ORIGINAL

Effect of anti-immunoglobulin antibodies provides new insights into immune response to HCV

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Abstract : To determine the effect of anti-immunoglobulin antibodies on the measurement of the humoral immune response in hepatitis C virus (HCV) infected patients. Anti-immunoglobulin antibodies were defined using sheep immunoglobulins as a target to characterize distinct changes in patterns of immunoglobulin levels. Serum immunoglobulin A, G and M concentrations were measured by ELISA in 45 patients with recent-onset HCV infection and 45 matched normal individuals. It was found that normal individuals had mean IgA, IgG and IgM levels of 2.67 mg/ml, 9.39 mg/ml and 1.77 mg/ml, respectively while HCV infected patients had mean levels of 3.19 mg/ml, 10.76 mg/ml and 1.94 mg/ml. These represented significant increases in immunoglobulin levels in the sera of HCV patients compared to normal individuals ($p<0.0001$, $p<0.00004$ and $p<0.0004$). Anti-immunoglobulin antibodies lead to an overestimation of serum immunoglobulin levels in HCV patients. Interestingly, the mean levels of immunoglobulins A, G and M in HCV infected sera, determined after purification from anti-sheep immunoglobulins, was 2.73 mg/ml, 9.55 mg/ml and 1.79 mg/ml. Therefore, there was no significant difference in HCV patients compared to normal individuals ($p<0.42$, $p<0.36$ and $p<0.44$). The presence of circulating immune complex in serum during the early phase of infection may contribute to immunopathological effects in the infected host and provide some new insights into antibody response to HCV. **J. Med. Invest. 52 : 172-177, August, 2005**

Keywords : *hepatitis C virus ; antibodies*

INTRODUCTION

An understanding of the natural history of hepatitis C virus (HCV) infection has improved in recent years. However, immunological responses to hepatitis C virus infection have not been fully studied. The disease it causes is characterized by silent onset in most infected individuals, a high rate of viral persistence and the potential for development of ever-worsening chronic liver disease, ranging from chronic hepatitis to cirrhosis and occasionally to hepatocellular carcinoma. Such progression, when it occurs, is also most commonly a silent process that may take 20-40, and occasionally even more, years to reach its end. Because of these characteristics, it has been exceedingly difficult to accurately assess the natural history (1, 2).

Hepatitis C virus infects an estimated 170 million persons worldwide (1, 3). The prevalence of HCV infections is 1 to 2%, although certain geographical regions, age groups and ethnic groups have much higher rates of infection (4). The natures of circulating HCV particles and their association with immunoglobulins or lipoproteins as well as the characterization of cell entry have all been subject to conflicting reports (5).

Acute infection with HCV is characterized by a disturbingly high propensity to progress to chronic

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hepatitis that, in a proportion of cases, may evolve into cirrhosis and hepatocellular carcinoma (6, 7). It is generally accepted that B-cells infected with HCV clonally expand and produce autoantibodies. When antigen-bound, these autoantibodies, along with anti-HCV antibodies that target viral epitopes on the surface of cells are known to circulate as immune complex (IC). These IC may participate in the pathogenesis of HCV (8). Immunoglobulins, which bind other immunoglobulins or antibodies, add another facet to the abnormal immune response of HCV infected patients. In addition, when there is too much autoreactive immunoglobulin, autoantibody response may be dominant.

The present study was therefore designed to investigate the presence of these anti-immunoglobulin antibodies, using as a target sheep immunoglobulins, in HCV infected patients in an attempt to clarify some immuno-pathogenetic aspects of B cell activation during hepatitis C virus infection and to characterize distinct changes in patterns of immunoglobulin levels in hepatitis C infected patients.

PATIENTS AND METHODS

Anti-human IgA (G, M) antiserum (raised in rabbit), human IgA (G, M), rabbit anti-human IgA (G, M) conjugated to horseradish peroxidase (HRP), and tetramethylbenzidine were purchased from Sigma (Sigma-Aldrich Company Ltd, UK) and all other chemicals were supplied from BDH (VWR International Ltd, UK).

Patients

Informed patient consent was obtained in every case and the use of blood or scientific studies was approved by the local Ethical Committee. The study population consisted of 45 normal adult individuals (group 1) as a control group, 45 patients with recent-onset acute HCV infection (group 2) and the same HCV-infected individual serum samples pre-absorbed with sheep immunoglobulins (group 3) were used for analysis of immunoglobulin levels by enzyme-linked immunosorbent assay (ELISA). Both group 1 and 2 were matched for sex and age (median age, 39 years [range, 21 to 84 years]). Medical history, physical examination and routine laboratory investigations were completely normal in all subjects of group 1. They didn't use any medication prior to this study. Screening for hepatitis B virus or human immunodeficiency virus infection was negative in both patient and control subjects. Sera were stored frozen at -80°C until used.

At the initial consultation, all patients presented were tested positive for HCV RNA by polymerase-chain-reaction assay, had elevated serum alanine amino-transferase levels and had been diagnosed with acute HCV infection. If 1 or more of the following criteria was met : known or suspected exposure to HCV within the preceding 4 months, documented with a known previous negative HCV test, or a serum alanine amino-transferase level of more than 350 U/L, with a documented normal level during the year before infection. Only 45 patients with HCV fulfilled these conditions for admission to this study.

Human immunoglobulin measurement by ELISA

Coating antibody [anti-human IgA (IgG, IgM) antiserum] was diluted 1 in 1000 in 1x coating buffer (0.02 M Tris-HCl, 1.5 M NaCl pH 9.0) and 100 μl was added to each of the wells of a microtiter plate (9, 10). After overnight incubation at 4°C the plate was washed 4 times with PBST20 (0.1% (w/v) [Tween 20 in 1x PBS (phosphate buffered saline ; 0.25 M NaCl, 0.0268 M KCl, 0.081 M Na_2HPO_4 and 0.0146 M KH_2PO_4)]. Sites unoccupied by antibody were blocked by addition of 5% (w/v) Marvel (dried skimmed milk) in PBS for 1 h at room temperature followed by washing 6 times with PBST20. The human serum samples were initially diluted 1 in 2000 in 1x PBS, and 2 fold serial dilutions subsequently performed on the plate. Diluted samples were allowed to bind to the first antibody and the plate was then washed 6 times in PBST20.

Rabbit anti-human IgA (IgG, IgM) conjugated to HRP [second antibody] was diluted 1 in 1000 in 1x PBS, 100 μl was added to each well of the microtiter plate, incubated at room temperature for 1 h and then washed 6 times in PBST20. The amount of bound second antibody was determined by adding 200 μl of the substrate solution [tetramethylbenzidine 6 mg/ml in 0.1 M sodium acetate buffer pH 6.0] to each well. After incubation in the dark at room temperature for 20 min, the reaction was stopped by adding 50 μl of 10% (w/v) H_2SO_4 to each well. The optical density of each sample was read with an ELISA plate reader with a 450-nm filter. A standard curve was constructed by plotting absorbance against concentration for the standard solutions and the concentration of immunoglobulin (mg/ml) in the samples was determined.

Electrophoresis of immuno-precipitates on SDS polyacrylamide gel

Human serum samples were immuno-precipitated with anti-human IgG developed in rabbit in the pres-

ence of sheep immunoglobulins. Serum samples [25 μl] were diluted with sheep immunoglobulins [475 μl] and, in addition, 75 μl of anti-IgG with 1x PBS [425 μl]. After dilution, the antiserum and serum were mixed to give a final volume of 1 ml and incubated for 1 hour at room temperature. The precipitate was removed by centrifugation at 13,000 rpm for 5 min in micro centrifuge and then washed with 100 μl 1x PBS. The antibody-antigen precipitate was dissolved in 50 μl of 2x Laemmli sample buffer (0.125 M Tris-HCl pH (6.8), 0.1% (w/v) SDS, 20% (w/v) glycerol, 0.005% (w/v) bromophenol blue) and then incubated at 95°C for 3 min (11) A fraction of this mixture [25 μl] was electrophoresed overnight on a discontinuous 10% polyacrylamide gel containing 0.1% (w/v) SDS at a constant voltage of 45 V at room temperature. Following electrophoresis, proteins in gel were visualized by staining with Coomassie blue staining (12).

Purification of HCV infected sera from the effect of antibodies

Sheep immunoglobulins were isolated previously from sheep serum by affinity chromatography using the appropriate sepharose-bound antibody (Amersham Pharmacia Biotech UK Ltd). The final purified antibody preparation contains only antigen-specific active antibody plus a small amount of denatured antibody resulting from elution procedure. Sheep immunoglobulins, 200 μl, at a concentration of 10 mg/l in PBS, pH 7.2 were mixed with 200 μl of human serum samples from each of 45 HCV infected individuals (diluted 1 in 10) to minimize further cross-reactivity to human sera. The absorption was carried out for 1h at 37°C, followed overnight at 4°C. The HCV infected sera were clarified by centrifugation at 10000 x g for 15 min at 4°C before testing (13). The absorption of HCV infected sera with sheep immunoglobulins completely removed the positive reaction of these sera (group 3), and then the concentration of immunoglobulin present in each of these samples was determined by ELISA as described above.

Statistical analysis

After tabulating the data, the arithmetic mean for each group was calculated. The variation or variability in each group was represented by the standard deviation (SD). The means of the groups were compared to see if the differences were significant. Student's *t*-test was used to assess the significance of the difference between groups.

RESULTS

It was necessary to confirm the possibility that HCV infected sera bind and co-precipitate with sheep immunoglobulins using immuno-precipitation reaction. Results (Figure 1) showed that sheep immunoglobulins precipitated more antibodies in HCV infected sera (lanes 4-6) than in normal sera (lanes 1-3). Visual examination of the intensities of heavy chain bands showed that sheep immunoglobulins bind and co-precipitate with the antibody-antigen complex that reveals differences in band intensities.

Furthermore, ELISA measurements showed that group 2 had a high level of serum immunoglobulin compared to group 3. The absorption of HCV infected sera with sheep immunoglobulins was carried out to eliminate the positive reaction of these sera and the dramatic increase determined by ELISA was varied between different HCV infected samples.

It was found (Table 1) that normal individuals had mean IgA, IgG and IgM levels of 2.67 mg/ml, 9.39 mg/ml and 1.77 mg/ml, respectively while HCV infected patients had mean levels of 3.19 mg/ml, 10.76 mg/ml and 1.94 mg/ml. These represented

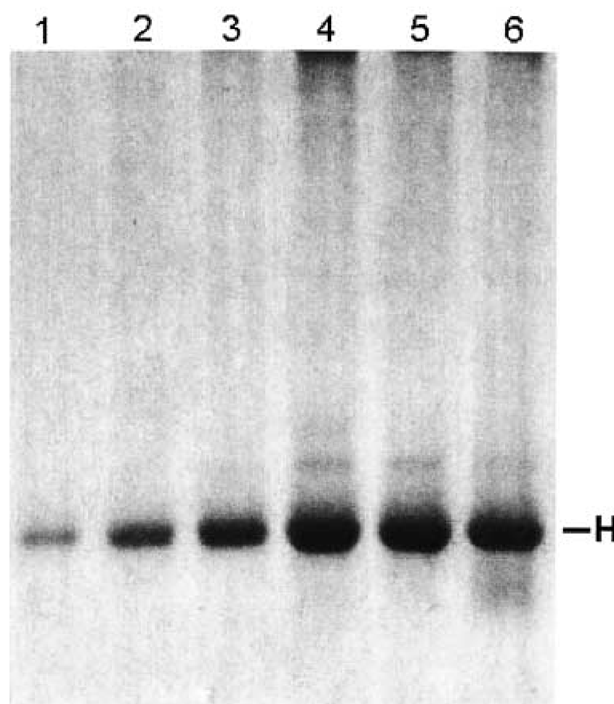

Figure 1. **Immuno-precipitation and polyacrylamide gel electrophoresis of human serum samples with anti-human IgG. Three normal serum samples (lanes 1-3) and three HCV infected sera (lanes 4-6) were immuno-precipitated with anti-IgG developed in rabbit in the presence of sheep immunoglobulins. H indicates positions of immunoglobulin heavy chains. The precipitates washed, dissolved in Laemmli sample buffer and analyzed by 10% SDS polyacrylamide gel electrophoresis.**

Table 1. Statistical analysis of IgA, IgG and IgM measured by ELISA.

Study Group	Case	Serum IgA Level (mg/ml)	Serum IgG Level (mg/ml)	Serum IgM Level (mg/ml)
1 (n=45)	Normal	2.67±0.37	9.39±0.83	1.77±0.14
2 (n=45)	HCV infected	3.19±0.46*	10.76±0.65^{+}	1.94±0.15$^{\#}$
3 (n=45)	HCV infected (Purified)	2.73±0.40**	9.55±0.72^{++}	1.79±0.13$^{\#\#}$

Values are MEAN±SD : *p<0.0001, **p<0.42 ; ^{+}p<0.00004, ^{++}p<0.36 ; $^{\#}p$<0.0004, $^{\#\#}p$<0.44 compared with normal individuals.

significant increases in immunoglobulin levels in the sera of HCV infected patients compared to normal individuals (p<0.0001, p<0.00004 and p<0.0004). However, the mean levels of immunoglobulins A, G and M in HCV infected sera (Figures 2, 3 & 4) determined after purification from anti-sheep immunoglobulins, was 2.73 mg/ml, 9.55 mg/ml and 1.79 mg/ml, therefore, there were no significant differences in the sera of HCV infected patients compared to normal individuals (p<0.42, p<0.36 and p<0.44). Our results in this study did not address any statistical significant differences in sex variation (data not shown).

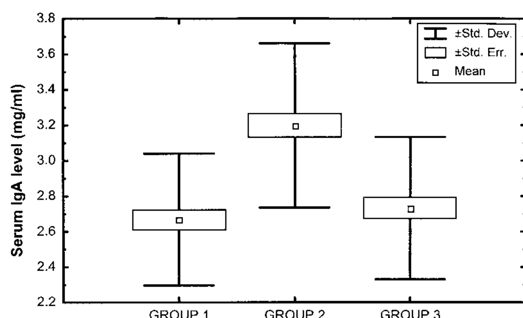

Figure 2. Serum IgA levels in groups of HCV infected and unaffected control. Comparison of average serum IgA (mean ± SD).

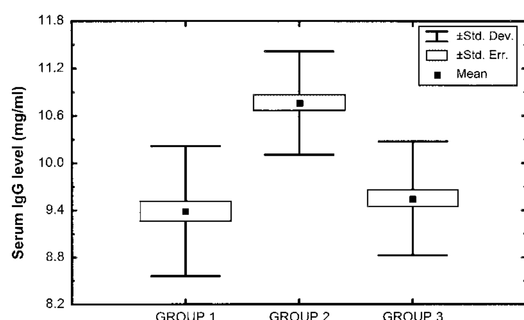

Figure 3. Serum IgG levels in groups of HCV infected and unaffected control. Comparison of average serum IgG (mean ± SD).

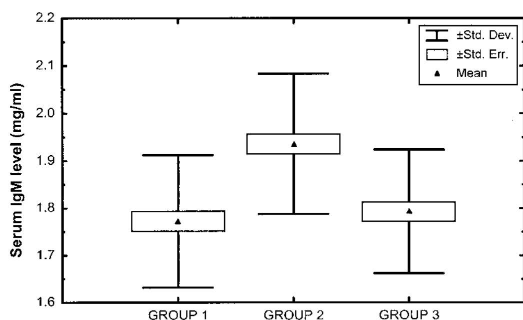

Figure 4. Serum IgM levels in groups of HCV infected and unaffected control. Comparison of average serum IgM (mean ± SD).

DISCUSSION

There is compelling evidence that HCV can infect immune cells, such as macrophages, B cells and T cells. It has been previously reported that HCV core, the first protein expressed during the early phase of viral infection, contains the immuno-modulatory function of suppressing host immune responses (8, 14). This altered function of immune cells caused by HCV infection may explain the ineffective immune response to HCV. Despite this abundance of reports, most researchers consider that whether the role of immunological factors is primarily pathogenetic, co-causative, secondary or simply chronologically associated to pathogenetic events has not been definitively answered (15). This problem has directed research towards other possible immunological factors likely to be present in HCV infected patients, in the attempt to elucidate further the complex immunopathogenetic interactions of the disease : our finding of anti-immunoglobulin antibody in HCV infected sera, leading to inaccuracies in immunoglobulin estimation by immunoassay, represents a move in this direction. In order to overcome this problem of interfering due to the effect of anti-sheep immunoglobulin antibodies on ELISA, the HCV infected human sera were pretreated with sheep immunoglobulins to eliminate cross-reaction of other irrelevant antibodies found in HCV infected sera and exclude false positive values, thereby preventing unnecessary evaluation and treatment.

ELISA measurements showed that group 2 had a

higher level of serum IgA, IgG and IgM compared to group 3. The dramatic increase determined by ELISA was varied between different HCV infected samples, hence this proves that it is not reliable to use the human HCV infected sera directly without pre-absorption. Thus, this sort of interaction led to over-estimation of immunoglobulin levels in HCV infected sera, which in turn produces unreliable results. The concept is that different antibodies recognize different epitopes together with the fact that the antibody molecules are dimeric means that anti-sheep immunoglobulin antibodies found in HCV infected sera is able to form a cross-linked structure when mixed with antiserum. Hence, an antibody in antiserum binds to antibodies in HCV infected sera is not indicative that the bound antibody is the antigen. Thus, it appears that there was interactions arise during the analysis of immunoglobulins.

Anti-sheep immunoglobulins antibodies found in HCV infected sera greatly overestimate serum immunoglobulin concentration measurements in HCV infected patients, regardless of the source of antibody used. The possibility of interference with the antigen-detection immunoassay for HCV infected patients by anti-sera developed in sheep or goat was previously investigated and concluded that antigen detection ELISA for captures and detection might misdiagnose HCV infected serum immunoglobulin concentration (results not shown). Results indicate that this bias will be avoided if reagents for capture and detection are derived from different species such as rabbit. This is in agreement with previous reports (16-18).

A mechanism that may account for this study is that the presence of these anti-sheep immunoglobulins antibodies in HCV infected patients may reflect the increase production of autoantibodies and then, lead to humoral immune abnormalities. This is best explained by suggesting that there is an interaction producing spurious immuno-precipitation as well as a circulating immunoglobulin which is capable of binding other autologous immunoglobulins may well interact with other immune factors, thus participating in vivo in the complex immunopathological events which occur in HCV infected sera. Moreover, this study indicates the risk factor of antibodies reacting with human immunoglobulins in sera from HCV infected patients which may play a major pathogenetic role by the generation of autoantibodies.

Finally, the presence of circulating immune complex may help to explain the immunological abnormalities and extra-hepatic disorders observed in HCV infection, thereby providing an opportunity for early intervention that may be used for predicting disease in at-risk populations. Furthermore, the presence of circulating immune complex in serum during the early phase of infection may contribute to the persistence of HCV and its many immunopathological effects in the infected host. The findings in this study may provide some new insights into antibody response to HCV.

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