
Original article

Cytoplasmic rods and rings autoantibodies developed during pegylated interferon and ribavirin therapy in patients with chronic hepatitis C

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Background: Serum autoantibodies are frequently detected in patients with chronic HCV infection, reflecting the wide spectrum of immune reactions related to this virus. In the present study, a novel autoantibody to cytoplasmic rods and rings (RR) in chronic HCV patients was characterized.

Methods: Sera from 75 previously untreated HCV patients were investigated by indirect immunofluorescence using HEP-2 cell substrate before and during pegylated interferon (PEG-IFN)/ribavirin (RBV) therapy. HEP-2 cells were cultured and fixed either following standard protocols or with the addition of RBV in culture medium.

Results: In 15 out of 75 (20%) patients, analysis revealed the presence of antibodies to rod-like cytoplasmic structures ranging approximately 3–10 μm in length and rings approximately 2–5 μm in diameter. These RR structures became detectable in >95% of cells after addition of RBV in culture medium, whereas they were absent in untreated cells. Anti-RR antibodies were found in sera collected during PEG-IFN/RBV treatment only, but never detected before antiviral therapy nor in control groups. More importantly, these anti-RR antibodies were more often detected in non-responder/relapsers than in responder patients (33% versus 11%; P-value =0.037).

Conclusions: An RBV-induced autoantibody was identified to a new cytoplasmic autoantigenic structure developed in HCV patients after PEG-IFN/RBV and this same structure can be induced by RBV in vitro culture. Owing to the onset of anti-RR antibodies in PEG-IFN/RBV-treated patients and their association with a treatment failure, studies are deemed necessary to clarify whether anti-RR plays a role in the response to PEG-IFN/RBV therapy.

Introduction

Serum organ and non-organ specific autoantibodies are frequently detected in patients with chronic HCV infection, reflecting the wide spectrum of immune reactions associated with HCV [1–10]. Experimental studies have in fact demonstrated the existence of an interaction between the HVR1-E2 region of the virus and CD81 receptors on the surface of B lymphocytes, which promotes B cell proliferation and activation, thereby stimulating antibody production [11,12]. In addition, a significant homology motif between HCV polyproteins and tissue autoantigens has been postulated to suggest ‘molecular mimicry’ as a mechanism that may also play a role in priming specific autoantibodies in patients chronically infected by HCV [13–16]. Finally, several clinical studies have reported deterioration of pre-existing autoimmune diseases in patients receiving interferon (IFN) therapy for hepatitis C, as well as de novo induction of autoimmune phenomena in HCV-infected patients on IFN-α therapy. In this setting, the most common phenomenon associated with IFN therapy is the de novo induction of serum tissue antibodies or boosting of the titre of pre-existing autoantibodies that, in some patients, ends with the development of an autoimmune disease [17–26]. The immune properties of IFN, at least in part, rely on its ability to enhance the expression of HLA class I and II antigens on cell membranes, promote T-cell activation...
and the subsequent release of cytokines, mechanisms that could account for the onset of autoimmune reactions during IFN therapy in patients with chronic hepatitis C [27].

To date, there is little data on autoimmunity in HCV patients being treated with the combination pegylated (PEG)-IFN plus ribavirin (RBV) [28–30]. One main reason for this is that high titre of serum autoantibodies remains a widely accepted criteria against starting IFN therapy in patients with chronic hepatitis C, mainly as a consequence of the strict criteria of exclusion adopted in the design of registration trials rather than for the evidence of adverse reactions in practice. In other studies, the presence of tissue autoantibodies was associated with an increased risk of treatment failure [31–34]. We describe a novel autoantibody developed in HCV patients under treatment with PEG-IFN and RBV and its correlation with the outcome of treatment.

Methods

Patients

From 2004 to 2007, 156 previously untreated patients with chronic hepatitis C were consecutively treated with PEG-IFN/RBV at the Liver Center, Istituto Clinico Humanitas, Rozzano, Italy. Seventy-five patients with sera available in our laboratory for autoantibody tests at baseline and during treatment were investigated. All subjects had histologically proven, compensated liver disease characterized by elevated serum alanine aminotransferase (ALT) activity, anti-HCV antibodies and serum HCV RNA for at least six months. Excluded were patients with autoimmune diseases, infection with HBV or HIV, and with general contraindications to using either IFN or RBV [35]. Patients were treated with subcutaneous injections of PEG-IFN-α2a (Pegasys; Roche, Basel, Switzerland) 180 μg once weekly coupled with oral RBV (Copegus; Roche, Basel, Switzerland) at a dose of 1,000 mg per day for patients weighing 75 kg or less and 1,200 mg per day for those weighing more than 75 kg. All patients gave their written informed consent to receive therapy and permission for use of their medical records. HCV-1- and HCV-4-infected patients were treated for 48 weeks. HCV-2 and HCV-3 patients were treated for 24 weeks. Patients were classified as non-responders (NR) if HCV RNA was still detectable at week 24 of therapy, as relapers (REL) if HCV RNA was detected after the end of treatment in patients with a virological response, and as sustained virological responders (SVR) if HCV RNA was undetectable in the 24 weeks after the completion of therapy. As controls, sera were obtained from 105 primary biliary cirrhosis patients, 43 with primary sclerosing cholangitis, 56 with autoimmune hepatitis, 100 untreated HBV-related chronic active hepatitis patients, 100 with hepatocellular carcinoma and 100 blood donors. This study meets and is in compliance with all ethical standards in medicine and informed consent was obtained from all patients according to the Declaration of Helsinki.

Cell culture and indirect immunofluorescence studies

Different cell lines, such as normal human epidermoid larynx carcinoma HEp-2, human colon cancer cell line HCT116, human cervical cancer cell line HeLa, myelogenous leukaemia cell line K562, oral squamous cell carcinoma CAL 27 and rat kidney fibroblasts NRK, were cultured and prepared with multiple fixation methods that included acetone, -20°C, for 5 minutes, or 3% paraformaldehyde and 0.1% triton-X fixation as commonly used in many standard cell biology laboratories. For the induction of RR, RBV (Sigma–Aldrich, St Louis, MO, USA; R9644) was solubilized in water to a stock of 50 mM and was added to cultured cells seeded in monolayer at a final concentration of 2 mM for 24 h. RR were detected using patient sera and anti-IMPDH2 (anti-inosine monophosphate dehydrogenase 2; Proteintech, Chicago, IL, USA; 12948-1-AP). Anti-nuclear (ANA), anti-smooth muscle (SMA), anti-mitochondrial (AMA) and anti-liver kidney microsomal (LKM) antibodies were detected by IIF at a serum dilution of 1:40 or higher in PBS and incubated for 45 min using as substrates commercial HEp-2 (INOVA Diagnostics, San Diego, CA, USA) for ANA, whereas cryostat sections of rat kidney were used to detect SMA, AMA and LKM antibodies. Slides were then rinsed in PBS two times for 10 min. Goat anti-human-IgG conjugated to fluorescein isothiocyanate (Cappel, ICN Biomedicals Inc., Aurora, OH, USA) or Alexa 488 goat anti-human-IgG (Invitrogen, Carlsbad, CA, USA) was used as secondary antibody diluted 1:100 in PBS and incubated on slides for 30 min. Slides were then rinsed again in PBS 2 x for 10 min, mounted with cover slip and observed with a fluorescence microscope. IIF slides were read by a single investigator (GC) under standardized experimental conditions.

Statistical analysis

The following 18 biological and clinical variables were analysed for statistical significance: sex, age, ALT, aspartate transaminase (AST), gamma-glutamyl transpeptidase (GGT), bilirubin level, prothrombin time, HCV genotype, HCV viraemia level, presence of autoantibody at baseline (ANA, SMA, LKM and
AMA), treatment outcome (long-term response and relapse/non-response), steatosis, cirrhosis, diabetes mellitus and HCC development during post-treatment follow-up. Data are expressed as number and percentage or mean and range, unless otherwise stated. The differences between different groups were compared using Student’s t-test and Mann–Whitney U test for continuous data; χ² and Fisher’s exact test for categorical data as appropriate. A P-value of <0.05 was considered to be statistically significant. All analyses were made with Stata10 (StataCorp, College Station, TX, USA).

Results

A total of 75 HCV-infected patients were treated with standard of care PEG-IFN-α2a plus RBV regimens. Forty four patients (59%) were male, with a mean age of 49 ±11 years. Histological evidence of cirrhosis was present in 8 subjects (11%). The overall prevalence of serum autoantibodies at baseline was detected in 26 subjects (35%); the prevalence of ANA, SMA and anti-LKM-1 was 24, 8, and 3% respectively. None of the patients were AMA-positive. No patient met the criteria of probable or definite autoimmune hepatitis on the basis of the International Autoimmune Hepatitis Group score [36]. Demographic, clinical and laboratory features of the patients are shown in Table 1.

The IIF study on HEP-2 cells substrate revealed the presence of antibodies reacting with a cytoplasmic structure in 15 patients (20%) with HCV who received PEG-IFN/RBV (Table 2). Human prototype serum Ir2006 recognized rods ranging approximately 3–10 μm in length and rings ranging approximately 2–5 μm in diameter (Figure 1A). These cytoplasmic structures, here referred to as ‘rods and rings’ or RR, were found in more than 95% of HEP-2 cells. Usually there were only one or two RR per HEP-2 cell. Rods may align either close to the nuclear envelope (Figure 1C) or extend perpendicularly from the nucleus (Figure 1B), while rings are found at a low frequency (approximately 10% of all cells) only in the cytoplasm (Figure 1D).

Colocalization experiments with known markers showed no association with any known cytoplasmic organelles, including Golgi complex, mitochondria, centrosomes and GW bodies which are cytoplasmic foci known to mediate gene expression via the small RNA-guided translation silencing process [37] (WCC et al., unpublished observations). Table 2 summarizes the relevant clinical features of the 15 patients who tested positive for anti-RR. Interestingly, anti-RR autoantibodies were found in the sera collected in HCV patients during PEG-IFN-α2a plus RBV treatment only (15/75, 20%) whereas it was never detected before antiviral therapy (0/75) or in any of the control groups listed in Methods.

Although the INOVA HEP-2 cell substrate was consistently positive (>95% of cells) for RR structures, home grown HEP-2 cells using standard conditions were mainly negative. In fact, among the few commercial ANA slides tested, none other than the INOVA slides showed >1% cells with RR, making these other products extremely difficult for the accurate detection of anti-RR. Preliminary experiments were performed to determine whether IFN or RBV treatment would

### Table 1. Clinical, serological and immunological features of the patients at baseline and response to treatment

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Total patients (n=75)</th>
<th>Patients with RR Ab (n=15)</th>
<th>Patients without RR Ab (n=60)</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>Male</td>
<td>44 (59)</td>
<td>9 (60)</td>
<td>35 (58)</td>
<td>NS</td>
</tr>
<tr>
<td>Female</td>
<td>31 (41)</td>
<td>6 (40)</td>
<td>25 (42)</td>
<td></td>
</tr>
<tr>
<td>Age, years ±SD</td>
<td>49 ±11</td>
<td>48 ±11</td>
<td>49 ±12</td>
<td>NS</td>
</tr>
<tr>
<td>ALT, UI/l (range)</td>
<td>112 (23–408)</td>
<td>123 (32–408)</td>
<td>110 (23–389)</td>
<td>NS</td>
</tr>
<tr>
<td>Genotypeα</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCV RNAα</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANA</td>
<td>18 (24)</td>
<td>4 (27)</td>
<td>14 (23)</td>
<td>NS</td>
</tr>
<tr>
<td>ASMA</td>
<td>6 (8)</td>
<td>3 (20)</td>
<td>3 (5)</td>
<td>NS</td>
</tr>
<tr>
<td>LKM</td>
<td>2 (3)</td>
<td>1 (7)</td>
<td>1 (2)</td>
<td>NS</td>
</tr>
<tr>
<td>AMA</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>NS</td>
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<td>Steatosis</td>
<td>16 (21)</td>
<td>2 (13)</td>
<td>14 (23)</td>
<td>NS</td>
</tr>
<tr>
<td>Cirrhosis</td>
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<td>0 (0)</td>
<td>8 (13)</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes</td>
<td>5 (7)</td>
<td>0 (0)</td>
<td>5 (8)</td>
<td>NS</td>
</tr>
<tr>
<td>Treatment outcome</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SVR</td>
<td>45 (60)</td>
<td>5 (33)</td>
<td>40 (67)</td>
<td>0.037</td>
</tr>
<tr>
<td>REL/NR</td>
<td>30 (40)</td>
<td>10 (67)</td>
<td>20 (33)</td>
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Values presented as n (%) unless otherwise indicated. *Patients were classified according to their genotype, but no statistical correlation was found among the two groups. †Patients' viraemia at the baseline were stratified as <400,000 UI/l, 400,000–800,000 UI/l, >800,000 UI/l. These findings were not statistically relevant. Ab, antibody; ALT, alanine aminotransferase; AMA, anti-mitochondrial antibody; ANA, anti-nuclear antibody; ASMA, anti-smooth muscle antibody; LKM, anti-liver kidney microsomal; NS, not statistically significant (P>0.05); REL/NR, relapers/non-responders; RR, rods and rings; SVR, sustained virological response.
Table 2. Clinical and laboratory characteristics of the 15 patients who seroconverted to RR antigen during pegylated interferon-α2a/ribavirin therapy

<table>
<thead>
<tr>
<th>Patients</th>
<th>Sex</th>
<th>Age</th>
<th>Genotype</th>
<th>HCV RNA, IU/l×10^5</th>
<th>SVR</th>
<th>ANA</th>
<th>ASMA</th>
<th>AMA</th>
<th>LKM</th>
<th>Anti-RR titre</th>
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<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>59</td>
<td>1a</td>
<td>0.3</td>
<td>Yes</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>1:320</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>46</td>
<td>1b</td>
<td>4.0</td>
<td>No</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>&gt;1:320</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>55</td>
<td>2a/2c</td>
<td>3.0</td>
<td>No</td>
<td>1:160</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>1:160</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>44</td>
<td>3a</td>
<td>7.0</td>
<td>No</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>1:320</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>45</td>
<td>3a</td>
<td>6.0</td>
<td>Yes</td>
<td>Neg</td>
<td>1:160</td>
<td>Neg</td>
<td>Neg</td>
<td>1:320</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>41</td>
<td>2a/2c</td>
<td>7.0</td>
<td>Yes</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>1:160</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>58</td>
<td>1b</td>
<td>5.7</td>
<td>No</td>
<td>1:160</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>1:320</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>36</td>
<td>1b</td>
<td>7.0</td>
<td>No</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>&gt;1:320</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>41</td>
<td>1b</td>
<td>3.0</td>
<td>No</td>
<td>Neg</td>
<td>1:160</td>
<td>Neg</td>
<td>Neg</td>
<td>1:160</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>56</td>
<td>1b</td>
<td>7.0</td>
<td>No</td>
<td>1:80</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
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<tr>
<td>11</td>
<td>M</td>
<td>42</td>
<td>1b</td>
<td>7.0</td>
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<td>Neg</td>
<td>Neg</td>
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<td>1:160</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>55</td>
<td>2a/2c</td>
<td>7.0</td>
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<td>Neg</td>
<td>1:160</td>
<td>Neg</td>
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<td>13</td>
<td>F</td>
<td>60</td>
<td>1b</td>
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<td>&gt;1:320</td>
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<tr>
<td>14</td>
<td>M</td>
<td>47</td>
<td>2a/2c</td>
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<td>No</td>
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<td>Neg</td>
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<td>&gt;1:320</td>
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<tr>
<td>15</td>
<td>M</td>
<td>17</td>
<td>2a/2c</td>
<td>3.5</td>
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<td>1:160</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>&gt;1:320</td>
</tr>
</tbody>
</table>

*Speckled. †Vessel smooth muscle antibody. AMA, anti-mitochondrial antibody; ANA, anti-nuclear antibody; ASMA, anti-smooth muscle antibody; F, female; LKM, anti-liver kidney microsomal; M, male; Neg, negative; RR, rods and rings; SVR, sustained virological responder.

Figure 1. Cytoplasmic rods and rings structure stained by sera from HCV patients

INOV A HEP-2 cells stained with human anti-rods and rings (RR) serum (H2006/Alexa 488 goat anti-human-IgG [green]. Nuclei were counterstained with DAPI (blue). Arrows indicate rods or rings. (A) 20× magnification of HEP-2 cells; (B) HEP-2 cell with rod perpendicular to the nucleus; (C) HEP-2 cell with rod adjacent to the nucleus; (D) HEP-2 cell with ring in cytoplasm. Scale bar 10 μM.
Figure 2. Rod and ring structure can be induced with ribavirin

HeLa cells were stained with human anti-rods and rings (RR) serum It2006/ Alexa 488 goat anti-human-IgG (green). HeLa cells are shown (A) untreated or (B) treated with 2 mM of ribavirin for 24 h. Nuclei were counterstained with DAPI (blue). Scale bar 10 μM. (A) shows HeLa cells that were untreated gave primarily diffuse cytoplasmic staining and no detection of RR; however (B) after treating cells with 2 mM of ribavirin for 24 h, ribavirin appeared to induce the aggregation of the protein into RR.

induce the formation of RR in in vitro culture. Several attempts of IFN treatment in HeLa or HeLa cells did not yield expression of RR when treated cells were stained by prototype anti-RR serum It2006. In contrast, RBV treatment readily induced RR formation. Figure 2A shows HeLa cells that were untreated gave primarily diffuse cytoplasmic staining and no detection of RR; however after treating cells with 2 mM of RBV for 24 h, RBV appeared to induce the aggregation of the protein into RR (Figure 2B). Furthermore, different cell lines such as normal rat kidney fibroblasts NRK or immortalized human cancer cell line such as HCT116, HeLa, K562, CAL 27 have been examined after RBV treatment and RR were induced in all cells stained with anti-RR positive sera (WCC et al., unpublished observations). Thus, current data suggest that the RR staining pattern is not unique to HeLa cells but also detected in various immortalized human cell lines and also in cells derived from rodents.

The prevalence of anti-RR antibody is significantly higher in non-responders/relapsers (10/30, 33%) than in responder patients (5/45, 11%; Fisher P-value = 0.037; Table 1). In other words, the prevalence of REL/NR in anti-RR-positive group (10/15=67%) is higher than that of anti-RR-negative group (20/60=33%; Fisher P=0.037). Interestingly, seven anti-RR-positive patients had HCV genotype 2a/2c or 3 (five genotype 2 and two genotype 3); of those seven easy to cure patients, three were non-responders or relapsers. None of the anti-RR-positive patients had cirrhosis.

Discussion

This study describes a novel serum antibody (anti-RR) reacting against a cytoplasmic structure that can be detected by IIF using HeLa cells as substrate in HCV patients treated with PEG-IFN-α2a plus RBV. The overall prevalence of anti-RR antibodies was 20% of HCV treated patients. RR are the first autoantigen described in literature arising during this treatment. In fact, only few drugs are reported in hepatology to induce cytoplasmic autoantigens but all of them are organ-specific like anti-LKM-2 [38], anti-gastric parietal cells [18], anti-thyroid [8,39], anti-pancreatic islet cells [26], anti-glutamic acid decarboxylase [22] and anti-adrenal cortex [26]. Interestingly, anti-RR antibodies were detected in PEG-IFN/RBV-treated patients only. In fact, baseline sera of HCV patients and of controls did not show any reactivity for anti-RR antibodies, thus suggesting an important role of PEG-IFN/RBV in triggering RR seroconversion.

Although INOVA HeLa cells express RR and are suitable for the detection of anti-RR antibodies, a few other pre-made HeLa slides marketed for ANA testing are negative for RR staining using the same prototype serum and other anti-RR positive sera (data not shown). Our novel data show that many cell lines treated with RBV in vitro induced the formation of RR (Figure 2B); the induction led to greater than 95% of cells expressing RR compared to untreated cells not expressing RR. Induction of RR could happen also in vivo in human hepatocytes during antiviral therapy and these changes may induce the immune system resulting in the production of anti-RR.

In general, the most common autoantibody encountered in HCV-treated patients is ANA. Even if all published studies describe only fluorescence ANA patterns without focusing on their identity, it is reasonable to think that their specificities are heterogeneous like in HCC [4], being directed against different nuclear antigens, suggesting a nonspecific reaction of the immune system to nuclear antigens [30,40]. By contrast, the production of anti-RR antibodies in HCV sera is quite different than ANA. In fact, the most important difference is that ANA appears independently from HCV therapy although anti-RR develop only during treatment and are apparently directed exclusively against a single antigenic structure; thus, anti-RR appears to mimic reactivity of serum autoantibodies which develop in the course of systemic autoimmune disease where each disease is associated with a specific profile of autoantibody specificities [41,42]. For example, systemic lupus erythematosus is characterized by a high prevalence of ANA directed to the same structures such as DNA and splicosomal proteins. In this context, the high prevalence of anti-RR antibodies that react to the same cytoplasm structure suggests their direct biological role in HCV treated patients. This hypothesis is supported by our data in which anti-RR positivity seems to alter antiviral response with significantly higher prevalence of anti-RR in non-responders/relapsers than sustained
responders (33% versus 11%; P-value =0.037). This evidence is enhanced in HCV genotype 2 and 3 patients where approximately half of anti-RR positive patients failed to respond to therapy, further suggesting a correlation between anti-RR antibodies and response to antiviral therapy.

In our recent study (WCC et al., unpublished observations), at least one autoantigen recognized by the prototype anti-RR is the enzyme inosine monophosphate dehydrogenase 2 (IMPDH2), which catalyses the oxidation of inosine monophosphate to xanthosine monophosphate in the rate-limiting step in the de novo guanine synthetic pathway responsible for the GTP production. It is intriguing that IMPDH2 is the enzyme known to be inhibited by RBV in vitro and at the same time RBV induced the formation of RR in all cell lines examined to date, including primary mouse cells such as fibroblasts and endothelial cells. We speculate that RR can also form in vivo in the hepatocytes during RBV treatment; in some predisposed patients, IMPDH2 condensation into RR may induce the immune system to develop antibodies. This observation is extremely important also for immunologists to understand the development of drug-induced autoantibodies; in fact, this is the first study that elucidates a mechanistic action of a drug to induce autoantibodies through a topographic rearrangement of cytoplasmic proteins.

As this is a retrospective study, our sera were collected at different times after starting treatment. For this reason we are not able to determine the kinetics of rising anti-RR and we do not know whether these autoantibodies disappear after the end of therapy. With all the caveats of the limited sample size of the present study, one could speculate that anti-RR reflect an immune reactivity to cytoplasmic structure of the liver cells that interact with IFN/RBV therapy. Our current observations in HCV patients undergoing antiviral therapy need to be expanded to clarify the association between anti-RR and response to treatment.

Acknowledgements

Erwin Lam, Natasha Deming and Pabina Dhawan are acknowledged for the early work helping to develop this study. We thank Emanuela Morenghi for support with statistical analyses and Minoru Satoh, University of Florida, for critical reading and editing. This work was supported, in part, by a grant from the National Institute of Health (grant AI47859). WCC is supported by Bridges to Doctorate, University of Florida Graduate School, and University of Florida Alumni Graduate Fellowship. No additional external funding was received for this study.

Disclosure statement

MC has received research support, honoraria and/or consulting fees from Roche, MSD, Bayer, Achillion, Tibotec, Gilead Sciences and BMS. All other authors declare no competing interests.

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Accepted 29 September 2011; published online 1 December 2011