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Diagnostic Value of Immunoglobulin A and M antibodies as a disease Marker for hepatitis A Infection

[İmmünogloblin A ve M Antikorlarının Hepatit A Enfeksiyonu Yönünden Teşhis Edici Değeri]

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ABSTRACT

This study was conducted to assess the effect of anti-immunoglobulin antibodies on the measurement of the humoral immune response in hepatitis A virus (HAV) infected patients. Serum samples from 47 patients with acute hepatitis A and from 47 age/sex matched healthy adult subjects were tested for IgA, IgG and IgM by ELISA. Anti-immunoglobulin antibodies were defined using goat immunoglobulins as a target to characterize distinct changes in levels of interacting immunoglobulins. Initial results obtained before removal of antibodies that interacted with goat immunoglobulins suggested that HAV patients had increased levels of IgA and IgM in their sera. It was found that normal individuals had mean IgA, IgG and IgM levels of 2.52 ± 0.22 mg/ml, 9.86 ± 0.94 mg/ml and 1.75 ± 0.13 mg/ml, respectively while HAV patients had mean levels of 2.89 ± 0.20 mg/ml, 10.03 ± 1.05 mg/ml and 1.97 ± 0.13 mg/ml ($p < 0.0002$, $p < 0.41$ and $p < 0.0001$). However, the mean levels of IgA and IgM in HAV infected sera, after purification from antibodies that interacted with goat immunoglobulins, were 2.49 ± 0.21 mg/ml and 1.73 ± 0.14 mg/ml. Therefore, there was no significant difference in HAV patients compared to normal individuals ($p < 0.55$ and $p < 0.56$). 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 HAV.

Key Words: Hepatitis A virus, Antibodies, IgA, IgM, IgG

ÖZET

Bu çalışma hepatit A enfeksiyonlu (HAV) olgularda humoral cevabın ölçüsü olarak anti–immunoglobulin antikorlarının kullanımını değerlendirmektedir. 47 akut hepatit A olgusu ile yaş ve cinsiyet yönünden eşleştirilmiş sağlıklı 47 kontroldan toplanan serumlarda ELISA metodu ile IgA, IgG ve IgM ölçülmüştür. Keçi immünoglobulinleri kullanılarak anti-immünoglobilin antikorları ayrıştırılmış ve immünoglobulin düzeylerindeki değişimler belirlenmiştir. Keçi immünoglobinleri ile etkileşen antijenlerin ayrılmasından önceki ön sonuçlar HAV hastalarının serum IgA ve IgM düzeylerinin yükselmiş olduğunu göstermektedir. Normal şahıslarda ortalama IgA, IgG ve IgM düzeyleri sırasıyla 2.52 ± 0.22 mg/ml, 9.86 ± 0.94 mg/ml ve 1.75 ± 0.13 mg/ml, HAV hastalarında ise ortalama 2.89 ± 0.20 mg/ml, 10.03 ± 1.05 mg/ml ve 1.97 ± 0.13 mg/ml olarak bulunmuştur ($p < 0.0002$, $p < 0.41$ ve $p < 0.0001$). Buna rağmen, HAV enfeksiyonlu hastalarda keçi immünoglobulinleri ile etkileşen antijenlerin ayrıştırılmasından sonraki ortalama IgA ve IgM düzeyi 2.49 ± 0.21 mg/ml ve 1.73 ± 0.14 mg/ml.dir. Bu yüzden, HAV hastaları ile normal şahıslar karşılaştırıldığında anlamlı bir farklılık ($p < 0.55$ ve $p < 0.56$) gözlenmemiştir. Enfeksiyonun erken döneminde dolaşımda bulunan immün kompleks, hastadaki immünopatolojik etkilere katkıda bulunabileceği gibi HAV enfeksiyonunda gözlenen antijen cevabına da ışık tutabilir.

Anahtar Kelimeler: Hepatit A virüsü, Antikorlar, IgA, IgM, IgG

INTRODUCTION

Hepatitis A virus (HAV) causes acute viral hepatitis in humans by an immuno-pathogenetic mechanism (1). Spread of infection is generally person-to-person or by oral intake after fecal contamination of skin or mucous membranes; less commonly, there is fecal contamination of food or water (2, 3). However, the mechanism by which the virus first enters the bloodstream and reaches the liver as well as the pathogenetic mechanism leading to a relapsing disease remains unclear. The symptoms of HAV infection are often mild or unnoticed. Therefore, the true prevalence of HAV infection is difficult to determine (4). The immunology of hepatitis A is important for two reasons. First, specific diagnostic tests for the confirmation of HAV as the etiologic agent are dependent on the production of antibody by the humoral immune response. The humoral immune response also leads to the development of circulating immune complexes (CIC) (5) with associated symptoms and signs in some patients (6, 7, 8). Second, clearance of viral infection and the disease manifestations associated with this process is almost certainly produced by the cellular immune response. Immunoglobulin M (IgM), IgG, and IgA antibodies directed against conformational epitopes on the HAV particle are induced and can usually be detected by the onset of clinical illness. The hepatitis A-specific IgM response is limited to the initial infection except in rare instances and thus becomes a useful marker of acute disease. IgA is also produced for a limited period. Its role in immunity is uncertain. The IgG response to HAV is delayed compared with IgM and IgA responses but is long-lived and accounts for resistance to reinfection (9, 10).

Diagnosis of acute hepatitis A is based on the detection of the IgM antibody to HAV (HAV-IgM) in patients who present with clinical features of hepatitis. Nevertheless, since many cases of hepatitis A are asymptomatic, HAV-IgM can be found without clinical symptoms or biological abnormalities (11). IgM antibodies directed against specific viral antigens can be detected due to nonspecific polyclonal activation of memory cells from a previous infection with an unrelated agent. Immune cells may become activated during viral infections or immune diseases (12, 13). Thus, Anti-HAV IgM detection could also correspond to immune reactivation in some cases.

Immunoglobulins, which bind other immunoglobulins or antibodies, add another facet to the abnormal immune response of HAV infected patients. The human antibodies, which reacted in this way, were termed anti-ruminant antibodies (14, 15, 16).

The present study was therefore designed to investigate the presence of these anti-immunoglobulin antibodies in hepatitis A infected patients. Anti-immunoglobulin antibodies were defined by goat immunoglobulins as a target to determine the effect of anti-immunoglobulin antibodies on the measurement of the humoral immune response in HAV infected patients in an attempt to characterize distinct changes in patterns of immunoglobulin levels in hepatitis A infected patients.

MATERIALS 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, Gillingham, UK) and all other chemicals were supplied from BDH (VWR International Ltd, Leicestershire, UK).

Subjects

Sera were collected from 47 patients with clinical diagnosis of acute hepatitis. Serological diagnosis was based on the detection of anti-hepatitis A virus (anti-HAV) IgM, hepatitis B virus (HBV) markers (anti-HBV core IgM, HBV surface antigen and HBV early antigen), anti-hepatitis C virus (anti-HCV) IgG, and anti-HEV IgG. Forty-seven patients diagnosed with hepatitis A that fulfilled these conditions and were admitted to the study. Forty-seven participants unaffected by HAV were selected as a control group. Affected and unaffected groups were matched for age and sex (median age, 34 years [range, 24 to 49 years]). Medical history, physical examination and routine laboratory investigations were completely normal in all unaffected subjects. They did not use any medication prior to this study. All sera were collected within three months and stored in small aliquots at -80 ^{o}C until tested under code. Informed patient consent was obtained in every case and the use of blood for scientific studies was approved by the local Ethical Committee.

Electrophoresis of immuno-precipitates on polyacrylamide gel

Human serum samples were immuno-precipitated with anti-human IgA developed in rabbit in the presence of goat immunoglobulins. Human serum samples [25 µl] were diluted with 1x PBS [475 µl] and, in addition, 75 µl of anti-IgA antiserum 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 h at room temperature. The precipitate was removed by centrifugation at 13,000 rpm for 5 min in microcentrifuge and then washed with 100 µl 1x PBS. The antigen-antibody precipitate was dissolved in 50 µl of 2 x Laemmli sample buffer (0.125 M Tris-HCl pH (6.8), 4 % (w/v) SDS, 20 % (w/v) glycerol, 10 % (v/v) β-mercaptoethanol, 0.005 % (w/v) bromophenol blue) and then incubated at 95 ^{o}C for 3 min (17). A fraction of this mixture [25 µl] was electrophoresed overnight on a 10 % SDS polyacrylamide gel at a constant voltage of 45 V at room temperature. Following electrophoresis, proteins in gel were visualized by staining with Coomassie blue staining (18).

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 (19, 20). After overnight incubation at 4 °C the plate was washed 4 times with PBST20 (0.1 % (w/v) [Tween 20 in 1 x 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 were 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 secondary is the word that is mosty used 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.

Purification of HAV infected sera from the effect of antibodies that interact with goat immunoglobulins

Goat immunoglobulins were isolated previously from goat serum by affinity chromatography using the appropriate sepharose-bound antibody. The final purified antibody preparation contains only antigen-specific active antibody plus a small amount of denatured antibody resulting from elution procedure (Tago, 4100 series Ab). Goat immunoglobulins, 200 µl, at a concentration of 10 mg/l in PBS, pH 7.2 were mixed with 200 µl of human serum samples (diluted 1 in 10) to minimize further cross-reactivity to goat immunoglobulins. The absorption was carried out for 1 h at 37 °C, followed overnight at 4 °C. The human sera were clarified by centrifugation at 10000 x g for 20 min at 4 °C before testing (21, 22). The absorption of human sera with goat immunoglobulins completely removed the positive reaction of anti-immunoglobulin antibodies, 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 HAV infected sera bind and co-precipitate with goat immunoglobulins using immuno-precipitation reaction. Eight serum samples from HAV infected patients, eight sera from unaffected control participants were immuno-precipitated in the presence of goat immunoglobulins, and the immuno-precipitates were electrophoresed on a polyacrylamide gel (Figure 1). Results showed that goat immunoglobulins precipitated more antibodies in HAV infected sera (lanes 1-8) than in normal unaffected sera (lanes 9-16). Visual examination of the intensities of heavy chain bands showed that goat immunoglobulins bind and co-precipitate with the antigen-antibody complex that reveals differences in band intensities and co-migrates with the IgA heavy chain.

It is not possible to differentiate between IgA and other immunoglobulin heavy chains using polyacrylamide gel electrophoresis, therefore ELISA measurements were carried out with or without pre-treatment with goat immunoglobulins. Sera from individuals unaffected or affected with HAV where allocated to two groups (A and B). Each serum sample in group A (from normal unaffected individuals) was divided into two and assigned to groups 1 and 2 and each sample in group B (from HAV affected individuals) was assigned to groups 3 and 4. Groups 1 and 3 were untreated while groups 2 and 4 were treated as outlined in Materials and Methods (Purification of HAV infected sera from the effect of antibodies that interact with goat immunoglobulins). Unaffected and HAV affected sera were pre-treated with goat immunoglobulins to examine whether this would affect the immunoglobulin levels measured by ELISA. Results

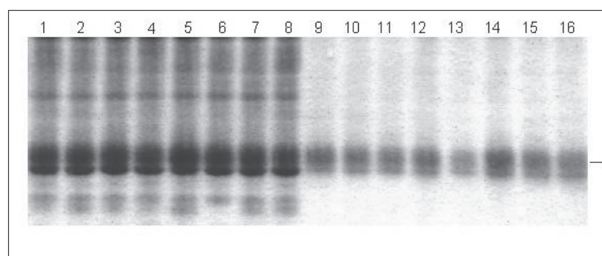

Figure 1. Immuno-precipitation and polyacrylamide gel electrophoresis of human serum samples with anti-human IgA. Eight HAV infected serum samples (lanes 1-8) and eight normal sera (lanes 9-16) were immuno-precipitated with anti-IgA developed in rabbit in the presence of goat immunoglobulins. The precipitates were washed, dissolved in Laemmli sample buffer and analyzed by 10 % SDS polyacrylamide gel electrophoresis.

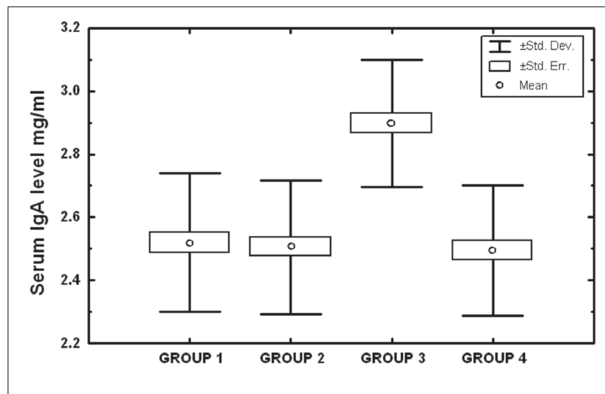

Figure 2. Serum IgA levels in groups of HAV infected sera and unaffected control measured by ELISA. Comparison of average serum IgA (mean ± SD).

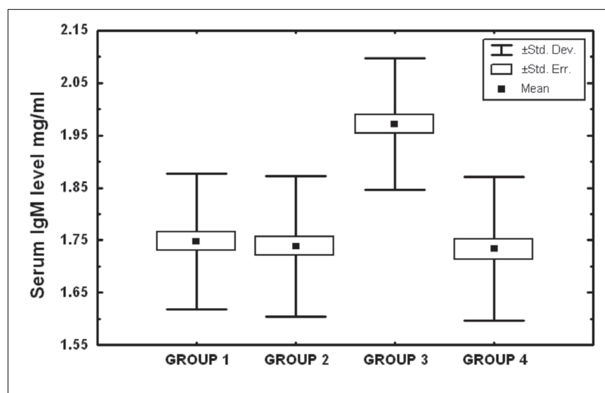

Figure 3. Serum IgM levels in groups of HAV infected sera and unaffected control measured by ELISA. Comparison of average serum IgM (mean ± SD).

(Figure 2 and 3) demonstrated that pre-treatment of sera with goat immunoglobulins prior to ELISA affected both IgA and IgM levels, but that were dramatically reduced the levels of IgA and IgM in the sera from HAV patients (group 4) while sera from unaffected control individuals (group 2) were barely affected by this treatment.

The quantitative analysis of serum IgA and IgM level (mean ± SD) found that group 1 had mean IgA and IgM levels of 2.52 ± 0.22 and 1.75 ± 0.13 mg/ml, respectively which was lower than group 3 (2.89 ± 0.20 mg/ml and 1.97 ± 0.13 mg/ml). This represented significant increases in both IgA and IgM level in the sera of group 3 compared to group 1 ($p < 0.0002$ and $p < 0.0001$). However, the mean levels of both IgA and IgM in the sera of group 4 were 2.49 ± 0.21 mg/ml and 1.73 ± 0.14 mg/ml and there were non-significant differences between group 4 and group 1 ($p < 0.55$ and $p < 0.56$). In addition, the effect of goat immunoglobulins treatment on IgA and IgM levels in the sera of the control group (group 2) was previously investigated and concluded that the IgA and IgM level were within the normal level after treatment (2.51 ± 0.21 mg/ml and 1.74 ± 0.13 mg/ml; $p < 0.22$ and $p < 0.17$). On the other hand, non-significant difference was found in IgG level (Table 1) of group 3 compared to normal individuals of group 1 ($p < 0.41$). Hence, pre-treatment of sera from normal or HAV infected individuals with goat immunoglobulins leads to removal of the differences in IgA and IgM detected by ELISA. Our results in this study did not address any statistical significant differences in sex variation (data not shown).

DISCUSSION

We had access to serial serum samples collected over 3 months from 47 hepatitis A patients. The demonstration of antibodies that interact with goat immunoglobulin in HAV infected sera initiated a controversial debate on the value of goat immunoglobulin antibodies as a risk factor in HAV. Our data suggest a high frequency of goat immunoglobulin/antibody cross reaction among HAV infected patients. The diagnosis of acute hepatitis A is most commonly made through the detection of immunoglobulin M (IgM) anti-hepatitis A antibody (23, 24), although in various test results for IgM antibody to hepatitis A virus were falsely positive (25). Hence, a sensitive IgM test should be able to identify most disease cases from late-convalescent phase serum specimens.

In addition, during the first days of infection, the avidity indicator may not be presented due to the insufficient amount of specific IgG. Immunoglobulin G maturation occurs after a variable delay depending on the individual so avidity measurements cannot accurately date primary infection. Previous study demonstrated

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

Study Group	Case	Serum IgA Level (mg/ml)	Serum IgM Level (mg/ml)	Serum IgG Level (mg/ml)
1 (n = 47)	Normal (untreated)	2.52 ± 0.22	1.75 ± 0.13	9.86 ± 0.94
2 (n = 47)	Normal (Pretreated)	2.51 ± 0.21*	1.74 ± 0.13#	___
3 (n = 47)	HAV infected (untreated)	2.98 ± 0.20**	1.97 ± 0.13##	10.03 ± 1.05+
4 (n = 47)	HAV infected (Pretreated)	2.49 ± 0.21***	1.73 ± 0.14###	___

Values are MEAN ± SD: *p <0.17, **p <0.0002, ***p <0.55; #p <0.22, ##p <0.0001, ###p <0.56; +p <0.41 compared with normal individuals

that HAV virions are partially associated with IgA molecules (26). Because IgA molecules and antigen-IgA complexes are eliminated from the blood by liver functions, IgA molecules not only may assist HAV entry into hepatocytes but also may direct HAV to the liver. This mechanism, which may depend on the strength of the IgA response during the first contact with the virus (27), would protect the virus from the neutralizing activities of low-avidity IgM and IgG antibodies present in this early phase of the clinical course of the infection and would thereby enable re-infection of the liver. The relevance of this mechanism, by which HAV-IgA complexes may overcome the intestinal barrier and contribute to infections of the liver, results from the fact that HAV-IgA complexes are infectious for hepatocytes and that significant amounts of intestinal HAV-IgA are present during acute infections, which are also partly transmitted. Besides supporting the primary infection, this mechanism may play a role in relapsing infections by establishing an enterohepatic cycle for HAV (28, 29).
This problem has directed our research towards other possible immunological factors likely to be present in HAV patients, in an attempt to elucidate further the complex immuno-pathogenetic interactions of the disease: Our finding of goat immunoglobulin/antibody interaction in HAV sera, leading to inaccuracies in IgA and IgM estimation by ELISA represents a move in this direction. In order to overcome this problem of antibody interaction with goat immunoglobulin, the sera were pre-incubated with goat immunoglobulin to eliminate this interaction. Interestingly, after removal of antibodies that interact with goat immunoglobulin, the IgA and IgM levels appear to be normal between the affected and unaffected groups (Figure 2, 3 and Table 1) hence, after pre-treatment with goat immunoglobulin, IgM levels alone do not appear to be a reliable measure of HAV infection.
The presence of antibodies that interact with goat immunoglobulin in HAV infected patients may reflect the increased production of autoantibodies and then, lead to humoral immune abnormalities. This is best explained by suggesting that there is an interaction producing false immuno-precipitation, as well as a circulating immunoglobulin which is capable of binding other autologous immunoglobulins and may well interact with other immune factors.
In conclusion, this study indicates that the presence of antibodies reacting with goat immunoglobulin in sera from HAV infected patients may play a major pathogenetic role by the generation of autoantibodies. However, the time course for the development of antibodies before onset of clinical HAV infection is unknown, which might be most sensitive or specific for predicting future development of the disease activity. The high prevalence of elevated goat immunoglobulin/antibody interactions in HAV infected patients enhances the clinical usefulness of this valuable marker due to polyclonal B cell activation or autoantibodies generation. Furthermore, characterization of the autoantibody evidence continues to be an attractive and important tool to get access for accurate diagnosis and to gain insight into antibody response to HAV infection. Finally, the presence of HAV antibodies may help to explain the immunological abnormalities and extra-hepatic disorders, thereby providing an opportunity for early intervention that may be used for predicting disease in high-risk populations.

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