AS was significantly induced in a dose-dependent manner in the hepatocytes isolated from mice given rmIFN-β (Fig. 2). The enzyme activity per mg protein in hepatocyte extracts was 3.2- and 5.7-fold higher in mice given 1.2 and 5 μg/mouse of rmIFN-β, respectively, compared with control mice, and this correlated well with the dose-dependent increase in the antiviral state against VSV in the hepatocytes (r=0.80; Fig. 3).

Antiviral responses of IFNs have been demonstrated in PBMCs from healthy volunteers, cancer patients, and patients with chronic hepatitis B. Many studies have shown a good correlation between the induction of 2-5AS and the development of the antiviral state in PBMCs, suggesting that 2-5AS plays a role in the antiviral action of IFNs. PBMCs, however, are not the primary tissue infected with hepatitis virus, and little has been reported about what happens in liver which is the target organ of the virus. Therefore, the present study was conducted in mice to investigate the antiviral responses of IFNs in liver.

The present ex vivo assay can be used for the evaluation of galactosylated IFN-α targeted to liver and of long-acting IFN preparations including pegylated IFNs and gelatin microspheres. Our recent study found galactosylated IFN-α to be distributed largely into target parenchymal cells in liver immediately after intravenous and intraperitoneal injections (unpublished data). Further studies will use the present assay to examine the nature of...
and extent of the antiviral state localized in liver.

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REFERENCES