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Original Research Article

Hospital-Based, Observational Study Assessing Relations of Regulatory T Cells with Hepatitis Markers in Chronic Hepatitis B Virus Infection

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Abstract

Aim: The aim of this study was to assess the Treg in peripheral blood of chronic hepatitis B (CHB) virus infected patients and to evaluate the presence of a possible relation between them and hepatitis B markers.

Methods: The Hospital-based, observational study was conducted in the Department of Medicine, Assam Medical College for the period of one year (July 2018 to June 2019). The study was conducted on 63 patients fulfilling the inclusion criteria attending the outpatient Department or admitted in the Department of Medicine, Assam Medical College, and Hospital during the study period.

Results: In the present study, the maximum cases were in the age group between 30-39 years. Among the study population, 69.84% patient were male and 30.16% were female. HBsAg and HBsAb had 100% biomarkers. The percentages of CD4+CD25+, CD4+CD25+Foxp3+, CD4+CD25high, CD4+CD25 highFoxp3+, and CD4+CD25—Foxp3+ T cells were significantly higher in CHB patients than in healthy controls. The percentages of CD4+ CD25+ Foxp3+, CD4+CD25high Foxp3+, and CD4+CD25—Foxp3+ T cells secreting IL-10 were higher in CHB patients than in healthy controls but the difference in the percent- age of CD4+ CD25+ Foxp3+ IL10+ T cells between the two groups was not statistically significant. The percentage of CD4+CD25+T cells was significantly higher in CHBeAg+ve patients than in CHBeAg-ve ones. Otherwise, no statistically significant differences were observed between CHBeAg+ve patients and CHBeAg-ve ones in the other measured cell percentages.

Keywords: HBV, Immune regulation Foxp3, Treg, HBV markers

Introduction

Hepatitis B virus (HBV) is a common noncytopathic DNA virus. Infection with HBV in adults results frequently in a self-limiting, acute hepatitis, which confers protective immunity and causes no further disease. In 10% of infected adults, HBV leads to a chronic infection. Chronic HBV infection is an important risk factor for the development of cirrhosis and hepatocellular carcinoma. Worldwide, 350 million people suffer from chronic HBV infection, and approximately 1 million people die annually from HBV-related liver disease. [1,2]

T helper 1 type cytokines such as interferon γ (IFN-γ) and interleukin 2 are involved in cell-mediated immunity and play a crucial role in the protection against intracellular pathogens, including HBV. [3] In patients with an acute self-limiting HBV infection, a multi-specific CD4+ and CD8+ T-cell response with a type 1 cytokine profile is important for control of the infection. [4] These multispecific T-cell responses are maintained for decades after clinical recovery. [5] In contrast, patients with a chronic HBV infection lack such a vigorous multispecific response. These patients have a weak or undetectable virus-specific T-cell response. [4] The precise mechanism responsible for this T-cell hypo-responsiveness or tolerance is still unknown. One scenario that has not been explored in relation to chronic HBV infection is the potential role of host-mediated immunosuppressive mechanisms that might be activated in the face of persistent antigenic exposure.

Peripheral T cells contain an immunoregulatory subpopulation that expresses CD4, CD25 (the interleukin 2 receptor chain), and CD45RO, as well as the cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4). These regulatory T cells (Treg) are capable of inhibiting the effector functions of CD4+, CD8+, and natural killer T cells. [6-9] Treg express the fork head/winged helix transcription factor gene (FoxP3). Retroviral gene transfer of FoxP3 converts naıve T cells into CD4+ CD25+ Treg capable of

suppressing the proliferative response of other CD4+ T cells. [10]

The aim of this study was to assess the Treg in peripheral blood of chronic hepatitis B (CHB) virus infected patients and to evaluate the presence of a possible relation between them and hepatitis B markers.

Materials and Methods

The Hospital-based, observational study was conducted in the Department of Medicine, Assam Medical College for the period of one year (July 2018 to June 2019). The study was conducted on 63 patients fulfilling the inclusion criteria attending the outpatient Department or admitted in the Department of Medicine, Assam Medical College, and Hospital during the study period. HBV infected patients, 13 years and above, attending Department of Medicine Assam Medical College and Hospital, during the period of one year were taken up for the study.

Exclusion criteria of the study include patients co-infected with hepatitis C virus (HCV), patients who were pregnant or in menstruation at time of sample collection and patients that received anti- viral drugs, steroids or other immune suppressive drugs during the preceding 6 months. Also, patients who had other cause of chronic liver injury such as excessive alcohol intake.

They were negative for known serologic markers of hepatitis B surface antigen (HBs Ag) and anti-bodies to HCV and human immunodeficiency virus (HIV). All participants gave informed consent before blood donation. All patients were subjected to history taking, clinical examination, liver function tests and abdominal ultrasonography. The study protocol was approved by the local ethical committee. A 6-ml quantity of blood was obtained from each subject and divided into two aliquots, one without anticoagulant was used for assessment of Liver functions (alanine aminotransferase (ALT), aspartate aminotransferase (AST), Total bilirubin and albumin levels) and for the detection of hepatitis B markers (HBs Ag, HBs Ab., HBe Ag, HBe Ab. and HBc IgG Ab.) by ELISA test (purchased from CTK Biotech, Inc., United States). The other part was heparinized and was used to quantify circulating Treg before in vitro culture in human peripheral blood in chronic hepatitis B patients in comparison with that of controls and to assess interleukin-10 (IL-10) secretion by Treg and measurement of IFN-γ production by T cells after in vitro culture. For this purpose, a four-color flow cytometry analysis by a fluorescence-activated cell sorter using a set of fluorochrome-labeled monoclonal antibodies against Treg surface and intracellular markers was accomplished.

Phenotypic analysis of Tregs

Block IgG was added at 4°C to 50 µl of whole blood together were added after 10 minutes to PE-cy7 conjugated anti-CD25 and APC conjugated anti-CD4 (e-Bioscience, San Diego, United States) and the mixture was incubated for 15 minutes at 4°C in dark. After addition of lysing solution and washing with PBS, cells were fixed by addition of fix solution and incubated at room temperature for 15 minutes. Following incubation, cells were washed with PBS then Perm solution and FITC conjugated anti-FoxP3 (e-Bio- science, San Diego, United States) were added and the mixture was incubated at room temperature for 30 minutes to be ready for analysis by flow cytometry. Appropriate isotype controls were also prepared and processed in a similar manner. All antibodies were used at concentrations titrated for optimal staining.

CD4+CD25+ and CD4+CD25high T cells percentages were determined within total peripheral blood mononuclear cells (PBMCs) and CD4+CD25+T cells, respectively. The expression of Foxp3 was quantified within CD4+CD25+, CD4+CD25high and CD4+CD25— T cells. Cut-offs for positive populations were determined by using isotype control staining as appropriate.

Induction and detection of intracellular cytokines by flow cytometry

Heparinzed blood samples were incubated with phorbol myristate acetate (PMA) (Applichem, Germany) (1 mg/1 ml DMSO) and ionomycin (MP. Biomedicals, Inc., United States) (25 mg/1 ml DMSO) as a positive polyclonal stimulus, for a period of 4 hours at 37°C, simultaneously Brefeldin A (Applichem, Germany) (1 mg/200 µl DMSO) was added, to block cytokine secretion at the Golgi. Cytokine production was evaluated through intracellular staining. A 50-µl quantity of the previously prepared mixture was incubated with FITC conjugated anti-CD69 and PE conjugated anti-CD3 for 15 minutes at 4°C in the dark. In another two tubes (one for IL-10 staining and another for IFN-γ staining) block IgG was added at 4°C and after 10 minutes 50 µl of the prepared mixture together with PE-cy7 conjugated anti-CD25 and APC conjugated anti-CD4 were added and the mixture was incubated for 15 minutes at 4°C in the dark. After addition of lysing solution and washing with PBS, cells were fixed by addition of fix solution and the mixture was incubated at room temperature for 15 minutes. Following incubation, in the IL-10 tube, the surface stained, fixed cells were incubated with Perm solution, FITC conjugated anti-Foxp3 and PE conjugated anti- IL-10 (e-Bioscience, San Diego, United States) and in the IFN-γ tube the surface stained, fixed cells were incubated with Perm solution and the PE conjugated anti–IFN-γ (e-Bioscience, San Diego, United States). The tubes were incubated at room temperature for 45 minutes to be ready for analysis by flow cytometry.

Appropriate isotype controls were also prepared and processed in a similar manner.

For assessment of lymphocyte activation CD3+CD69+ cells were assessed within the viable PBMCs gate, IL-10 producing cells were quantified within CD25+Foxp3+, CD25highFoxp3+, and CD25—Foxp3+ T cells and IFN-γ–producing cells were quantified within the CD4+CD25+ and CD4+CD25— T cells.

Statistical Analysis of the Data

Statistical package for social sciences (SPSS), version 16 was used for data analysis. Percentages of cells were expressed as mean ± standard error (SE). Differences in mean values of sur- face, intracellular markers and cytokine expression between the different groups of subjects were calculated using the independent t test. Mann–Whitney U test was used for comparison between subgroups. p Values were considered significant when less than 0.05. Relations were analyzed by linear regression and Pearson correlation.

Results

Table 1: Patient characteristics

AGE GROUP *(in years)*	**NUMBER** *(n)*	**PERCENTAGE** *(%)*
<20	3	4.76
20–29	13	20.63
30–39	15	23.81
40–49	13	20.63
50–59	9	14.29
60–69	7	11.11
≥ 70	3	4.76
Mean ± S.D.	41.41 ± 15.46 *years*	
SEX		
Male	44	69.84
Female	19	30.16
Liver function tests		
ALT (U/L)a AST (U/L)a	28 ± 5	
Albumin (g/dl)a	31 ± 8	
Total bilirubin (mg/dl)	5 ± 0.7	
ALT (U/L)a AST (U/L)a	0.8 ± 0.6	
Hepatitis B markers		
HBsAg	63 (100%)	
HBcAb	63 (100%)	
HBsAb	27 (42.85%)	
HBeAg	21 (33.33%)	
HBeAb	28 (44.44%)	

In the present study, the maximum cases were in the age group between 30-39 years. Among the study population, 69.84% patient were male and 30.16% were female. HBsAg and HBsAb had 100% biomarkers.

Table 2: Percentages of T cells with regulatory activity in the studied groups

Parameter	Cases (*n*=63)	Controls (*n*=30)	*p* value
$CD4^+CD25^+$ (%)	6 ± 0.5	4 ± 0.4	0.03
$CD4^+CD25^+Foxp3^+$ (%)	12 ± 1.6	3 ± 0.8	0.001
$CD4^+CD25^{high}$ (%)	6 ± 0.8	2 ± 0.7	0.04
$CD4^+CD25^{high}Foxp3^+$(%)	45 ± 3.5	8 ± 3.6	0.01
$CD4^+CD25^-Foxp3^+$ (%)	5 ± 1.0	2 ± 0.8	0.02

The percentages of CD4+CD25+, CD4+CD25+Foxp3+, CD4+CD25high, CD4+CD25 highFoxp3+, and CD4+CD25—Foxp3+ T cells were significantly higher in CHB patients than in healthy controls.

Table 3: Percentage of Treg cells secreting IL-10 and CD4+ T cells secreting IFN-γ in the studied groups

Parameter	Cases (n = 63)	Controls (n = 30)	*p* Value
$CD4^+CD25^+Foxp3^+ IL10^+$ (%)[a]	81 ± 3.7	66 ± 5.8	0.4
$CD4^+CD25^{high} Foxp3^+ IL10^+$ (%)[b]	82 ± 3.5	55 ± 8.3	<0.001
$CD4^+CD25^- Foxp3^+ IL10^+$ (%)[c]	62 ± 6.4	18 ± 7.2	0.017
$CD4^+CD25^+IFN-^+$ (%)[d]	7 ± 1	15 ± 1.5	0.7

The percentages of CD4+ CD25+ Foxp3+, CD4+CD25high Foxp3+, and CD4+CD25—Foxp3+ T cells secreting IL-10 were higher in CHB patients than in healthy controls but the difference in the percent- age of CD4+ CD25+ Foxp3+ IL10+ T cells between the two groups was not statistically significant.

Table 4: Percentage of T cells with regulatory activity in CHBeAg+ve, CHBeAg-ve, CHBsAb+ve, and CHBsAb-ve patients

Parameter	CHBeAg+ve	CHBeAg-ve	*p* value	CHBsAb+ve	CHBsAb-ve	*p* value
$CD4^+CD25^+$(%)	8 ± 0.9	6 ± 0.4	0.007	7 ± 0.7	6 ± 0.5	0.92
$CD4^+CD25^+Foxp3^+$ (%)	13 ± 2.5	13 ± 1.6	0.5	12 ± 2.1	13 ± 1.8	0.8
$CD4^+CD25^{high}$ (%)	7 ± 1.4	6 ± 1	0.3	6 ± 1.4	7 ± 1	0.2
$CD4^+CD25^{high}Foxp3^+$ (%)	47 ± 6.5	42 ± 4.8	0.5	47 ± 5.9	41 ± 5	0.5
$CD4^+CD25^-Foxp3^+$ (%)	8 ± 2.4	4 ± 0.9	0.6	5 ± 1.5	6 ± 1.4	0.6
$CD4^+CD25^+Foxp3^+IL10^+$ (%)	84 ± 5.7	78 ± 4.6	0.90	84 ± 4.3	77 ± 5.5	0.7
$CD4^+CD2^{high}Foxp3^+IL10^+$(%)	81 ± 5.6	80 ± 4.3	0.7	85 ± 3.3	77 ± 5.4	0.92
$CD4^+CD25^-Foxp3^+IL10^+$(%)	59 ± 11.1	61 ± 7.7	0.9	63 ± 9	58 ± 8.8	0.8
$CD4^+CD25^+IFN\text{-}\gamma^+$ (%)	5 ± 1.6	7 ± 1.3	0.2	4 ± 1	9 ± 1.6	0.03
$CD4^+CD25^-IFN\text{-}\gamma^+$(%)	5 ± 1.6	7 ± 1.6	0.6	3 ± 0.8	9 ± 1.9	0.07
$CD4^+IFN^+$ (%)	10 ± 2.5	12 ± 2.2	0.6	7 ± 1.6	14 ± 2.6	0.1

The percentage of CD4+CD25+T cells was significantly higher in CHBeAg+ve patients than in CHBeAg-ve ones. Otherwise, no statistically significant differences were observed between CHBeAg+ve patients and CHBeAg-ve ones in the other measured cell percentages.

Discussion

Hepatitis B virus (HBV) still remains a major global public health problem. It can cause chronic liver disease and puts people at high risk of death from cirrhosis of the liver and liver cancer. [11] Infection with HBV in adults usually results in a self-limiting, acute hepatitis. However, in 10% of infected adults, HBV leads to a chronic infection which is an important risk factor for the development of liver cirrhosis and hepato-cellular carcinom. [12] In patients with an acute self-limiting HBV infection, a multispecific CD4+ and CD8+ T-cell response with interferon-γ (IFN- γ) production is important for control of the infection. Patients with a chronic HBV infection exhibit a weak or undetectable virus-specific T-cell response. [4] The mechanisms responsible for the T cell tolerance in chronic HBV infection are not completely understood. [13]

In the present study, the maximum cases were in the age group between 30-39 years. Among the study population, 69.84% patient were male and 30.16% were female. HBsAg and HBsAb had 100% biomarkers. The percentages of CD4 +CD25+, CD4+CD25+Foxp3+, CD4+CD 25 high, CD4+CD25 highFoxp3+, and CD4+CD25—Foxp3+ T cells were significantly higher in CHB patients than in healthy controls. In this study, a non significant increase in IL-10 secretion by CD4+CD25+Foxp3+ T cells was observed in CHB patients. Mean- while, the secretion of IL-10 by CD4+D25highFoxp3+ and CD4+CD25—Foxp3+ T cells was significantly increased in CHB patients compared with normal controls. These findings more or less match those of an earlier study [14] in which IL-10 secretion by CD4+Foxp3+ T-cell population was significantly increased in HBV-infected patients in comparison with healthy subjects. Although another study did not find that difference [15], this may be because the investigators used an ELISA test for detection of IL-10, which may be less sensitive than flow cytometry.

The percentages of CD4+ CD25+ Foxp3+, CD4+CD25high Foxp3+, and CD4+CD25—Foxp3+ T cells secreting IL-10 were higher in CHB patients than in healthy controls but the difference in the percent- age of CD4+ CD25+ Foxp3+ IL10+ T cells between the two groups was not statistically significant. The percentage of CD4+CD25+T cells was significantly higher in CHBeAg+ve patients than in CHBeAg-ve ones. Otherwise, no statistically significant differences were observed between CHBeAg+ve patients and CHBeAg-ve ones in the other measured cell percentages. IFN-γ production by CD4+CD25— susceptible T cells and hence, IFN-γ production by the whole CD4+ T-cell population was reduced in CHB patients in comparison with normal controls. This was somewhat consistent with some previous studies [16,17] in which IFN-γ secretion by PBMCs was suppressed in vitro in the presence of Tregs in a dose dependent manner. Taking all into consideration, these findings suggest that these Tregs are capable of inhibiting the HBV immune response, which could contribute to persistence of HBV infection. Few studies have discussed the relation between Treg cells and hepatitis B markers. Most of them focused on HBeAg may be because of its known relation with high transmissibility, infectivity and active viral replication. Our results suggest that there is a relation only between HBeAg and activated CD4+CD25+T cells but not necessarily with Tregs. This was supported by the finding of a moderate positive correlation between the percentage of CD4+CD25+T cells and HBeAg. This was

consistent with some studies [17] and contradictory to others. [15]

Regarding HBsAb, to the best of our knowledge, no previous study has discussed the relation between HBsAb and the Tregs. The percentage of CD4+CD25+, CD4+CD25—, and total CD4+ T cells secreting IFN-γ were lower in CHBsAb+ve patients than in CHBsAb-ve ones. However these differences were significant only in CD4+CD25+ T cells, indicating that patients co-expressing both HBsAg and HBsAb had lower immune response than patients negative for HBsAb and this may explain the unfavorable outcome of hepatitis B infection in those patients that was mentioned by earlier studies. [18,19]

Conclusion

In conclusion, taking these findings all together, there is strong evidence that patients with CHB virus infection have a higher per- centage of Tregs in their peripheral blood compared with healthy controls and have lower levels of IFN-γ–secreting CD4+T cells, which may be caused by Tregs and which may contribute to viral persistence. In addition, CD4+CD25+Foxp3+ T cells may have close relation with hepatic inflammation as was evidenced by their relation to ALT levels. It was not possible to say that Tregs were the cause of immune suppression in CHB patients and CD4+ Foxp3+ T cells negative for CD25 could represent an important arm in immunosuppression in this group of patients.

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