Usefulness of specific IgG avidity for diagnosis of hepatitis A infection

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SUMMARY

Aim — Diagnosis of acute hepatitis A virus (HAV) infection is classically based on the detection of HAV-IgM. Nevertheless, HAV-IgM can be positive for patients with polyclonal stimulation of their immune system (i.e. immune reactivation). To improve the diagnostic yield, an avidity test for HAV-IgG antibodies was developed and tested.

Methods — Avidity tests were performed in 128 sera: 11 selected samples from patients with past infection, 15 acute hepatitis A, 10 vaccinated subjects and 4 patients with immune reactivation as well as 84 HAV-IgM positive unselected sera, provided by routine laboratories.

Results — Patients with past infection had avidities over 70%, whereas avidities in patients with acute hepatitis A were below 50% during the first month following the onset of symptoms. As expected, patients with immune reactivation had avidities over 70% consistent with past infection. The results obtained for the 84 unselected sera allowed reconsidering the diagnosis of acute hepatitis A for nearly a third of patients.

Conclusion — This test could improve the diagnosis of acute hepatitis A infection, particularly in elderly patients.

RÉSUMÉ

Intérêt de l’avidité des IgG spécifiques pour le diagnostic de l’hépatite A

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Le diagnostic d’hépatite A aiguë repose classiquement sur la détection d’IgM anti-virus de l’hépatite A (VHA). Mais les IgM anti-VHA peuvent être positives par stimulation polyclonale non spécifique du système immunitaire chez des malades déjà immunisés vis-à-vis du VHA (réactivation immunitaire). Pour aider au diagnostic différentiel, un test de mesure de l’avidité des IgG anti-VHA a été mis au point et testé sur 128 sérums : 11 ont été obtenus de sujets ayant une infection ancienne, 15 d’infections aiguës (19 échantillons), 10 de sujets vaccinés, 4 de réactivations immunitaires et 84, positifs en IgM anti-VHA, ont été adressés par des laboratoires privés et hospitaliers. Les sujets immunisés par infection ancienne, ainsi que les malades ayant une réactivation immunitaire, avaient des IgG spécifiques avec un indice d’avidité supérieur à 70 %. Les malades à la phase aiguë de la maladie (< 30 jours), avaient des IgG spécifiques avec un indice d’avidité inférieur à 50 %. L’étude des 84 sérums positifs en IgM anti-VHA a remis en cause le diagnostic d’hépatite A aiguë chez presque un tiers des malades. La mesure de l’avidité des IgG anti-VHA est utile et simple à réaliser pour confirmer une hépatite A aiguë, en particulier chez le sujet âgé.

Introduction

Hepatitis A virus (HAV) infection is the leading cause of acute viral hepatitis worldwide [1]. Due to constantly improving hygiene conditions, the prevalence of anti-HAV antibodies has greatly declined in the general French population: only 11.5% of 20-year-old military recruits were immunized in 1997 versus 50% in 1978 [3]. The incidence of HAV infection is estimated at 5,000 to 13,700 new cases per year [2]. Lower natural immunity increases the number of subjects who could become infected during adulthood when the disease is usually symptomatic, and more severe with a risk of fulminating hepatitis. During epidemic outbreaks, 70% to 80% of adult infections are symptomatic, while the proportion is 40% to 50% in children aged more than six years and only 10% among children aged less than six years [4-6].

In patients with a clinical presentation suggestive of acute hepatitis, the diagnosis of HAV infection is generally based on the detection of anti-HAV-IgM. Subjects with other viral infections can however be anti-HAV-IgM positive due to cross-reactivity or polyclonal stimulation of the immune system in previously immunized subjects (immune reactivation). Search for viral genome in serum or feces with reverse transcriptase polymerase chain reaction (RT-PCR) can be used to confirm acute infection, but the duration of viremia and viral excretion are variable, ranging from 5 to 391 days [7-9] for viremia, and from a few days to three months for viral excretion [10]. Moreover, detection of viral genome is not a routine laboratory technique. A technique which could be used in the routine setting and which can distinguish anti-HAV IgM due to acute HAV infection from anti-HAV IgM resulting from immune reactivation is needed.

In this context, IgG avidity tests have been found to be a very useful tool in a large number of viral infections in immunocompetent subjects such as: rubella [11], cytomegalovirus [12], varicella-zoster virus [13], herpes virus 6 [14], Epstein-Barr virus [15], parvovirus B19 [16] and hepatitis C virus [17]. Specific IgG synthesized during the acute phase of the viral infection have low avidity. Avidity increases then with maturation, leading to highly avid IgG. Measurement of avidity might thus enable distinction between acute infection and immune reactivation.

We developed a technique to measure anti-HAV IgG avidity and test its usefulness in the diagnosis of acute HAV infection.

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Patients and methods

Study population

One hundred twenty-eight samples were obtained from five groups of patients:

— **Group 1**: 11 sera from patients immunized against HAV by natural infection: positive for total anti-HAV antibodies (IgM+IgG), and negative for anti-HAV IgM;

— **Group 2**: 19 sera from 15 patients with symptomatic acute hepatitis A confirmed by the presence of anti-HAV IgM in serum and, for 11 of 12, positive detection of viral genome in serum. For three subjects, four samples were obtained after the onset of the clinical signs, so the time course of IgG levels could be established;

— **Group 3**: 10 sera from subjects vaccinated against HAV sampled 3 to 84 months after vaccination;

— **Group 4**: 4 sera which were anti-HAV IgM positive but viral genome negative. In these patients the presence of anti-HAV IgM was attributed to immune reactivation following hepatic cytolysis due to other causes than HAV. One of these patients, an 81-year-old man with active chronic hepatitis of unknown cause, was also positive for anti-CMV IgM and anti-EBV IgM. Two other patients, an 87-year-old man and a 78-year-old woman, had hepatic cytolysis related to anti-tuberculosis treatment. The fourth patient was a 57-year-old woman with malignant hematological disease who had hepatic cytolysis attributed to chemotherapy.

— **Group 5**: 84 sera from unselected anti-HAV IgM positive patients tested by routine laboratories in an epidemiological (N = 74) or difficult diagnostic (N = 10) context.

All sera were stored at -20°C for serology tests and at -80°C for molecular biology tests.

Total anti-HAV and anti-HAV IgM serology

Sera from the first four groups of patients were tested for total antibodies using the EIT-AB-HAVK-3 kit (Diasorin, Saluggia, Italy) and for anti-HAV IgM using the Vidas IgM technique (bioMérieux, Marcy l’Etoile, France). Among the sera from group 5 patients, 76 were tested for anti-HAV IgM using the HAVAB-M version 2 AxSYM (99.59% sensitivity and 100% specificity) (Abbott, Abbott Park, IL), four using the Vidas IgM technique and four using the Enzygnost Anti-HAV IgM technique (sensitivity 99.3%, specificity 100%) (Dade Behring, Marburg, Germany). The sensitivities of these methods were established by the manufacturer such that a positive result indicates acute HAV infection. Anti-HAV IgM are detectable in serum on average 8 to 12 weeks after peak elevation of serum transaminases.

Anti-HAV IgG serology

An ELISA kit using plates covered with HAV antigen (VAI Elisa plates, Viral Antigen Inc, Memphis TN) was used in accordance with the manufacturer’s instructions. Briefly, serum samples and negative and positive controls were diluted 1/21 in the dilution solution provided by the manufacturer. One hundred µL of diluted samples and controls were deposited in wells and incubated for 30 min at room temperature. The plate was washed manually three times with a washing solution containing PBS 1X, BSA 1%, and Tween-20 0.05%. Then 100 µL of rabbit anti-human IgG IgG conjugated with alkaline phosphatase were added, diluted 1/500 in the washing solution (Dakocytomation, Trappes, France) were added. After incubation for half an hour at room temperature and three washings, 100 µL of substrate (Sigma p-NPP® diluted in 5 mL 0.1M diethanolamine) were added in each well. After incubation for 15 min in obscurity at room temperature, the reaction was stopped with 100 µL stopping solution (1M NaOH) and absorbance was read at 405 nm.

The threshold level for anti-HAV IgG was set at ±3 standard deviation (SD) of the mean absorbance recorded for the negative control deposited in triplicate for each test.

Measurement of anti-HAV IgG avidity

Anti-HAV IgG avidity was measured with the same technique as described above using a washing buffer containing 6M urea (18). Each sample was deposited in duplicate. One well was washed three times using the washing solution without urea and the other using the washing solution with urea, with 5-min contact for each washing. Before adding the conjugate, all wells were washed a last time using the washing solution without urea.

When the optical density (OD) of the samples was above the threshold level, avidity was calculated as:

\[
\text{avidity index} = \left( \frac{\text{absorbance with urea}}{\text{well absorbance without urea}} \right) \times 100
\]

Ten sera were tested twice to determine the technique reproducibility (coefficient of variability 7.5%).

RT-PCR detection of viral genome

Viral RNA was extracted from 140 µL sera using the QiAmp Viral RNA® technique (Qiagen, Courtaboeuf, France). Ten µL of extract RNA were then amplified by one-step RT-PCR (Qiagen) using probes described elsewhere (19). A 512-base pair fragment of the VP1/2A region of the hepatitis A viral genome was thus amplified. The sensitivity of this technique, established from a WHO HAV RNA standard provided by the National Institute of Biological Standards and Controls (NIBSC, Hertfordshire, UK), was 43 IU/mL.

Statistical analysis

Results, presented as mean ± SD were analyzed with the Pearson and Student t-tests.

Results

Results obtained with sera from the first four groups of patients are presented in figure 1.

The avidity index for anti-HAV IgG from patients immunized against HAV by past infection was greater than 70% (m = 86 ± 10%). The avidity index could not be calculated for 4 of the 15 patients with acute hepatitis A because the IgG titer was below the threshold value. For the eleven other patients, the anti-HAV IgG avidity index varied from 19% to 66% (mean = 36 ± 16%). This mean was significantly different (P < 0.001) from the group with past infection. Among the 8 sera sampled less than 30 days after occurrence of first clinical signs of acute HAV infection, the avidity indexes were all below 50% (28 ± 12%). Search for viral genome was performed in 12 subjects with acute infection and was positive in 11. In vaccinated subjects, the avidity index varied from 20% to 71% (44 ± 17%) independently of vaccination date. The four sera from patients with documented immune reactivation showed avidity indexes above 70% (88 ± 10%).

The avidity indexes for patients with acute hepatitis A are presented in figure 2 by the number of days between serum sampling and onset of clinical signs. The time course of the avidity

Fig. 1 – IgG anti-HAV avidity index according to clinical data.

Avidité des IgG anti-VHA en fonction du contexte clinique.
index could be established for three patients. The time to maturation of anti-HAV IgG showed interindividual variability but all indexes were above 70% 2 to 6 months after the onset of clinical signs.

The results of anti-HAV IgM positive sera tested in routine laboratories are summarized in Table 1. Twenty sera had an anti-HAV IgG level below the threshold value preventing calculation of the avidity index. The RT-PCR procedure was performed on 16 of these sera to search for viral genome and was positive in 14. Anti-HAV IgG positive sera from 30 patients had an avidity index below 50% (23 ± 11%) and RT-PCR was positive in 28 of 29 tested sera. Mean age of these 50 patients was 26 years and mean optical density for IgM was 4.5. Twenty-five patients were positive for specific IgG with an avidity index greater than 70% (85 ± 9%). Search for viral genome was negative in the 23 tested sera. Mean age of this group was 57 years and mean optical density for anti-HAB IgM was 1.7. Nine IgG positive patients had avidity indexes ranging from 50% to 70%. Search for viral genome was performed in six of these patients: two were positive for viral genome with avidity indexes of 50% and 51%, and four were negative with avidity indexes greater than 56%.

### Discussion

The avidity index of specific IgG has been found to be a useful tool for distinguishing primary infection from past infection for a larger number of viral diseases [11-17]. Specific IgG synthesized during the acute phase of infection have weak avidity while IgG with a high avidity index are indicative of past infection.

The results of the present study demonstrate that anti-HAV IgG in immunocompetent persons have the same maturation pattern as observed for other viral infections with weak avidity following primary infection and strong avidity in subjects immunized by past infection. During the first days of infection, the avidity index may not be available due to the insufficient amount of specific IgG. IgG maturation occurs after a variable delay depending on the individual so avidity measurements cannot accurately date primary infection. However, avidity indexes greater than 70% can exclude HAV infection in the two months before and indexes below 50% indicate acute infection. Avidity indexes ranging from 50% to 70% have to be interpreted with caution since we found a positive viremia in a small number of these subjects.

Vaccinated subjects exhibited variable avidity indexes, often lower than subjects immunized by natural infection. This finding is similar to that reported for rubella where specific IgG were found to mature slower and present a weaker avidity in vaccinated subjects compared with individuals immunized by natural infection [20].

Our avidity test allows distinguishing anti-HAV IgM positivity resulting from recent infection from potential IgM positivity resulting from polyclonal stimulation of the immune system in patients previously immunized against HAV. In our four patients with documented immune reactivation, the anti-HAV IgG exhibited strong avidity (index > 70%) similar to subjects with past infection. Analysis of the anti-HAV IgM positive sera provided by routine laboratories demonstrated the frequency of immune reactivation which can question the diagnosis of acute hepatitis A. We found that only 50 of the 84 subjects tested (60%) had specific IgG with weak or non-calculable avidity compatible with acute hepatitis A. RT-PCR confirmed the presence of virus in 42 of the 45 tested sera. Inversely, anti-HAV IgG exhibited strong avidity in 25 sera (30%) and search for viral genome was negative in all of those tested. Because of the specificity displayed by commercial IgM assays, the IgM positivity in these cases did certainly not result from cross reactions but more likely from a polyclonal stimulation of the immune system. Higher mean age in this group of subjects and lower optical densities for specific IgM support this conclusion.

Measuring avidity led to a reconsideration of the diagnosis of acute HAV infection for nearly one-third of the patients with search for another cause. This proportion of immune reactivation in the general population could appear to be high, but is in

### Table I

<table>
<thead>
<tr>
<th>Vidity index (%)</th>
<th>Mean IgM anti-HAV OD</th>
<th>Mean age (range) years</th>
<th>Number of patients</th>
<th>RT-PCR VP 1/2A</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; cut-off</td>
<td>4.5 (1.1)</td>
<td>26 (6-53)</td>
<td>20 (24 %)</td>
<td>14</td>
</tr>
<tr>
<td>&lt; 50 %</td>
<td>4.5 (1.1)</td>
<td>25 (8-61)</td>
<td>30 (36 %)</td>
<td>28</td>
</tr>
<tr>
<td>50-70 %</td>
<td>3.7 (2.9)</td>
<td>50 (12-93)</td>
<td>9 (10 %)</td>
<td>2</td>
</tr>
<tr>
<td>&gt; 70 %</td>
<td>1.7 (0.9)</td>
<td>57 (19-86)</td>
<td>25 (30 %)</td>
<td>0</td>
</tr>
</tbody>
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Densité optique (DO) des IgM anti-VHA, moyenne d'âge, indice d'avidité des IgG anti-VHA et résultats de la recherche du génome viral par RT-PCR des 84 malades ayant des IgM anti-VHA positives (groupe 5).
agreement with a recent French report showing that 82% of individuals aged over 50 years are anti-HAV IgG positive [21]. Further large-scale prospective work would however be necessary to confirm these results.

In light of our findings, the results of anti-HAV IgM tests should be interpreted with caution, particularly in older subjects and if the optical density is low, close to the threshold,. In this context, measurement of anti-HAV IgG avidity is a useful tool applicable in routine setting to distinguish between acute infection and polyclonal immune stimulation.

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