Liver and biliary

Relationship of HLA protein display to activation of 2-5A synthetase in HBe antigen or anti-HBe positive chronic HBV infection

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SUMMARY In this study we have examined the density of HLA class I protein display on the hepatocytes of patients with HBe antigen (HBeAg) or anti-HBe (HBeAb) positive chronic hepatitis B virus (HBV) infection and related this to the level of interferon activation of these cells determined by measuring hepatic oligo 2-5A synthetase activity. In HBeAg positive patients the density of HLA class I (HLA-I) protein was not significantly increased above that found in uninfected liver, but levels of 2-5A synthetase were twice normal. In anti-HBe positive patients, HLA-I density was markedly increased in the presence of normal 2-5A synthetase activity. These data are consistent with type I (α or β) interferon activation of the hepatocytes in HBeAg positive patients and type II (γ) interferon activity in anti-HBe positive subjects.

The interferon system has potent antiviral effects in man inducing an antiviral state in uninfected cells. Alpha and beta (type I) interferons are structurally similar, act through a common receptor¹ and are produced in response to viral infection of most cell types.² Gamma interferon (type II) is a lymphokine acting through a different receptor³ and is produced exclusively by T lymphocytes in response to mitogenic or antigenic stimuli.^{4,5}

In vitro production of alpha and beta interferon can be stimulated using polynucleotides and the antiviral state can be shown to be related to interferon induced increases in 2-5A synthetase activity which results in production of oligo adenylylate nucleotides in the cell.⁶ In addition interferon has been shown to enhance synthesis and expression of HLA I on the surface of cells⁷ even those that do not usually display HLA I antigens – for example, hepatocytes.⁸ Both types I and II interferon exhibit these properties but the latter is 100-fold more potent in stimulating HLA I antigen expression.⁹

We have measured the level of expression of HLA I protein on the hepatocyte membranes and of 2-5A synthetase activity in liver tissue from HBeAg and HBeAb positive HBV carriers. We also obtained liver biopsies from 3 HBeAg positive

patients after initiation of treatment with lymphoblastoid interferon (α) (Wellferon) and measured the same variables.

Methods

PATIENTS

Forty patients with chronic HBV infection, 21 HBeAg positive and 19 anti-HBe positive were studied. The majority¹⁵ of the HBe antigen positive patients were homosexuals who had been HBV carriers for at least six months and in many cases for several years. The onset of the carrier state and therefore its duration, were usually not known. The anti-HBe carriers were referred because of the identification of abnormal liver function tests: eight of these were homosexual. All the patients were anti-delta negative by RIA. In the HBeAg positive group, 10 patients had chronic persistent hepatitis, 10 chronic active hepatitis and one cirrhosis. In the anti-HBe positive group, eight patients had chronic persistent hepatitis, six chronic active hepatitis, and five cirrhosis. The time of seroconversion from HBe antigen to antibody was not known. Many patients had, however, been known to be anti-HBe positive for several years. None of the patients had received therapy with immunosuppressants or antiviral compounds in the 18 months before the study. Many of the HBeAg positive patients were being investigated before antiviral therapy.

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Liver specimens obtained by percutaneous needle biopsy were rapidly frozen in isopentane and stored in liquid nitrogen. In 24 cases, samples of the biopsies were also stored at -70°C for 2-5A synthetase assay.

In addition 2-5A synthetase activity was measured in liver biopsies from three HBeAg positive patients (CAH) after receiving two doses of lymphoblastoid interferon (10 mega units/ m^2 /day by intramuscular injections). The material was obtained by delaying the routine diagnostic/prognostic biopsy for 24 hours after the second injection of interferon. Thus these patients had only one biopsy, which is in accordance with normal practice in HBV carriers with increased transaminases. These three patients were receiving interferon as part of a randomised trial to determine whether a three month course of interferon has therapeutic value in ameliorating the course of the disease. These studies were approved by the Hospital Ethics Committee and informed consent obtained from the patients. All biopsies were prepared for routine histological examinations and also for immunofluorescence studies of HLA display.

2'5' OLIGOADENYLATE SYNTHETASE ASSAY

Liver biopsy specimens were homogenised in lysis buffer containing 0.5% Nonidet-P 40. The liver extract was clarified by centrifugation for three minutes in an Eppendorf microfuge, and then frozen at -70°C until assayed. For enzyme assay, 40 μl poly (rI):(rC) agarose beads (PL Biochemicals) which had been mixed with 40 μl liver extract were incubated at 30°C for 18–20 hours in the presence of ATP (2.5 mM) and 2.5 μCi of [^3H]ATP.¹⁰

The incubated reaction mixture was then heated at 85°C for five minutes. [^3H] Labelled 2-5A was extracted by binding to DEAE-cellulose and the radioactivity of the entire sample measured after dilution into 10 ml liquid scintillant. The protein content was determined by the Lowry technique. The enzyme activity was expressed as nmol 2-5A/mg protein in the homogenate per hour. In each assay, three normal liver extracts (obtained from transplant donors) were assayed, and because of intertest variation the 2-5A synthetase levels were expressed as a percentage of these mean enzyme activities.¹⁰

MEASUREMENT OF HLA-I DISPLAY

Cryostat sections 5 μm thick were cut from frozen biopsies and air dried at room temperature overnight. Sections were then fixed for five minutes in chloroform-acetone solutions (1:1), washed with phosphate buffered saline (PBS) pH 7.2 for 10

minutes, air dried at room temperature for two hours and then stored at -70°C until stained.

HLA class I was detected using W6/32 monoclonal antibody (Sera Laboratories, Sussex, England) by indirect assay using rabbit antimouse Ig, fluorescein conjugated (Dako, Denmark), as a second layer reagent.¹¹ Fixed sections were warmed to room temperature and rehydrated for 15 minutes in a PBS bath. Sections were incubated for 30 minutes at room temperature in a humidified chamber with 10 μl aliquots of the monoclonal antibodies. Slides were then washed with PBS for 30 minutes and 10 μl of the second layer reagent was added for a further 30 minutes. After washing with PBS for 30 minutes, slides were mounted in PBS glycerol with p-phenylenediamine and examined using a Zeiss microscope equipped with an IV/2 epifluorescence condenser. Control sections were incubated either with the fluorescein conjugated antibody alone or with normal human serum. All slides treated in this way were negative. HLA class I staining intensity was estimated semiquantitatively using a Zeiss microscope fitted with a Zeiss photometer 03, as previously described.¹² The measurements were standardized using a fluorescein standard and expressed in arbitrary units as the intensity of fluorescence per unit length of hepatocyte membrane. The margin of the cell, distant from the sinusoid, was used so that fluorescence from the intensely staining endothelial and Kupffer cells was not recorded. Saturating concentrations of the monoclonal antibody and second layer reagent were identified and used for subsequent examinations. The results were expressed as the mean $\pm$ standard error of the mean (SEM) of eight areas selected randomly in the lobule.

STATISTICAL ANALYSIS

This was undertaken using the Mann Whitney U test.

Results

HLA-I protein display on hepatocytes is shown in Figure 1. In patients with HBeAg positive disease the HLA-I antigen display was significantly lower ($M \pm SD = 3.73 \pm 1.14$) than that in HBeAb positive patients ($M \pm SD = 7.69 \pm 1.55$). The HLA-I display in the HBeAg positive group was not significantly related to the histological activity, although those with CAH showed most display in areas of inflammatory activity (Figure 1). In the HBeAb positive group, patients with chronic active hepatitis had significantly more HLA-I display than patients with chronic persistent hepatitis (Figure 1).

Hepatic 2-5A synthetase is shown in Fig. 2. In

Fig. 1 Relationship of HLA I protein display on hepatocytes to the histological diagnosis in the HBeAg and HBeAb positive patients (CPH=chronic persistent hepatitis, CAH=chronic active hepatitis, CIR=cirrhosis). The dashed line represents the upper limit of the normal range (mean $\pm$ SD = 3.5 ± 1.5) obtained in four normal controls.

HBeAg positive patients this was significantly higher ($M \pm SD = 214.28 \pm 71.43$) than in normal controls ($M \pm SD = 107.14 \pm 28.57$) ($p < 0.005$) and anti-HBe positive patients ($M \pm SD = 92.86 \pm 42.85$) ($p < 0.0001$). Anti-HBe positive subjects and normal controls did not differ significantly. The level of enzyme activity was not related to histological stage of the liver disease (Fig. 2).

After initiation of lymphoblastoid interferon therapy a high ($10 \times$ normal) level of 2-5A synthetase activity was observed with a moderate increase ($2 \times$ normal) of HLA display on the hepatocytes (Table).

Table Increase of 2-5A synthetase and HLA I protein on the hepatocytes in three patients after two doses of lymphoblastoid IFN. 2-5A synthetase levels are expressed as percentage of the mean enzyme activities of three normal liver extracts obtained from transplant donors. HLA I display is expressed as mean fluorescence intensity per unit length of hepatocytes membrane $\pm$ SEM.

Patient	2-5A Synthetase (% normal hepatic activity)	HLA I Display (mean fluorescence intensity $\pm$ SEM)
1	1470	11.2 ± 2.3
2	782	7.9 ± 1.8
3	697	6.6 ± 1.4

2-5A SYNTHETASE ACTIVITY IN CHRONIC HBV CARRIERS

Fig. 2 Relationship of 2-5A synthetase activity in the liver to the histological diagnosis in the HBeAg and HBeAb positive patients (CPH=chronic active hepatitis, CIR=cirrhosis). The values are expressed as percentage of mean enzyme activities of normal liver samples.

Discussion

These data confirm our earlier observation that patients with chronic HBV infection who are HBeAg positive have a low density of HLA-I protein on their hepatocytes. In contrast, patients who are anti-HBe positive have a significantly higher degree of HLA-I expression.¹² This was initially thought to reflect the production of alpha interferon during and after the HBe antigen/antibody seroconversion¹³ but in this study the demonstration that 2-5A synthetase levels in the HBeAg positive patients are significantly higher than those in the anti-HBe positive patients suggests that interferon production is occurring during the phase of viral replication. This is probably type I (α and/or β) interferon which is a powerful inducer of 2-5A synthetase¹⁴ but a weak inducer of HLA-I protein expression.¹⁵ The 10-fold rise of 2-5A synthetase associated with only a two-fold increase in HLA-I expression in patients receiving lymphoblastoid interferon, supports the contention that

alpha interferon is more potent at inducing 2-5A synthetase than HLA-I expression in these patients.

An alternative explanation of the selective increase in 2-5A oligosynthetase might be that HBV replication selectively inhibits the ability of the hepatocyte to enhance HLA I display under type I interferon stimulation. This might be related to the mimicry by the virus of cellular interferon consensus sequences¹⁶ which are normally involved in the induction of transcription of HLA genes during interferon stimulation.

The high level of HLA-I protein display associated with minor or undetectable increases in 2-5A synthetase activity found in anti-HBe positive carriers is consistent with a state of γ interferon activation of the hepatocytes. This interferon is known to be a potent stimulant of HLA class I protein synthesis, and is capable of inducing HLA I display without producing changes in 2-5A synthetase activity.¹⁵ This suggests that HBeAg/antibody seroconversion, which usually coincides with clearance of hepatocytes containing replicating HBV,¹³ may be associated with γ interferon production presumably from T cells specific for HBV antigens. Recent data would suggest that HBc¹⁷ or HBe antigens¹⁸ are recognised during this phase of the infection. The continued rise in HLA I display in the absence of increased 2-5A synthetase activity during the established anti-HBe positive phase of the chronic infection, if also due to continued interferon production, would infer that T-cells sensitised to nucleocapsid proteins continue to circulate and exposure to these proteins, displayed in small quantities in the liver, release γ interferon. Although further data are needed to examine this possibility directly, the fact that immunosuppression in these anti-HBe carriers may lead to reactivation of HBV¹⁹ and renewed expression of nucleocapsid proteins, is consistent with control of HBV replication being a balance between established cellular and humoral immunity and low level HBV replication and expression. Whether this is also true in patients with established immunity (anti-HBs positivity) after acute hepatitis B virus infection, remains to be determined.

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