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ARTICLE

Hepatitis C virus Infection in Patients with Essential Mixed Cryoglobulinemia, Multiple Myeloma and Chronic Lymphocytic Leukemia

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Increased prevalence of HCV infection in some lymphoproliferative diseases has been recently reported. In the present study, the frequency of anti-HCV antibody (Ab) together with hepatitis B surface (HBs) antigen (Ag) and anti-HBs Ab were determined in 42, 45 and 23 patients with essential mixed cryoglobulinemia (EMC), multiple myeloma (MM) and B-cell chronic lymphocytic leukemia (B-CLL), respectively. Thirty hospitalized patients with chronic rheumatoid arthritis (RA) were also included as a control. Specific antibodies to HCV antigens were detected by enzyme linked immunosorbent assay (ELISA) and positive results were confirmed by a recombinant immunoblot assay (RIBA). Our results demonstrated anti-HCV positivity in 69%, 11% and 4.3% of the EMC, MM and B-CLL samples tested, respectively. None of the RA patients were found to be anti-HCV positive. No significant differences were observed between the patients' groups regarding the frequency of HBs Ag and anti-HBs Ab. Considering the low incidence of HCV infection in the control group and the normal population, these results confirm and extend previous reports on the possible role of HCV infection in the etiology of EMC and further suggest involvement of this virus in a subset of MM. (Pathology Oncology Research Vol 7, No 2, 135–139, 2001)

Keywords: Hepatitis C virus, Lymphoproliferative disorders, Anti-HCV antibody

Introduction

The etiology of lymphoproliferative disorders is largely unknown. A variety of genetic and environmental factors have been proposed to be implicated. Viruses have so far been the most widely studied agents considered to be involved in the process of malignant transformation. Some viruses, such as human T-cell lymphotropic virus (HTLV-1), hepatitis B virus (HBV) and Epstein-Barr virus (EBV) have been found to be responsible in the pathogenesis of adult T-cell leukemia lymphoma (ATLL), hepatocellular carcinoma (HCC) and Burkitt's lymphoma (BL), respectively.[1-3] Recent cloning and characterization of the hepatitis C virus (HCV) genome and development of serologic techniques using recombinant proteins corresponding to different structural and non-structural antigens of the virus provided the opportunity to study contribution of this virus in different lymphoproliferative diseases.[4,5] HCV is primarily a hepatotropic virus known as the major cause of non-A non-B hepatitis worldwide.[6] Infection with HCV frequently leads to chronic liver disease, which may be followed by cirrhosis or HCC.[7,8] A striking association has been found between essential mixed cryoglobulinemia (EMC) and HCV infection in the last few years.[9-12] Approximately 50% of patients with EMC (also known as type II cryoglobulinemia) have a liver disease or abnormalities in liver function tests.[13] Because of the close relationship established between EMC and HCV infection, HCV has been proposed as the major etiologic agent of this benign lymphoproliferative disorder.

Association between HCV and some other lymphoproliferative disorders such as non-Hodgkin's lymphoma (NHL), low grade mucosal associated lymphoid tissue (MALT) lymphoma and acute lymphoblastic leukemia (ALL) has been reported very recently.[14-16]

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In the present study, the prevalence of HCV infection was determined in patients with EMC, multiple myeloma (MM), and B-cell chronic lymphocytic leukemia (B-CLL). The prevalence of HBV infection has also been studied to shed some insight into the relative risk of exposure of the same patients' groups to another closely related blood-born viral infection.

Materials and Methods

Clinical samples

Peripheral blood was collected from 42, 45 and 23 patients with EMC, MM and B-CLL, respectively. Thirty samples collected from patients with classical RA served as a control group. Serum was isolated from clotted blood and stored at –20°C until use.

Diagnosis of EMC was based on clinical manifestations, including typical syndromes such as purpura, arthralgia and weakness associated with circulating type II cryoglobulins.[17] Lytic bone lesions together with hypercalcemia, hypogammaglobulinemia and presence of more than 10% plasma cells in bone marrow and serum paraprotein were the major findings in the MM patients.[18] Diagnosis of B-CLL was achieved according to the criteria outlined by Rai et al.[19]

The B-lymphocyte origin of the leukemic cells in B-CLL was confirmed by immunophenotyping using monoclonal antibodies (MAbs) specific for the human leukocyte surface markers as described elsewhere.[20] The criteria for diagnosis of RA has been given elsewhere.[21]

Characterization of paraproteins and cryoglobulins

Characterization of the paraproteins and cryoglobulins was achieved by cellulose acetate electrophoresis (EP), Immuno-electrophoresis (IEP) and/or immunofixation (IF), as previously described.[22] Cryoglobulin was isolated from serum by centrifugation following 24 hour incubation at 4°C. Isolated cryoglobulin was tested for rheumatoid factor (RF) activity[22] and subjected to EP, IEP and/or IF. Cryoglobulins with a complex of monoclonal IgM having RF activity and polyclonal IgG were assigned type II cryoglobulins.[23] Paraproteins were detected by EP and their isotype determined by IEP and/or IF using isotype-specific polyclonal antisera.[24]

Detection of anti-HCV Antibody

a) Enzyme linked immunosorbent assay (ELISA)

Screening for anti-HCV antibody (Ab) was performed using a commercial ELISA kit (United Biochemical Inc. New York, USA) which detects antibodies to structural and nonstructural antigens. Briefly, serum samples were diluted (1:21) and incubated in plates precoated with recombinant HCV antigens. Positive and negative sera were used as controls. Appropriate dilution of horseradish peroxidase (HRP)-conjugated goat anti-human IgG was then added and the reaction revealed with orthophenylendiamine dihydrochloride (OPD) substrate. Optical densities (O.D.) were determined by a multiscan ELISA- reader (Organon Teknica, Boxtel, Belgium) at 495 nm. The cut-off was calculated as follows: Cut-off = 0.15 x SRC , where SRC = strongly reactive control.

b) Recombinant immunoblot assay (RIBA)

Anti-HCV positive samples were also tested by RIBA using a commercial kit (HCV Blot 3.0, Genelabs Diagnostics, Singapore). The technique was performed based on instruction manual from the manufacturer. Briefly, anti-HCV positive sera tested by ELISA were added at 1:100 dilution to nitrocellulose membrane strips preblotted with recombinant antigens from the capsid, NS3-1, NS3-2, NS4 and NS5 regions. In addition the blots contain IgG and anti-IgG control bands. Following extensive washing, the strips were incubated with HRP-conjugated anti-human IgG and revealed with diaminobenzidine tetrachloride (DAB) substrate. The intensity of each band was compared to reference bands. Samples were considered positive for anti-HCV Ab if 1^+ or greater reactivity to two or more HCV antigens were visualized.

Screening of HBV markers

HBsAg and anti-HBs Ab were detected by sandwich ELISA using commercial kits (Behringwerke AG, Marburg, Germany).

Statistical analysis

Analysis of data was performed using Chi-square test. The results were considered significant if p values were less than 0.05.

Results

Serum and purified cryoglobulin from all EMC, MM and CLL patients were subjected to electrophoresis. A sharp band was detected as monoclonal band in all EMC and MM samples and only in 1 B-CLL sample (data not shown). All the purified cryoglobulins were constituted of a complex of monoclonal IgM with anti-human IgG (RF) activity and polyclonal IgG (type II cryoglobulin) (data not presented).

Prevalence of anti-HCV Ab was determined by ELISA and positive samples were double checked by RIBA. The results have been summarized in *Table 1*. Anti-HCV Ab was detected by ELISA in 29 out of 42 (69%) patients

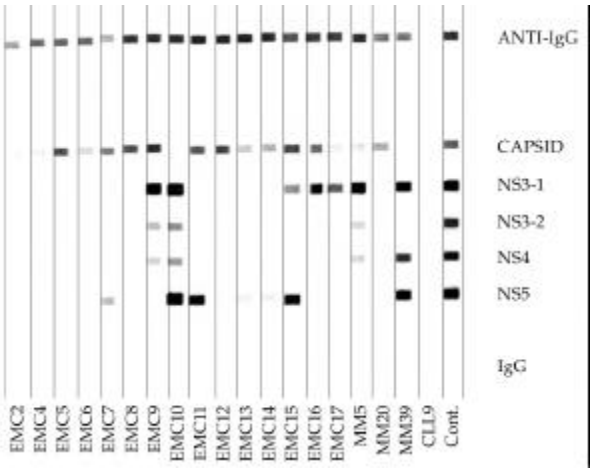

Figure 1. Immunoblotting of serum from EMC, MM and B-CLL patients

with type II cryoglobulinemia. Of 45 and 23 MM and B-CLL samples, 8 (18%) and 2 (8.7%) were found positive, respectively. One of the RA samples reacted weakly in the ELISA (OD = 0.32).

Seropositive samples were also tested by RIBA. The results obtained for some of the samples are illustrated in *Figure 1*. While all the 29 EMC samples (100%) reacted, only 5 (62%) of the MM and 1 (50%) of the B-CLL sera gave positive results (*Table 1*). The weakly positive RA sample was negative by RIBA.

The frequency of HBV markers was found to be similar in all subject groups (*Table 2*). Due to sample limitation some of the serum samples could not be tested for HBV markers.

To find out any association between anti-HCV reactivity and expression of a particular isotype in the MM paraproteins, the frequency of anti-HCV Ab positive samples was determined in paraproteins of different isotypes (*Table 3*). No significant differences were found when the results were compared with distribution of the same isotypes in the whole MM panel, regardless of anti-HCV reactivity.

Discussion

Recent findings indicate that in addition to its major hepatotropic character, HCV is also a lymphotropic virus, and can infect human lymphocytes and some other hematopoietic cells.[25,26] This suggests that HCV may also contribute to the development of some hematologic malignancies and lymphoproliferative disorders. This assumption has prompted many investigators to study the incidence of HCV infection in a variety of lymphoproliferative disorders. EMC has been the first disorder shown to be associated with HCV infection.[9] This finding was later confirmed by many investigators and extended to some other malignancies.[14-16] However, the results reported for lymphoproliferative diseases, other than EMC, are controversial.[27,28]

Our results also confirms, the proposed association between HCV infection and EMC. Sixty nine percent of our EMC samples were found to be anti-HCV Ab positive, by both ELISA and RIBA methods, indicating a very significant difference from the RA control group ($p<0.0001$) and the normal population. Inclusion of RA patients in this study as a control group is signified by the fact that these patients are usually elderly people suffering from a chronic inflammation requiring almost as frequent hospitalization as our malignant patients. This enables us to some extent to control for risk activities that might explain acquisition of blood-born infections in such patients. Although very similar, this figure is slightly lower than that of other reports, which may be explained by false negative results. Serologic tests have been demonstrated to give a lower positive results when compared with the more sensitive PCR technique. The overall prevalence of HCV RNA among patients with type II cryoglobulinemia was estimated to be 84% and 96%, whereas the frequency of anti-HCV Ab in the same groups of patients was 42% and 77.4%, respectively.[10,13] False negative results may also be attributed to the presence of immune complexes[10] or pro-

Table 1. **Frequency of anti-HCV Ab in the subject groups**

Subjects	*No. of patients*	*Anti-HCV Ab⁺*	
		ELISA	*RIBA*
EMC	42	29 (69%)	29 (69%)
MM	45	8 (18%)	5 (11%)
B-CLL	23	2 (8.7%)	1 (4.3%)
RA	30	1 (3.3%)	0 (–)

EMC: Essential mixed cryoglobulinemia, MM; Multiple myeloma, B-CLL: B-cell chronic lymphocytic leukemia, RA: Rheumatoid arthritis

Table 2. **Prevalence of HBs Ag and anti-HBs Ab in the subject groups**

Subjects	*No. of patients*	*HBsAg*	*Anti-HBs Ab*
EMC	32	1 (3%)	7 (21.8%)
MM	44	3 (6.8%)	13 (29%)
B-CLL	20	1 (5%)	5 (25%)
RA	30	2 (6%)	7 (23%)

Table 3. **Distribution of anti-HCV Ab within different isotypes of MM paraproteins**

Paraprotein	*IgG*	*IgA*	*IgM*	*IgD*
Whole (n=45)	33	8	1	3
Anti-HCV ⁺(n=5)	4	1	0	0

duction of low levels of normal immunoglobulins,[13] usually observed in many hematologic malignancies as a result of immunosuppression and infiltration of the malignant cells in the bone marrow. Presence of some HCV genotypes such as HCV3 or HCV4 which may not be detected by the commercial ELISA kits[29] and/or extensive mutations within the E2/NS1 region with subsequent loss of some B-cell epitopes[30,31] also explain failure of detection of HCV infection by serologic techniques.

False positive results have also been reported using commercial anti-HCV ELISA kits.[12] This limitation has paved the way for introduction of the immunoblotting technique as a confirmatory tool for the ELISA positive samples. In this study, though all the EMC samples which were positive by ELISA, also reacted in RIBA, anti-HCV reactivity of only 5/8 (62%) and 1/2 (50%) of the ELISA positive MM and B-CLL sera was confirmed by RIBA, respectively. This seems to be largely due to weak reactivity of some of the samples, since strongly reactive samples also gave positive results by RIBA. Non-specific interaction of some charged paraproteins with the pre-coated recombinant viral proteins could have lead to weak anti-HCV reactivity in the MM samples.

Despite the fact that only 62% of the ELISA reactive MM samples were confirmed by RIBA, HCV infection was found to be substantially higher in the MM patients (11%) compared to the RA control group ($p<0.06$) and normal population. Although prevalence of HCV infection in MM patients was substantially higher than in RA patients, (11% vz 0), however, due to the small sample size, the difference was not statistically significant. Less than 1% of the normal adult population of Iran (0.3%) have been reported to be anti-HCV antibody positive,[32] unpublished data from the Blood Transfusion Center of Iran). Our results, in line with others[33] indicate that HCV infection is not necessarily associated with cryoglobulinemic activity. Most of the data reported so far, however, suggest a strict association between HCV infection and monoclonal gammopathies with cryoglobulinemic activity.[13,33-35] The fact that about 80% of the anti-HCV+ MM samples contain paraproteins of the IgG isotype (*Table 3*), suggests that anti-HCV reactivity may also be found in gammopathies with heavy chain isotypes other than IgM and devoid of cryoglobulin activity.

The frequency of anti-HCV Ab was similar, though slightly higher in the B-CLL patients (4.3%) compared to the control group. However, due to the small sample size of the B-CLL patients studied and the fact that only one of these samples gave a positive result, firm conclusion can not be drawn.

High frequency of HCV infection in haematologic malignancies could also be attributed to frequent transfusion of blood or blood products to such patients due to suppression of haematopoisis.[8] Our clinical records, regarding transfusion of blood to the patients particularly the EMC patients, are not convincingly enough to allow us to address the argument, though some of available data does not support involvement of blood transfusion. Apart from 2 of the B-CLL patients who were in the stage 4 of the disease with anemic manifestations (without a history of blood transfusion at the time of blood sampling) the rest of the patients were mostly at stage 0 or 1 of the disease. Of the MM patients studied from whom a complete clinical records is available none show a history of blood transfusion.

Our findings regarding similar distribution of the anti-HBs Ab and HBs Ag (i.e. past and present HBV infection) within the subject and control groups suggest that the relative risk for acquisition of a blood-born viral infection is similarly represented in all groups, regardless of their particular diseases. This reiterates the significance of the role of HCV in pathogenesis of EMC and to a lesser extent MM.

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