ELISA in serodiagnosis of HCV infection

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Abstract: A high level of anti-HCV is generally associated with viral replication and the number of recognized epitopes appears to be correlated with the viral charge. Nevertheless, the absence of detectable antibodies in about 60% of patients during the acute phase of the disease and in 10% of chronically infected (generally immunocompromised subjects) are heavy handicaps for HCV serology. Moreover, low levels of anti-HCV antibodies can persist after complete recovery, and HCV viremia does not appear to be associated with the presence of a special antibody specificity. The immunoblots presented as ‘confirmatory test’ always appear to be less sensitive than the screening tests and therefore are unable to discriminate between post-infection antibodies and false-positive reactions, as rare as they can be. In these cases, as in non-responder patients, PCR appears essential. The possible reasons of immune response limitations and the possible improvements of HCV serology are discussed.

Key words: Hepatitis C virus; Serodiagnosis; ELISA; Hepatitis

Introduction

During the last 5 years, non-A, non-B hepatitis research has progressed from hepatitis C virus (HCV) cloning to assays for revelation of HCV antibodies and the subsequent use of the polymerase chain reaction (PCR) to detect the HCV genome.

During a first period (1989–1991), the currently available tests only detected the anti-C100 protein antibodies, but this assay rapidly reached its limitations since the test appears sometimes to become positive as long as 1 year after acute infection [1]. Furthermore, false-positive results were found especially in the sera of alcoholics in relation with the presence of anti-yeast antibodies, reacting with impurities contaminating the antigenic recombinant protein produced in yeast [2].

Since 1992, it is largely admitted that a second-generation testing has permitted to gain in sensitivity and specificity of anti-HCV antibody detection [1,2]. Therefore, our restatement of HCV serology will limit its matter to the second-generation tests, their failure and the improvements which could be foreseen in this field.

Tests for the study of the anti-HCV immune response

The situation of HCV is unique in the viral world as far as it remains the first virus identified by molecular biology [3]. As long as this virus will remain unseen and unavailable for large in vitro production by cell culture, recombinant proteins,
or synthetic peptides, will be the sole source of viral components utilizable as antigens for immunological investigations.

HCV shares a common genomic structure with flavivirus and particularly with pestivirus, which include a nucleocapsid and two putative envelope glycoproteins (E1, E2) at the 5' end, and four nonstructural proteins (NS2 to NS5) extending to the 3' end of the genome. Currently, six groups of HCV are characterized on the basis of genomic variations. Highly conserved sequences are found in the 5'-untranslated region and in the core. A variable region is found in the E2 domain, and the genomic variability excludes the use of the corresponding envelope proteins as target for antibodies [4]. This variability could be due to a strong selective pressure of the anti-virus immune response leading, to discard envelope proteins as antigen source for serological screening of HCV infection. Therefore, all new assays use capsid and nonstructural components and limit the use of envelope proteins to the definition of different virus variants.

The different proteins used as antigen are recombinant proteins or synthetic peptides. Under these conditions, the large majority of recognized epitopes are sequential (continuous) epitopes, particularly for synthetic peptides, thus minimizing the role of conformational (discontinuous) epitopes.

The sequential epitopes inferred in genomic data of HCV have been investigated extensively. Fifteen such epitopes have been characterized in the HCV structural proteins: 5 for C, E1 and E2 proteins, respectively [4–6], whereas with nonstructural proteins about ten sequential epitopes are known. The NS3 region appears poorer than the NS4 and NS5 regions. The capsid epitopes have been shown to react with 90% of anti-HCV-positive specimens, and the N-terminal region of NS5 with almost 80% [7].

ELISA screening

Currently, about 10 kits are commercially available for general screening of the presence of anti-HCV antibodies, most of them including recombinant proteins but some contain more or less long peptides. A comparative evaluation of the sensitivity and specificity of these different kits is difficult since these reagents are frequently being modified to improve their performances. Furthermore, the number of the proposed commercial preparations is always increasing.

In 1992, a French study compared the four most commonly used kits. The comparison involved all the associations of kits by pair in different groups of 400 hospitalized patients (Eur. J. Clin. Microb. Inf. Dis., in press). Significant differences appeared. For two kits, the observed discordances were negligible. A third kit showed weak, but significant differences, by comparison with the first ones. The fourth kit, the only displaying synthetic peptides, diverged very significantly from the others.

Analytical tests

While the screening enzyme immunoassay is recognized as a major serological advance that can result in a 70% reduction of transfusion-associated hepatitis, the specificity of positive results in ELISA is always questioned, particularly in random donor and other low-risk populations. Therefore, analytical assays, improperly named 'confirmation tests', have been developed. They involve separate deposits of different recombinant proteins, or synthetic peptides, on nitrocellulose strips or polystyrene beads. In the above-mentioned study, the discordant sera in screening tests, reexamined with analytical immunoblots from three distinct manufacturers, gave homogeneous results for only 35/80, among which six were positive by the three analytical tests; in four of these sera, viral RNA was detected by PCR.

The biological and clinical significance of analytical immunoblots (RIBA2) were recently analyzed within a blood donor population [8] and with patients investigated for routine diagnosis of HCV infections [9]. Nearly all blood donors giving a highly positive signal (> 5) with the screening test were also found positive by immunoblotting (61/67, among which 54 had viral RNA). When the positive signal was weak in the screening test, no serum reacted positively by immunoblot (and in no case, PCR was positive).
Kinetics of anti-HCV antibody response

In our hands, the early immune response was analyzed in a chimpanzee inoculated with the strain HUT (kindly given by R. Purcell). The first antibody which appeared was directed against antigen C33. It must be noted that this antibody was simultaneously detected by the isolated C33 recombinant protein on nitrocellulose (immunoblot) and only by one screening kit among the three tested. This kit contains a mixture of recombinant proteins on polystyrene beads as solid phase. The availability of C33 epitope(s) seems to be improved under these conditions, when compared to the wells of a plate with the same solid phase. Anti-NS4 antibodies appeared after anti-C33; anti-C22 was never detected but the monkey died early during the acute phase of the disease. The anti-C33 antibody was produced about 2 months before transaminase elevation, and anti-NS4 appearance was contemporary with hepatocyte alteration. A literature review [10–12] shows that the seroconversion time varies greatly according to the animal, but at least one antibody response is measurable at, or within 3 weeks, of the major alanine-aminotransferase peak. For this early detection, the anti-C33 response is still the most frequently observed.

In humans, the anti-HCV detection by the screening tests remains desperately late although introduction of the capsid subunit has somewhat reduced the time required for a positive reaction. It has been mentioned that the immune response could be stronger in post-transfusional hepatitis than in sporadic hepatitis. If this were true, this observation could reflect differences in the levels of viral replication. In countries without any systematic screening of blood donors, the risk to detect passively transferred antibodies must be considered.

Among recent studies on the kinetics of the anti-HCV response, only those using commercially available preparations will be considered. In the series from Van der Poel et al. [13], after documented infections following transfusion of blood products, all the nine studied patients seroconverted by the screening test within 26 weeks after inoculation. The latency between clinical manifestations and positivity of the screening test varied from 2 to 17 weeks. So-called indeterminate RIBA-3 results were found in this early phase of the HCV infection although anti-C33, like in monkeys, was frequently the antibody detected at first. Later, seven of the nine (78%) recipients who had become positive in immunoblot remained indeterminate. In a larger series of non-A, non-B acute hepatitis of different origins (Giuberti et al. [14]), in which 86% of patients seroconverted by the screening test, anti-C33 and anti-C22 were confirmed as first detectable markers of the HCV infection in 80% and 20% of the patients, respectively.

To conclude: (i) the anti-HCV human immune response differs qualitatively according to individuals, but the anti-C33 antibody appears as an early privileged antibody; (ii) the analytical immunoblot appears to be less sensitive than the screening test used; (iii) it also appears impossible to predict the course of infection from the specific antibody recognition pattern which is progressively developing.

In patients who showed early and persistent normalization of their transaminase levels and who became PCR-negative, all antibodies decreased beyond 6 months after the clinical onset, but a minority lost circulating antibodies in the following 5 years [15]. For most of these subjects.
scrotopositivity by screening tests was no more documented by analytical immunoblot tests. The loss of anti-HCV with normalization of liver functions was preferentially observed in patients with non-parenterally transmitted HCV [16].

The chronic evolution of about 60% of patients with post-transfusional hepatitis is generally accompanied by a high level of antibodies and by a large panel of anti-HCV specificities. Natural disappearance of HCV in patients with persisting chronic liver disease of CV antibodies have been found for as much as 12.8 years after the acute phase in some patients with normal transaminases. In immunocompromised patients (e.g. infected by the human immunodeficiency virus, or grafted) or co-infected by HCV and HBV, anti-HCV may be undetectable whereas viral RNA is displayed by PCR. Nevertheless, immunodepression does not seem to be able to explain the failure of antibody detection since HCV-RNA has been found in the serum of some blood donors involved in post-transfusional hepatitis with normal transaminases and without anti-HCV antibodies [17,18].

The significance of a low level of anti-HCV antibodies remains an unsolved problem. Immunoblotting appears to have no interest since a low sensitivity reaction is not able to serve as control for a more sensitive reaction. PCR seems essential to clarify the problem. If anti-HCV antibodies persist at a low level in the constant absence of viral RNA, the trace of post-infection can be evoked. The eventuality of cross-reaction also needs to be considered. Recently, cross-reactivity of sera of patients with flavivirus infections has been mentioned in Taiwan [19]. Nevertheless, we could not confirm such a cross-reactivity between strongly positive sera from HCV-infected patients with yellow fever, West-Nile, dengue, Japanese encephalitis viruses, and with the sera of the convalescent patients with the same diseases and HCV recombinant antigens (in collaboration with Dr. Deubel, Institut Pasteur, Paris). Furthermore sera from a pig or a sheep immunized with hog cholera virus or bovine mucosal disease virus (pestivirus), respectively, remained unreactive with the available HCV antigens (in collaboration with Dr. Laude, Jouy and Josas).

**Immunological diagnosis deficiencies of HCV infections: possible improvements**

The limitations of the immune response

The principal defects of anti-HCV serology lie in the fact that antibodies are not detected in about 60% of patients during the acute phase. Moreover, antibody detection fails in about 10% of chronically infected patients with viral RNA. Immunoblots have never been recommended in the absence of positive screening tests, although anti-C33 frequently seems to be involved before positivity of the screening tests. To explain the limitations of the anti-HCV immune response and to try to palliate them, the possible causes will be discussed.

(i) An intrinsic cause could be the weak viral charge inherent in HCV infection. Indeed, the number of infectious particles in this disease does not exceed 10^7/ml of plasma, whereas in hepatitis B this number usually reaches values of 10^9. If this is true, improvements of serological investigations will be difficult to achieve.

(ii) The infection of immunocompetent cells may be evoked. Indeed, the infection of macrophages, CD4 T cells, with replicative intermediates is now well-documented [20,21]. An impairing of the immune capacities could ensure. Nevertheless, an identical situation is seen in numerous other infectious diseases without affecting antibody production. Furthermore, no correlation could be found between the presence of HCV-RNA or antigen in the peripheral blood mononuclear cells and any serological markers of the HCV infection [20].

(iii) The frequent presence of cryoglobulinemia in HCV-infected patients could be related with the infection of immunocompetent cells. It has been postulated that an unusual immunological response could lead to the production of anti-HCV antibodies, undetectable by the currently available tests [22]. We believed that the elimination of anti-HCV antibodies with cryoprecipitating properties during centrifugation usually carried out at a temperature below 37°C could be a more plausible hypothesis. We tested this possi-
bility. The conservation of collected bloodsamples at 37°C did not increase the titer of the detected anti-HCV antibodies. A type II cryoglobulin from an HCV-infected patient was exhaustively purified and its constitutive IgM and IgG separated by gel-filtration at 37°C. A rheumatoid factor activity was found in the monoclonal IgM component but no significant anti-HCV activity could be evidenced in the polyclonal IgG fraction. By inhibition of specific antibodies, no HCV antigen could be characterized in both fractions. Thus, cryoprecipitation does not seem to be involved in cases where no antibodies can be detected.

(iv) The possibility of immune complexes associating with an excess of HCV antigens to specific antibodies is another hypothesis that must be considered. Indeed, a high in vivo infectivity of HCV in plasma appears to be correlated to a low density and to the impossibility to precipitate viral RNA by anti-immunoglobulin antibodies. On the contrary, with less infectious plasmas, this activity is found in a high density fraction where proteins are associated to the virus, and furthermore the infectivity can be precipitated by an anti-immunoglobulin serum [23]. A dissociating treatment could help to reveal the presence of anti-HCV antibodies. Nevertheless, for a disease in which the viral charge remains particularly weak, such a role of immune complexes would be surprising.

(v) The impossibility to detect anti-conformational antibodies, as far as recombinant proteins or polypeptides are used, could be the true explanation for the poor antigenic reactivity of the preparations. Anti-conformational antibodies are known to represent 60–70% of anti-virus antibodies [24]. Antibodies recognizing a quaternary structure (subunits association) play an important role in anti-HBV (envelope or capsid) antibody activities, particularly at the onset of the infection [25,26]. Recombinant proteins and the peptides used as antigen for the diagnosis are presumed to contain only few conformational epitopes. If a constitutive quaternary structure associating capsid subunits could be offered for diagnosis, a gain of sensitivity could be anticipated. The recent announcement of HCV propagation in cell cultures could help to this realization [27,28].

Possible short delay improvements

IgM antibody detection

The use of second-generation tests for IgM detection is presently submitted for evaluation [29–31]. The preliminary results suggest that IgM directed against the capsid protein appear shortly after the onset of hepatitis, reducing the delay for immunological characterization of the disease. Anti-HCV capsid protein IgM seem to be detected more frequently than anti-C100 or anti-C33 IgM in recent infections. In most cases, the acute phase IgM anti-capsid response was detected coincidentally or before active IgG. In individuals who resolved their HCV infection or progressed to chronicity, anti-HCV/IgM was produced and tended to disappear. All the data indicate that IgM against the HCV capsid can be a useful acute-phase marker in HCV infection.

Substitution of polystyrene for nitrocellulose blots for analytical tests

The detection of antibodies against an isolated HCV recombinant protein or against a polypeptide should be improved if these antibodies were investigated with the isolated protein on a solid phase rather than on nitrocellulose. Immunological reactions involving an antigen on polystyrene always display a higher sensitivity than the same reactions with the same antigen on nitrocellulose. Under these conditions immunoblots appear of poor interest, especially as the interpretation of immunoblotting appears too arbitrary and since a so-called indeterminate reaction can be significant during the early phase of infection.

Conclusion

The field of ELISA for serodiagnosis of HCV infection is still moving. The formidable advance realized during the last 5 years engenders optimism about total prevention of this blood-transmitted disease and a better control of infected subjects.
References


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