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Significance of Indeterminate Third-Generation Hepatitis C Virus Recombinant Immunoblot Assay

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Indeterminate hepatitis C virus (HCV) third-generation recombinant immunoblot assay (RIBA3.0; Ortho Diagnostic Systems) patterns were arbitrarily defined by the manufacturer as the detection of only one antibody out of the four that were sought, namely, c100 (NS4 encoded), c22 (core encoded), c33c (NS3 encoded), and NS5 (NS5 encoded). The aims of the present study were (i) to determine the prevalence of indeterminate RIBA3.0 patterns in patients consecutively tested for anti-HCV antibodies in a university hospital; (ii) to evaluate the significance of these patterns in terms of viral replication, liver disease, and risk factors for HCV; and (iii) to get an insight into the mechanism underlying this peculiar immune response. Among 3,074 serum samples consecutively tested for anti-HCV antibodies, 588 were found to be positive by screening assays. Fifty-nine of them (10%) were RIBA3.0 indeterminate and were compared with 59 RIBA3.0-positive ones. Thirty-one RIBA3.0-indeterminate and 53 RIBA3.0-positive serum samples were HCV RNA positive by PCR (53 versus 90%; $P < 10^{-6}$). RIBA3.0-indeterminate and RIBA3.0-positive patients with positive PCR results were not significantly different for the prevalence of risk factors for HCV infection and elevated serum alanine aminotransferase activities. Immunosuppression, attributable to coexisting human immunodeficiency virus infection, organ transplantation, or the administration of immunosuppressive drugs, was significantly more frequent in PCR-positive, RIBA3.0-indeterminate patients than in PCR-negative, RIBA3.0-indeterminate patients ($P < 0.001$) and PCR-positive patients with a positive RIBA3.0 result ($P < 0.01$). The distribution of HCV genotypes did not differ significantly between HCV RNA-positive patients with indeterminate or positive RIBA3.0 results. In conclusion, the prevalence of indeterminate RIBA3.0 patterns in virology laboratories is about 10%; in about half of these patients HCV replication is detected by PCR; the main factor responsible for indeterminate RIBA3.0 patterns could be immunosuppression, whereas HCV genotypes do not seem to play a major role.

The diagnosis of hepatitis C virus (HCV) infections usually begins with the detection in serum of specific antibodies by screening tests. Today, third-generation enzyme immunoassays are widely used. Confirmation is sought by immunoblot assays that usually detect the same antibodies as those detected by screening assays. They may be positive, confirming the presence of anti-HCV antibodies, or negative, suggesting a false-positive result of the enzyme immunoassay. There is also a third category of results, indeterminate, the definition of which may vary according to the composition of the peptides present on the blot and with each manufacturer's instructions (13). The study described here was performed with sera that were tested by using the third-generation recombinant immunoblot assay (RIBA), manufactured by Chiron Corporation (Emeryville, Calif.) and commercialized by Ortho Diagnostic Systems (Raritan, N.J.), with indeterminate results, defined as being positive for any one of the following four antigens: c100 (NS4 encoded), c22 (core encoded), c33c (NS3 encoded), and NS5 (NS5 encoded).

Previous studies showed that in laboratories that routinely perform viral diagnosis, indeterminate second-generation RIBA results were associated with active viral replication in most cases when the only antibody detected was directed against c22-3 (core) or c33c (NS3) recombinant proteins, so that these patterns were usually considered positive (2, 9, 11, 14). Conversely, isolated 5.1.1 or c100-3 patterns (NS4 encoded) were generally shown to be false positives and were usually considered negative (2, 9, 11, 14). The situation was quite different in blood banks, where 86 to 98% of the blood donors with an indeterminate second-generation RIBA pattern were HCV RNA negative by PCR, whatever the band pattern, likely corresponding to false-positive results of enzyme immunoassays (1, 5, 19).

The third-generation RIBA (RIBA3.0 HCV strip immunoblot assay [RIBA3.0]; Ortho Diagnostic Systems) was introduced in Europe in mid-1993 and since then has been widely used in virology laboratories and blood banks. Several studies showed that RIBA3.0 is able to resolve most of the indeterminate second-generation patterns observed in patients routinely tested for anti-HCV antibodies in hospital virology laboratories into positive or negative according to the manufacturer's instructions (11, 14). These results were likely due to the modifications introduced in the third-generation assays, and these modifications are likely responsible for the higher degrees of sensitivity and specificity of the third-generation assays (4, 6, 14, 21). However, some indeterminate patterns by second-generation assays remained unresolved by RIBA3.0; these patterns were mainly found in patients with severe immunosuppression attributable to human immunodeficiency virus (HIV) infection or organ transplantation (11, 14). Moreover, routine use of RIBA3.0 revealed a significant pro-

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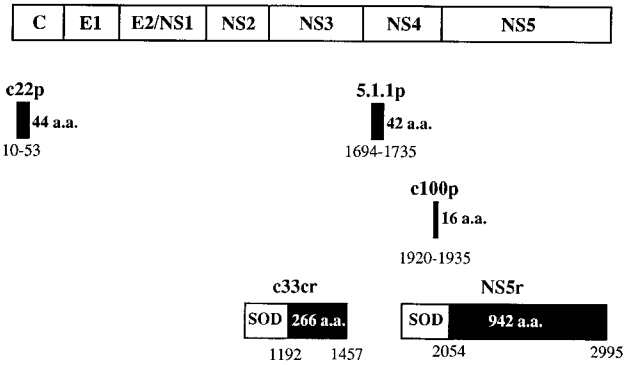

FIG. 1. HCV antigens present in RIBA3.0 (Ortho Diagnostic Systems). a.a., amino acids; SOD, superoxide dismutase; p, synthetic peptide; r, recombinant protein.

portion of indeterminate patterns, the significance of which was uncertain. The aims of the present study were (i) to determine the prevalence of indeterminate RIBA3.0 patterns in a university hospital; (ii) to evaluate the significance of these patterns in terms of viral replication, liver disease, and risk factors for HCV; and (iii) to get an insight into the mechanism that might underlie this particular immune response.

MATERIALS AND METHODS

Sera. Between 1 July and 31 December 1993, 3,074 consecutive requests for anti-HCV antibody detection were received in the virology unit of Hôpital Henri Mondor. The corresponding 3,074 serum samples were studied for the present work.

Anti-HCV serological assays. All of the 3,074 serum samples were routinely tested for anti-HCV antibodies by two different screening enzyme immunoassays, detecting antibodies directed to antigens encoded by both structural and nonstructural regions of the HCV genome (ELISA3.0 HCV [Ortho Diagnostic Systems] and Monolisa HCV [Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France]). For confirmation, anti-HCV antibodies were sought by RIBA3.0 in all of the sera found to be positive by screening assays. RIBA3.0, which detects antibodies directed to both structural (core, c22 synthetic peptide) and nonstructural (NS3, c33c recombinant protein; NS4, mixed 5.1.1 and c100 peptides; and NS5, NS5 recombinant protein) antigens (Fig. 1), was performed according to the manufacturer's instructions. The sera found to be indeterminate by RIBA3.0 constituted the study group. A control group of RIBA3.0-positive sera was also constituted by selecting the first RIBA3.0-positive serum immediately following the selection of an indeterminate one in each series of tests.

HCV RNA was sought by PCR, and the HCV genotype was determined for the RIBA3.0-indeterminate sera and the control RIBA3.0-positive sera. In addition, patient data were collected, including risk factors for HCV infection, alanine aminotransferase (ALT) activity in serum, and immune status of the patient (i.e., immunosuppression or not).

HCV RNA detection. HCV RNA was detected by a standardized HCV RNA PCR assay (Amplicor HCV; Roche Diagnostic Systems, Neuilly/Seine, France) according to the manufacturer's instructions (22, 23). Briefly, 100 μl of serum was incubated for 10 min at 65°C in a lysis buffer. Nucleic acids were then precipitated by the addition of isopropyl alcohol, and after a 15-min centrifugation at 13,000 × *g* and washing with 70% ethanol, the pellet was resuspended in 1 ml of specimen diluent. For amplification, 50 μl of the master mix was added to 50 μl of the samples from the study and control groups. The master mix contains buffer and reagents for both reverse transcription and amplification, which were performed with the thermostable enzyme *rTth*. In addition, amplification was performed by using dUTP instead of dTTP, a procedure that allows carryover prevention by the enzyme uracil-*N*-glycosylase (UNG; Amperase). The reaction mixtures were placed into a Perkin-Elmer thermal cycler 9600, and the successive steps of the reaction (action of UNG, reverse transcription, amplification, inactivation of UNG) were performed as follows: 50°C for 2 min, 60°C for 30 min, and 95°C for 1 min; 2 cycles at 95°C for 15 s and 60°C for 20 s; 38 cycles at 90°C for 15 s and 60°C for 20 s; 60°C for 4 min; and storage at 72°C. For detection of the amplified products, 100 μl of denaturing solution was added to each reaction mixture and 25 μl of the denatured amplicons was added to 100 μl of hybridization solution in microplate wells, and the plates were incubated for 1 h at 37°C. The wells were washed five times, and hybridizations were revealed by an enzymatic reaction. The A_{450} values were read. According to the manufacturer, a sample was considered positive if the well had an optical density greater than 0.4. Positive and negative controls for extraction were added to the amplification controls provided in the kit. To avoid false-positive results because of cross contamination, each sample was processed twice separately. In case of discrepant results, duplicate testing was repeated.

Determination of HCV genotype. The genotypes of the HCV isolate infecting PCR-positive patients of the two groups (RIBA3.0 indeterminate and positive) were determined by a modification of the Line Probe Assay (Inno-LiPA HCV; Innogenetics S.A., Ghent, Belgium) technique (17, 20). A combined Amplicor HCV–Inno-LiPA HCV technique was used. This technique was made possible because the amplification products of the Amplicor HCV reaction are biotinylated. Thus, PCR amplification of the 5′ noncoding region of the HCV genome was performed by the Amplicor HCV assay as described above. Thereafter, 20 μl of denatured amplified products was hybridized on LiPA strips as described previously (17). In the event that the HCV genotype could not be determined by the combined Amplicor HCV–Inno-LiPA HCV technique, another HCV genotyping procedure was used. That procedure was based on the hybridization of nested PCR products (20).

Statistical analysis. For comparisons, the chi-square test and the Fisher exact test were used when appropriate.

RESULTS

Among the 3,074 serum samples tested by the screening assays, 588 (19%) were found to be positive by enzyme-linked immunosorbent assay (ELISA) (i.e., strictly higher than the cutoff of the assay) and were thus tested by the RIBA3.0. The results of RIBA3.0 were as follows. (i) A positive pattern, characterized by the presence of at least two of four antibodies detected at a significant titer, was observed in 462 of the 588 serum samples (79%); (ii) 67 additional serum samples (11%) were found to be negative by RIBA3.0; (iii) finally, an indeterminate pattern, characterized by the presence of only one of the four antibodies at a significant titer, was found in 59 serum samples (10%), which constituted the study group. The indeterminate patterns were an isolated c100 in 3 cases (5%), c33c in 30 cases (51%), c22 in 25 cases (42%), and NS5 in 1 case (2%). Fifty-nine control serum samples with positive RIBA3.0 results were selected as indicated above.

Among the 59 serum samples with an indeterminate RIBA3.0 pattern, 31 (53%) were found to be HCV RNA positive by the Amplicor HCV assay, whereas 53 of the 59 serum samples (90%) with a positive RIBA3.0 pattern were HCV RNA positive ($P < 10^{-6}$). HCV RNA was present in 13 of 30 (43%) c33c-indeterminate serum samples and 16 of 25 (64%) c22-indeterminate serum samples (P was not significant). HCV RNA was also found in two of three c100-indeterminate serum samples but not in the serum from an asymptomatic blood donor with an NS5-indeterminate pattern, a result in keeping with previous findings suggesting that indeterminate NS5 patterns are false positives (15, 21).

As shown in Table 1, almost all (94%) HCV RNA-positive, RIBA3.0-indeterminate patients had an identified risk factor for HCV infection, that is, blood transfusion in 17 patients (55%) and intravenous drug use in 12 patients (39%). Two patients (6%) had no known cause of infection. Almost all (94%) PCR-positive, RIBA3.0-indeterminate patients also had elevated serum ALT levels on at least one determination during follow-up (range of available ALT determinations, 1 to 14). Both risk factors and elevated ALT levels were significantly more frequent in PCR-positive than in PCR-negative patients with indeterminate RIBA3.0 results (Table 1). Among the PCR-negative patients, risk factors were blood transfusion in all but one patient. Finally, immunosuppression was significantly more frequent in PCR-positive, RIBA3.0-indeterminate patients (77 versus 29%; $P < 0.0001$) (Table 1); among these patients, immunosuppression was due to HIV infection in 11 patients (46% of immunosuppressed patients), organ transplantation in 6 patients (25%), and immunosuppressive drugs in 7 patients (29%).

By comparison, in the group of 53 patients with positive RIBA3.0 results and positive for HCV RNA detection by PCR,

TABLE 1. Immunosuppression, risk factors, and elevated ALT levels in subjects with positive and indeterminate RIBA3.0 results

Characteristic	No. (%) of patients RIBA3.0 positive, PCR positive (n = 53)	P^a	No. (%) of patients RIBA3.0 indeterminate, PCR positive (n = 31)	P^a	No. (%) of patients RIBA3.0 indeterminate, PCR negative (n = 28)
Immunosuppression[b]	25 (47)	<0.01	24 (77)	<0.001	8 (29)
Risk factors[c]	48 (91)	NS[d]	29 (94)	<0.03	20 (71)
Elevated ALT level[e]	43 (83)	NS	29 (94)	<0.0001	11 (39)

[a] P values show significance between values in columns on either side.
[b] Immunosuppression attributable to HIV coinfection, organ transplantation, or immunosuppressive drugs.
[c] Identified risk factors for HCV infection, including blood transfusion and intravenous drug use.
[d] NS, not significant.
[e] ALT level higher than the upper normal value (45 IU/liter) on at least one determination.

a risk factor was found in 48 patients (91%), that is, blood transfusion in 31 patients (65%), intravenous drug use in 16 patients (33%), and occupational needlestick injury in 1 patient (2%). Elevated ALT levels on at least one determination during follow-up was found in 43 of these 53 patients (81%). These prevalences were not significantly different from those observed in the group of HCV RNA-positive, RIBA3.0-indeterminate patients. Twenty-five RIBA3.0-positive and PCR-positive patients (47%) were immunosuppressed because of HIV infection in 10 patients (40%), organ transplantation in 12 patients (48%), and immunosuppressive drugs in 3 patients (12%). Interestingly, immunosuppression was significantly rarer in HCV RNA-positive patients with positive RIBA3.0 results than in HCV RNA-positive patients with indeterminate RIBA3.0 results (47 versus 77%, respectively; $P < 0.01$).

Table 2 shows the distribution of HCV genotypes in HCV RNA-positive patients with indeterminate or positive RIBA3.0 results. These prevalences did not differ significantly between the two groups. It is of interest that four serum samples (13%) from the RIBA3.0-indeterminate group could not be typed by either the combined Amplicor HCV–Inno-LiPA HCV technique or the classical Inno-LiPA HCV technique, whereas only one serum sample (2%) from the RIBA3.0-positive group could not be typed by either method. Whether these patients were infected with HCV genotypes other than those usually found in industrialized countries remains to be determined.

DISCUSSION

The prevalence of indeterminate RIBA3.0 patterns in unselected sera consecutively tested for anti-HCV antibodies in our institution was 10%. Therefore, in 10% of the patients positive for anti-HCV detection by screening assays, the virologist was unable to give a clear, definitive interpretation of the results of serological assays, making necessary the use of HCV RNA detection for an accurate diagnosis of HCV infection. In the present study, we observed active viral replication in about half of the patients with indeterminate RIBA3.0 patterns. The RIBA3.0-indeterminate patients with positive PCR results had similar characteristics, e.g., a high proportion of risk factors for HCV infection and elevated ALT activity, compared with the patients with positive PCR results and a positive RIBA3.0 pattern. In contrast, the RIBA3.0-indeterminate patients with negative PCR results had significantly fewer risk factors for HCV infection and elevations in ALT levels than those with positive PCR results. All together, these results suggest that the patients with an indeterminate RIBA3.0 pattern associated with the positive detection of HCV RNA by PCR should be viewed like the patients with a positive RIBA3.0 pattern. It is of interest, however, that the RIBA3.0-indeterminate patients with negative PCR results also had a relatively high, although significantly lower, prevalence of HCV risk factors and elevated ALT levels. This was likely due to the peculiar situation of Hôpital Henri Mondor, which is highly specialized in the areas of organ transplantations and immunodeficiency syndromes, and to the fact that requests for HCV antibody detection were usually justified by signs of liver involvement. Two hypotheses might explain an indeterminate RIBA3.0 pattern without HCV RNA detection in these patients. (i) Some of them (especially at-risk patients) might have recovered from self-limiting acute HCV infection and lost part of their circulating antibodies or (ii) the remaining sera might display false-positive results by serological assays. In a few cases, the sensitivity of the PCR assay used in the present study might also be questioned in the case of very low amounts of circulating RNA.

In the patients with active viral replication and an indeterminate RIBA3.0 pattern, the question arises as to the mechanism by which they were not able to synthesize more than one of the various antibodies that we sought. Our study suggests an important role of immunosuppression in indeterminate RIBA3.0 patterns for those patients with associated viral replication, a factor already implicated in indeterminate patterns by the second-generation RIBA (2, 7, 10, 11, 14). The role of immunosuppression in indeterminate RIBA3.0 patterns is supported by several arguments. (i) Nearly 80% of the HCV RNA-positive patients with indeterminate RIBA3.0 patterns in our study were immunosuppressed, a prevalence significantly higher than that observed in HCV RNA-positive patients with positive RIBA3.0 patterns. Indeterminate patterns were associated with any kind of immunosuppression, including organ transplantation, HIV infection, or antitumor chemotherapy. (ii) Conversely, in our experience, indeterminate RIBA3.0 patterns are more frequent in HCV-infected immunosuppressed patients than in HCV-infected immunocompetent patients (unpublished data). (iii) Similarly, seronegative chronic HCV infection is frequently observed in immunosuppressed patients and is exceptional in immunocompetent ones (3, 8, 18). Apparent seroconversions and seroreversions have even been observed parallel to fluctuations in the immune status of immunosuppressed patients (18). The mechanism underlying indeterminate RIBA3.0 patterns in immunosuppressed patients with replicating HCV infection might be impairment of

TABLE 2. Distribution of HCV genotypes in the 31 patients with indeterminate RIBA3.0 patterns and the 53 patients with positive RIBA3.0 patterns in whom HCV RNA was also detected by PCR

RIBA3.0 pattern	No. (%) of patients infected with the following HCV genotype:					
	1a	1b	2a	3a	4a	Not typeable
Indeterminate (n = 31)	8 (26)	8 (26)	4 (13)	4 (13)	3 (10)	4 (13)
Positive (n = 53)	14 (26)	19 (36)	6 (11)	9 (17)	4 (8)	1 (2)

antibody production by B lymphocytes. Therefore, in immunosuppressed patients, HCV RNA detection appears to be mandatory for assessing the presence of HCV infection in the presence of symptoms of liver disease and indeterminate or negative results of third-generation anti-HCV serological assays. The Amplicor HCV assay, which was used in the present study, has been shown to be highly sensitive (12, 24). Our experience of rare false-positive results, likely due to cross contamination (unpublished data), makes it necessary to perform the test, including the extraction step, in duplicate. Under these conditions of use, the Amplicor HCV assay appears to be an accurate standardized test well adapted to the detection of HCV RNA in the particular clinical setting of indeterminate serological assays.

The role of peculiar HCV genotypes in patients with indeterminate RIBA3.0 patterns might also be questioned, since the HCV genotype has been shown to influence the antibody response in patients with chronic HCV infection (16). This hypothesis was, however, ruled out by our results, which showed a similar distribution of HCV genotypes among the patients with positive PCR results associated with indeterminate or positive RIBA3.0 patterns. This result bears out the high degree of sensitivity of RIBA3.0 for the detection of anti-HCV antibodies in patients infected by any of the main HCV genotypes found in industrialized countries (16). However, virus isolates from four patients with indeterminate RIBA3.0 patterns could not be typed by the Inno-LiPA HCV method, which has still been shown to be very sensitive. The possibility that a rare genotype might explain the indeterminate pattern in these few patients cannot be definitely ruled out.

Finally, the fact that indeterminate RIBA3.0 patterns were observed in some immunocompetent patients infected by frequently encountered HCV genotypes remained unexplained. The reason why these patients were not able to synthesize antibodies to the different viral antigens might be related either to the particular characteristics of the viral strains or to a genetic predisposition.

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