patients had neither anti-AChR antibodies by IP assay nor any clinical sign of MG. No detectable anti-AChR antibodies were found in these patients. Kyriatsoulis et al. (11) reported a high incidence of positivity in patients with primary biliary cirrhosis, but we could not detect any anti-AChR antibodies in these patients.

Our data indicate that this new assay is reliable in serum samples having relatively low or negative antibody titers by the IP assay and may have great potential for clinical use. However, we found that 10–15% of MG patients still have no detectable AChR antibodies: such cases will need re-evaluation with more specific and sensitive tests before any conclusions about them can be drawn.

This work was supported in part by a research grant for the Intractable Diseases from the Ministry of Health and Welfare, Japan.

References

Effects of Recombinant Leukocyte Interferon on Ribonuclease Activities in Serum in Chronic Hepatitis B

Hiroshi Teuji, Koichiro Murai, Kimihiro Akagi, and Masatochi Fujishima

Alkaline ribonuclease (RNase; EC 3.1.27.5) activities and 2',5'-oligoadenylate synthetase (2-5 AS; no EC no. assigned) activities in serum were measured in nine patients with hepatitis B e antigen-positive chronic hepatitis B before, during, and after treatment with recombinant human interferon α-2b for four weeks at daily doses ranging from 3 to 10 mega-units. Alkaline RNase activities in serum significantly increased from 65.8±9.5 units/L (mean±SD) to 84.3±11.9 units/L after the first week of therapy (P < 0.001). (One unit of RNase activity is defined as that increasing the absorbance at 260 nm by 1.0 in 1 min.) This increase persisted during and until two weeks after the end of the therapy, at which time the mean RNase activity in serum was still significantly increased (70.8±9.4 units/L, P < 0.01). Before therapy, phosphocellulose chromatography of RNase showed five active peaks of enzyme activity, which were similar to that observed even when RNase activity increased immediately after therapy. There was a close correlation between RNase activities and the logarithm of 2-5 AS activities, measured simultaneously in each patient. We conclude that recombinant α-interferon therapy increases RNase activities in serum, associated with the increased 2-5 AS activities.

The antiviral action of interferon (IFN) has been demonstrated to be mediated by activation of endonuclease, which degrades viral messenger ribonucleic acid (RNA).1 In IFN-treated cells, IFN-induced 2',5'-oligoadenylate synthetase (2-5 AS) catalyzes the synthesis of 2',5'-oligoadenylate (2-5 A) from ATP in the presence of double-stranded RNA, and 2-5 A activates endonuclease (1-3). Because IFN may inhibit viral replication in this way, endonuclease may play an important role in the antiviral action of IFN. Increased concentrations of ribonuclease (RNase; EC 3.1.27.5) activity in human serum have been reported in cases of renal failure (4, 5), pancreatic cancer (6), multiple myeloma (7), leukemia (8), and other diseases (9). However, the effect of IFN therapy on RNase activity in serum has not been previously reported.

Recently, recombinant human leukocyte IFN has been used for treating chronic hepatitis B virus (HBV) infection.

1 Nonstandard abbreviations: 2-5 AS, 2',5'-oligoadenylate synthetase; IFN, interferon; 2-5 A, 2',5'-oligoadenylate; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; HBeAg, hepatitis B surface antigen; ALT, alanine aminotransferase; AST, aspartate aminotransferase; and RNase, ribonuclease.
A variable response rate, usually seroconversion from hepatitis B e antigen (HBeAg) to antibody to HBeAg (anti-HBe), and occasionally complete clearance of the virus have been reported in IFN therapy (10–12). We therefore measured alkaline RNase activities in serum in a preliminary study of recombinant human leukocyte IFN therapy in patients with chronic hepatitis B. Here we report our findings and discuss the possibility that serum RNase could be used for monitoring IFN therapy.

Materials and Methods

Patients. Nine Japanese male patients with chronic hepatitis B were studied prospectively. All patients had been positive for hepatitis B surface antigen (HBeAg) and HBeAg in their serum for at least six months before IFN therapy. The clinical features of the nine patients in the study are summarized in Table 1. The mean age of the patients was 29 years (range 20–38 years). All had abnormal activities of alanine aminotransferase (ALT; EC 2.6.1.2) and aspartate aminotransferase (AST; EC 2.6.1.1) in serum, but normal concentrations of creatinine and urea nitrogen. The diagnosis was confirmed histologically in eight patients as chronic active hepatitis with various degrees of fibrosis. No patient had previously received any antiviral therapy, and none had any history of homosexuality, drug abuse, or positive reaction for antibody to human immunodeficiency virus.

Interferon. Recombinant α-IFN (α-2b) was supplied by Schering Plough Corp. (Kenilworth, NJ). The dose of IFN given intramuscularly was 3 mega-units on the first day, then 6 mega-units daily for each of two days, 10 mega-units daily for four days, and then 6 mega-units daily for each of the following 21 days. The 28-day cumulative dose of recombinant α-IFN was 181 mega-units.

Methods. Blood samples were obtained before (week 0) and during treatment (weeks 1, 2, 3, and 4) and after treatment (weeks 5, 6, 8, 12, 16, 20, and 24). Each blood sample was tested for RNase activity and 2-5 AS activity by week 12, by week 24 for other serum analyses and HBV markers. Concentrations in serum of AST, ALT, total bilirubin, alkaline phosphatase (EC 3.1.3.1), total protein, serum albumin, creatinine, and urea nitrogen were measured by standard methods with an AutoAnalyzer (Technicon Instruments, Tarrytown, NY). The presence or absence of HBeAg, antibody to HBeAg (anti-HBe), HBeAg, and antibody to HBeAg (anti-HBe) were determined by standard radioimmunoassay techniques (Abbott Laboratories, North Chicago, IL).

Substrate and measurement of RNase activity. The substrate solution was prepared by dissolving 3 g of yeast RNA (Sigma Chemical Co., St. Louis, MO) with 30 mL of 1 mol/L Tris · HCl buffer (pH 8.5) and dialyzed against 2 L of 50 mmol/L Tris · HCl buffer (pH 8.5) at 4 °C for 72 h with three changes of dialysis medium. The standard assay was performed with a reaction mixture (1.05 mL) containing 1 mL of 5 g/L yeast RNA and 50 μL of serum sample. After incubation for 30 min at 37 °C, we added 0.2 mL of 0.3 mol/L perchloric acid containing 12 mmol/L uranyl acetate and cooled the reaction tube in an ice bath for 15 min. The mixture was then clarified by centrifugation at 3000 × g for 15 min at 4 °C. The supernatant liquid (0.2 mL) was diluted with 3 mL of distilled water and the absorbance of this solution was read at 260 nm in a 10-mm-pathlength silica cell against a control assay. The absorbance of the reaction mixture for the sample, but without enzyme solution, was read at 260 nm as the control assay. The RNase activity was expressed as units per liter of serum, 1 unit of enzyme activity being defined as an increase in absorbance of 1.0 per minute.

Measurement of 2-5 AS activity. 2-5 AS activity in serum was measured by radioimmunoassay detection of the 2-5 A product (Eiken Immunochemical Laboratory, Tokyo, Japan) (13). The 2-5 AS activity was expressed as picomoles of product formed per minute per liter of serum.

Phosphocellulose column chromatography. We performed all the following procedures at 4 °C. We applied 2 mL of serum to a column (1 × 15 cm) of phosphocellulose (Brown Co., Berlin, NH) that had been equilibrated in 10 mmol/L sodium phosphate buffer, pH 6.5. After washing the column with equilibrating buffer to remove all unbound enzyme, we eluted the enzyme with NaCl linear gradient (0.2–0.9 mol/L), collecting 2-mL fractions at a flow rate of 15 mL/h.

Statistical analysis. Results are given as mean ± SD. Statistical significance was calculated by the paired Student's t-test for continuous data. Comparisons were done between pretreatment values and values at serial points during and after treatment. Bonferroni correction of the paired Student's t-test was used where appropriate, to describe changes over the whole time courses. Logarithmic transformation of 2-5 AS activity was applied for calculating correlation coefficients between RNase activities and 2-5 AS activities. The unpaired Student's t-test was used to assess the relationship between the effects of α-IFN on RNase activities or 2-5 AS activities in serum and the disappearance of HBeAg.

Results

Each patient received the complete course of recombinant α-IFN therapy. We observed no significant effect of recombinant α-IFN on the mean concentrations of AST, ALT, total bilirubin, alkaline phosphatase, total protein, serum albumin, creatinine, or urea nitrogen during therapy.

Alkaline RNase activity in serum. This IFN species did not possess alkaline RNase activity. Effects of recombinant α-IFN therapy on alkaline RNase activities in serum are shown in Figure 1. The mean activity of serum RNase was 65.8 ± 9.5 units/L before treatment, which significantly increased to 84.3 ± 11.9 units/L after the first week of therapy (P <0.001), and stayed constant at 82.6 ± 9.4
units/L through week 4. This high activity concentration was sustained until two weeks after the end of the therapy, when the RNase activity decreased to 70.8 ± 9.4 units/L (P < 0.01), still significantly higher than the pretreatment value. The mean serum RNase activity returned to pretreatment values by four weeks after the end of therapy. RNase activities were significantly higher than before therapy during the whole post-treatment period until the fourth week of therapy (Bonferroni correction, P < 0.01).

Phosphocellulose column chromatography. We performed phosphocellulose column chromatography for qualitative detection of increased RNase activity in serum after a four-week period of IFN therapy in one patient (no. 3). The activity of RNase in serum was 63.3 units/L before and 80.0 units/L, immediately after IFN therapy. Alkaline RNase in serum before treatment was eluted as five active peaks, such as is seen in normal individuals (14) (Figure 2, top). After four weeks of therapy with α-IFN, the patient's serum still contained five peaks of activity, eluted in the same positions and with each peak increased to the same degree (Figure 2, bottom). An additional species of RNase, indicative of an increase in activity, was not detected in the serum. The results for the other eight patients were similar to those for patient no. 3.

Correlation between RNase activity and 2-5 AS activity in serum. The mean 2-5 AS activity in serum was 6.3 ± 2.4 pmol/min per liter before treatment, and thereafter significantly increased to 51.7 ± 48.0 pmol/min per liter (P < 0.05) after the first week of therapy, to 85.3 ± 91.3 (P < 0.05) at the second week, then decreased to 46.5 ± 24.9 (P < 0.001) at the third week, 40.8 ± 26.4 (P < 0.005) at the fourth week, 23.1 ± 9.0 (P < 0.001) at the fifth week, and 8.9 ± 2.4 (P < 0.05) at the sixth week. This activity returned to pretreatment values by four weeks after the end of therapy.

The RNase activities and the 2-5 AS activities in serum were measured simultaneously in each patient. There was a close correlation between serum RNase activities and the common logarithm of 2-5 AS activities. Correlation coefficients (r), calculated for each patient, ranged from 0.726 to 0.953, and each was significant (P < 0.05 to < 0.001).

Relationship between RNase activity in serum and the disappearance of HBeAg. During a 24-week observation period (both during and after therapy), no patient showed any disappearance of HBeAg, although three patients (nos. 3, 4, and 8) showed a sustained clinical remission in which HBeAg disappeared from serum and aminotransferase activities decreased to normal values. In patient no. 3, AST and ALT activities in serum were within normal limits by the second week of α-IFN therapy, and HBeAg had disappeared by one month after therapy. Anti-HBe appeared two months after therapy and this continued during the observation period. Patient no. 4 showed normal aminotransferase activities at one month after therapy, and had no detectable HBeAg by two months after therapy. HBeAg was undetectable in serum from patient no. 8 after the first week of therapy, and aminotransferase activities were normal by two weeks after therapy. In patients no. 4 and 8, anti-HBe was not detected throughout the observation period. We compared values for these three responders with those for the remaining six patients (nonresponders). The mean activity of RNase in serum was slightly higher in responders than in nonresponders before, during, and after therapy; however, this difference was not statistically significant. In contrast, the mean activity of 2-5 AS in serum from responders was slightly lower than that from nonresponders during the whole observation period but, again, this difference was not significant between the two groups. Thus, we could not distinguish the responders from the nonresponders by either RNase activities or 2-5 AS activities in their sera.

Discussion

The present study demonstrated a significant increase in alkaline RNase activities in serum from patients with chronic hepatitis B receiving α-IFN therapy. These increased enzyme activities returned to pretreatment values by four weeks after the end of therapy. Although the mechanism of an increase of serum RNase activity remains to be clarified, several possibilities can be proposed. Of
these, impaired renal function must be considered because increased RNase activities have been observed in patients suffering from renal failure, and positive correlations between RNase and urea or creatinine concentration have been described (15). In our patients, however, the concentrations of creatinine and urea nitrogen in serum were within normal ranges, and remained unchanged during the IFN therapy. An alternative explanation for the increase in RNase activity may be associated with the protein nutritional condition. Increased activities of RNase have also been reported in children with protein malnutrition (9) and in adult patients receiving corticosteroid, which has protein catabolic effects (16). Furthermore, Shenkin et al. (16) have described an increase in RNase in plasma associated with a decrease in albumin in serum of patients with protein deficiency and normal renal function. In the present study, the concentrations of serum albumin were not affected by therapy, although subclinical protein malnutrition could not be excluded because anorexia is commonly seen in most series evaluating therapy with human IFN (10, 12).

IFNs have been demonstrated to induce activity of several enzymes, including RNase, in cells exposed to them. Reovirus messenger RNAs are degraded faster in an extract from IFN-treated mouse Ehrlich ascites tumor cells than in an extract from control cells (1), presumably because of endonuclease action. In activation of the RNase, both double-stranded RNA and ATP are required (2). Furthermore, this RNase is mediated by synthesized oligonucleotide without double-stranded RNA or ATP, and cleaves both cellular and viral messenger RNA, whether free or associated with polysomes. These observations can be explained by 2-5 AS, an intracellular enzyme induced by IFN, polymerizing ATP into a series of oligonucleotides characterized as 2-5 A in the presence of double-stranded RNA, followed by activation of RNase by 2-5 A. In the present study, we observed a close correlation between serum RNase activities and the logarithm of 2-5 AS activities. Therefore, the increase in RNase activities during IFN therapy could be considered to result from the increased 2-5 AS activities.

Results in this laboratory (14) have established the existence in normal human serum of five alkaline RNases which differ in optimal pH, molecular mass, heat stability, substrate specificity, and behavior toward metal ions. In the present study, alkaline RNase in serum was eluted as the five active fractions on phosphocellulose chromatography both before and immediately after therapy. A specific RNase indicating an increase in activity was not detected in the serum after IFN therapy.

Recently, several reports have demonstrated an increased 2-5 AS activity in peripheral mononuclear cells and serum from patients receiving IFN therapy (17, 18). Takeda et al. (19) showed that 2-5 AS activity in peripheral mononuclear cells was induced by IFN therapy more in patients whose HBeAg had been undetectable within one year of the end of therapy than in those with sustained HBeAg. In the present study, however, disappearance of HBeAg was unassociated with the activities of serum 2-5 AS or serum RNase before, during, or after therapy.

This preliminary study of recombinant human leukocyte IFN therapy for patients with chronic hepatitis B has shown an increase in alkaline RNase activity in serum. Although the number of patients analyzed was small, the results suggest that recombinant human α-IFN enhances RNase activities in serum through increased 2-5 AS activities. Further study will be needed to assess the exact relationship between the effects of α-IFN on RNase activities in serum and the therapeutic efficacy of α-IFN.

References