Expression of a Novel Protein by Regenerating Hepatocytes and Peripheral Blood Lymphocytes

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Regeneration and tolerance factor (RTF) is a protein with immunosuppressive activity and is normally present in the thymus and placenta. RTF was measured in the livers of patients with regenerating nodules due to alcoholic cirrhosis and hepatitis C. RTF was expressed in the regenerating nodules of 26 patients with alcoholic cirrhosis. All patients with chronic hepatitis C without cirrhosis failed to express RTF. Flow cytometry revealed upregulation of RTF on the lymphocytes from alcoholic cirrhosis and downregulation in hepatitis C disease.

Infection with the hepatitis C virus (HCV) is present in more than 1% of the U.S. population, with about 3.5 million carriers and 400 million people infected worldwide; thus, approximately 3% of the world population. HCV infection is more often observed in its chronic stage, at least in the Western hemisphere. Most hepatitis C infections (~80%) manifest clinically during the chronic stage. Hepatitis C infection accounts for most cases of parentally transmitted viral hepatitis. Parenteral drug users account for 30 to 40% of all cases. In over 56% of hepatitis C cases, according to the Centers for Disease Control and Prevention CDC, no risk factors have been identified. Persistent HCV infection not only results in chronic hepatitis but also precedes cirrhosis and liver cancer. On the other hand, alcoholic cirrhosis is the end-stage disease of alcoholic liver disease (ALD). This is still the most common cause of liver cirrhosis in our society. Thus, studies dealing with the pathogenesis of both conditions are of special interest.

Regeneration and tolerance factor (RTF), whose gene was originally known as J6B7 (3), is located on the distal portion of human chromosome 12. By the labeled streptavidin biotin (LSAB+) kit peroxidase method we searched for RTF expression in the livers of patients with regenerating nodules who had alcoholic cirrhosis and hepatitis C, with or without cirrhosis. All specimen evaluations were based on morphologic criteria, and investigators were not aware of the final diagnosis prior to their histological assessment.

Because patients with chronic liver diseases are often immunocompromised we decided to investigate the expression of RTF in human liver in both conditions. Earlier studies from this laboratory have shown that the distribution of lymphocytes in the trophoblast-maternal interface is mimicked by findings in the peripheral blood (4). We found that in addition to being expressed in the surface markers, RTF is also expressed on lymphocytes in the peripheral blood. Because patients with chronic liver disease have peculiar patterns of expression on the lymphocytes in the organs involved, we began to study the expression of RTF on lymphocytes in the peripheral blood of our patients with ALD and HCV disease.

We examined 31 patients with HCV infection and 28 patients with alcoholic cirrhosis. The HCV infection included 10 patients with cirrhosis and 21 patients with chronic hepatitis. The histological material was obtained from patients under study in our local hospitals. From these groups of patients blood was available for flow cytometric studies from 14 patients with alcoholic cirrhosis and 24 HCV-seropositive individuals. In addition, 31 healthy individuals were used as controls.

The liver needle biopsy specimens from the 28 patients with alcoholic cirrhosis with negative serology for viral markers were in addition investigated by the immunoperoxidase method. All cases of alcoholic cirrhosis were well-established disease class B and C of the Pugh classification, with regenerative nodules (6). In the cases of patients with HCV infection, 10 exhibited regenerative nodules and 21 showed chronic hepatitis only. HCV genotypes were not available.

Flow cytometric analysis of RTF, CD38, and HLA-DR expression on T cells. Flow cytometric analysis showed that RTF expression on CD4+ or CD8+ T cells is significantly different between individuals with alcoholic cirrhosis (n = 14) and HCV-seropositive patients (n = 24). The results demonstrated that surface expression of RTF on T cells is upregulated in patients with alcoholic cirrhosis and downregulated in HCV-seropositive individuals compared to that of healthy subjects (Table 1). As shown in Table 1, the mean channel fluorescence (MCF) of RTF expression on CD4+ and CD8+ T cells from individuals with ALD was significantly higher than the MCF of RTF expression in HCV-seropositive individuals (P < 0.001).
No correlation between viral load or current disease severity and RTF was identified. Since the increased cell surface expression of RTF by T cells was induced following activation (1, 4), we therefore investigated the expression of other activation markers (CD38 and HLA-DR). In contrast to the differential expression of RTF on T cells in individuals with ALD and HCV individuals, the percentages of CD4+ T cells expressing CD38 or HLA-DR were not significantly different between individuals with ALD and those with HCV (Table 2). Similar findings were obtained for the percentages of CD8+ T cells expressing CD38 or HLA-DR markers.

### RTF expression in regenerating hepatocytes by immunohistochemistry.

By immunohistochemistry the RTF protein was shown to be expressed in the regenerating nodules of 26 patients with alcoholic cirrhosis, and only two such patients were negative for RTF expression. In patients with hepatitis C it was expressed in only two patients who had cirrhosis with regenerative nodules and the remainder of patients, were negative for RTF expression. All patients with chronic hepatitis C without cirrhosis failed to express this protein. We cannot explain the difference in expression patterns of RTF between the two groups of patients, but the difference may be of potential significance for diagnosis and prognosis in the understanding of the differences between the two disease processes.

The expression of the RTF protein only in regenerative nodules of patients with alcoholic cirrhosis and in two patients with hepatitis C cirrhosis leads us to suspect a role for it in the regeneration process of hepatocytes. Furthermore, the inhibition caused by the protein in the mixed lymphocyte cultures allows us to designate it as a RTF. The lack of expression in chronic HCV liver injury without cirrhosis further supports this postulate.

The percentage of CD4 or CD8 cells in the peripheral blood expressing RTF in HCV-seropositive individuals is low. However, flow cytometric analysis of the peripheral blood showed that RTF expression is significantly higher in the lymphocytes of individuals with alcoholic cirrhosis than in those of patients with seropositive HCV infection. The liver-infiltrating lymphocytes failed to express RTF. This is not a surprising observation, because for over a decade now multiple studies comparing lymphocyte populations within the liver to those in the peripheral blood have revealed no correlation.

The highly elevated expression of RTF on T lymphocytes of individuals with alcoholic cirrhosis is similar to that seen in human immunodeficiency virus (HIV) infection (7). Previously we reported that the increased RTF expression is a correlate of HIV-associated immune system activation (2). Whether alcohol-induced liver cell injury that evokes immunologic reactions leads to the activation of RTF-expressing T cells or does not is still unknown. In contrast to patients with HIV and ALD, patients with HCV and ALD, patients with HCV infection had very low levels of RTF expression on T lymphocytes. A study by Prince and Fang (5) demonstrated that HCV-seropositive individuals did not exhibit lymphocyte subset alterations suggestive of the immune activation caused by chronic viral infections.

Furthermore, taken together with its expression during HIV infection, RTF may be a marker for partially activated or anergized cells, which are known to occur in the conditions studied here. Finally, it is hypothesized that RTF expression by regenerating hepatocytes prevents these cells from undergoing apoptosis and thus maintains them arrested in the S phase of the cell cycle. This keeps these cells dividing in order to restore or in an attempt to maintain liver function.

An interesting point that deserves further consideration, especially in cases of HCV disease, is that RTF, when expressed, does it only in some, not all, of the regenerating nodules of cirrhosis due to hepatitis C. In alcoholic cirrhosis most regenerative nodules express RTF, although this issue has not been studied extensively enough yet as to allow us to draw any conclusions about its significance in ALD. Further studies are needed concerning the expression of RTF in serial biopsy specimens from patients with hepatitis C at different stages of the disease to see if regenerating nodules continue to express RTF only in a segment of the population with HCV disease and to see what possible clinical or prognostic correlates exist.

| Parameter | ALD (n = 14) | HCV (n = 24) | Normal (n = 31) | P* (ALD vs HCV) | P (ALD vs normal) |
|-----------|-------------|-------------|----------------|----------------|------------------|
| % of CD4+ T cells expressing RTF | 15.3 ± 2.4b | 5.8 ± 0.5 | 8.6 ± 0.8 | <0.001 | <0.005 |
| MCF of CD4/RTF | 0.9 ± 0.09 | 0.5 ± 0.03 | 0.7 ± 0.05 | <0.001 | <0.01 |
| % of CD8+ T cells expressing RTF | 41.9 ± 6.6 | 10.6 ± 1.5 | 17.4 ± 2.0 | <0.001 | <0.001 |
| MCF of CD8/RTF | 1.7 ± 0.2 | 0.7 ± 0.05 | 1.2 ± 0.1 | <0.001 | <0.01 |

*P values were determined by unpaired t test.

### TABLE 2. Flow cytometric analysis of CD38 and HLA-DR expression on T cells from ALD and HCV+ individuals

| Parameter | ALD (n = 14) | HCV (n = 24) | Normal (n = 31) | P* (ALD vs HCV) | P (ALD vs normal) |
|-----------|-------------|-------------|----------------|----------------|------------------|
| % of CD4+ T cells expressing CD38 | 43.2 ± 3.0b | 43.3 ± 3.1 | 60.7 ± 2.0 | NS | <0.05 |
| % of CD4+ T cells expressing HLA-DR | 6.1 ± 0.9 | 6.2 ± 0.6 | 4.9 ± 0.5 | NS | NS |
| % of CD8+ T cells expressing CD38 | 4.6 ± 1.7 | 6.9 ± 0.8 | 34.0 ± 4.5 | NS | <0.001 |
| % of CD8+ T cells expressing HLA-DR | 7.8 ± 1.3 | 9.7 ± 1.7 | 12.5 ± 1.7 | NS | NS |

* P values were determined by unpaired t test.

b Data are expressed as mean ± standard error.

NS, not statistically significant.
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