Conference on chronically-evolving viral hepatitis

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The third international conference on chronically-evolving viral hepatitis was held on 4th–7th October 1992 in Pisa, Italy. This series of biannual meetings is organized by the European Society against Viral Diseases. Approximately 200 clinicians, epidemiologists and laboratory scientists assembled to present and to discuss new data and developments on hepatitis viruses type B, C and D. Furthermore, several invited speakers reviewed the current knowledge on molecular virology, pathogenesis, and therapy of chronic viral hepatitis.

Immune pathogenesis

H.C. Thomas from the Royal Free Hospital, London, described models of how hepatitis B virus (HBV) may escape immune elimination and how antiviral therapy with interferon may stimulate the immune system and may suppress viral replication. The aminoterminal portion of the HBV polymerase has the ability (besides its function as primer of viral DNA synthesis) to suppress the induction of interferon-responsive genes by interferon. This finding may explain why interferon is ineffective in patients with a very high level of viral replication. The interferon therapy in patients with moderate viral replication is justified by the fact that most patients seem to have a deficiency in interferon production. Interferon acts against HBV predominantly by an immunological mechanism. It enhances expression of antigen-presenting HLA molecules which are the targets for cytotoxic T-lymphocytes. Thus, interferon therapy of chronic HBV infection often causes a flare-up of serum transaminases. It is most successful in patients who already have an ongoing cytotoxic immune reaction and elevated transaminases. In persons who were infected perinatally, interferon therapy is usually ineffective, possibly because such patients are immune tolerant against HBV antigens. A potential tolerogenic factor produced by HBV may be the HBeAg. Due to its molecular weight it can pass the placenta and induce deletion of HBe/cAg-specific T-lymphocyte clones during 'education' of the fetal immune system.

The central importance of T-cell immunity in the resolution of HBV infection has been postulated for long, but only recently C. Ferrari and his collaborators have presented conclusive evidence for this theory. They found high levels of CD4- and CD8- positive T-lymphocytes specific for HBc/e proteins or peptides in the blood of patients who cleared the infection. In patients who developed chronic HBV infection, significantly less T-cell activity against HBc/eAg was found. T-cell immunity against the *surface* proteins seemed to play a minor role. Using partial peptides of the

HBc/eAg they could determine the fine specificity of the T-cell-response, e.g., HLA-A2 molecules presented most efficiently the sequence 18–27 of the HBc/e protein to CD8-positive cytotoxic T lymphocytes.

S. Abrignani analysed the T-cell response to recombinant HCV proteins in patients with various degrees of HCV-associated liver disease. Similar to HBV, a T-cell response against the core protein of HCV was found to correlate with a better prognosis of the HCV infection. T-cell responses against the other HCV proteins were found in many patients irrespective of the clinical state. It appears that T-cell-mediated cytotoxicity contributes significantly to pathogenicity and – possibly – to resolution of the HCV infection. M. Ballaré (Novara) studied the role of HCV in 8 patients with type II essential mixed cryoglobulinemia and liver disease. All patients had anti-HCV and HCV RNA. The cryoprecipitate contained a larger amount of HCV RNA than the supernatant. Bone marrow biopsies showed infiltrates of lymphoplasmoid cells. It was suggested that HCV may be an important etiological factor in this immune complex disease. It appears now that all viruses of chronic hepatitis develop their pathogenicity mostly by the host's immune response. In concordance with this theory are the findings from R. Repp, Giessen, Germany. He reported on asymptomatic carriers of HBV who became infected while being on multidrug anticancer therapy. These patients had high replication of HBV but no clinical or histological symptoms of hepatitis. Attempts to vaccinate such patients against hepatitis B failed during that therapy. It appeared that even the B-cell and/or T-cell clones which recognizing the hepatitis B vaccine were depleted by the ongoing chemotherapy, because these patients remained hyporeactive against the hepatitis B vaccine even after complete resolution of the oncologic disease. These findings give a hint of how immune tolerance could be generated on purpose: simultaneous application of an antigen and immuno-suppressive drugs would destroy the antigen-specific clones.

Oncogenesis

One of the most serious sequelae of chronic viral hepatitis is the development of hepatocellular carcinoma (HCC). There is no doubt that chronic inflammation and regeneration per se is a risk factor for developing HCC, but at least in HBV-associated HCC, a specific viral contribution is suspected. Insertional mutagenesis has been detected in a small proportion of human HCC cases, but cannot explain the majority of cases. Besides activation of cellular proto-oncogenes, inactivation of cellular tumor suppressor genes has been an important mechanism of viral oncogenesis in general. M. Höhne, Giessen, reported that the X protein of HBV transforms immortalized hepatocytes in vitro, and causes retention of the cellular tumor suppressor protein p53 in the cytoplasm where it cannot maintain its control function on completion of DNA repairs before transition from G1 to S phase during the cellular growth cycle. In keeping with this conclusion, the X-transformed hepatocytes show an increased rate of DNA rearrangements and progression towards malignancy.

p53 also seems to be a direct target of the fungal carcinogen aflatoxin. Alfatoxin-induced mutations of p53 at codon 249 were found in 67% of HCCs from Senegal, as reported by P. Coursaget (Tours). Aflatoxin adducts to DNA were also found in HCCs from Peking ducks (L. Cova et al., Lyon).

X protein of HBV has also been expressed as a liver-specific transgene in various mouse strains. Oncogenicity of X protein is not a regular feature, but transactivation

of enhancers like that of HIV has been found (C. Balsamo, Rome). For development of HCC several steps are probably necessary.

The biochemical activities of X protein are not yet understood. Both a kinase-dependent and a free-radical-dependent pathway of transcription activation by X have been reported. M. Höhne found that the p53 in his X protein-transformed cell lines was not mutated, but hyperphosphorylated. Thus, X protein seems to alter cell-cycle-controlling kinases.

A kinase was also found in HBV particles which phosphorylates the core protein. Using specific inhibitors and activators of various cellular protein kinases, M. Kann (Giessen) showed that the HBV-particle-associated kinase is most likely a proteolytic fragment of protein kinase C, known also as protein kinase M. He showed furthermore that in vitro phosphorylation by either protein kinase A or C abolished the ability of core protein to package RNA. Thus, the phosphorylation may regulate the release of the viral genome after infection.

The oncogenicity of HCV and HDV is even less understood than that of HBV but an interesting observation on HDV was reported by G. Tappero (Torino). He found that HDAg-expressing cells accumulate c-myc in the nucleus.

Diagnosis

Diagnosis of chronic HCV infection – and surprisingly also of HBV infection – is still the objective of intensive research.

G. Colloredo-Mels (Torino) reported on the serological patterns of reactivated HBV infection. It appears that most chronically-infected patients experience, between the silent phases, several episodes of HBV replication which are followed by a flare-up of transaminases and a subsequent rise of IgM-anti-HBc. The latter marker is most stable and allows for a more reliable diagnosis of chronic hepatitis B than transaminases or HBV DNA which may be often normal or negative at occasional examinations. It is, however, necessary to determine IgM-anti-HBc by a sensitive semi-quantitative assay. Many commercial assays for IgM-anti-HBc are adjusted in order to recognize only acute infections where the titers are much higher. A correlation between low-level IgM-anti-HBc and chronic inflammation was also observed by G. Marinos and colleagues from King's College, London.

Detection of viral genomes by PCR has opened up new possibilities and revealed the viral etiology in a large number of previously cryptic cases of chronic liver disease. A. Uy et al. (Göttingen, Germany) found that the majority (60%) of HBsAg-negative anti-HBc positive patients with chronic liver disease had HBV genomes in the blood. The presence of anti-HBs or anti-HBe did not correlate with the detection of HBV DNA and could not prevent viremia. In anti-HBc and anti-HBs positive blood donors with normal transaminases, HBV DNA was rarely detectable.

A. Uy reported a low variability of the viral preS genes in HBsAg-positive patients but a high variability of the preS2 region in HBsAg-negative patients. In that respect, HBsAg-negative HBV infection is similar to persistent HCV infection. A. Weimer (Chiron Corporation, Emeryville) described the high variability of the HCV envelope proteins E2 which has a region (E2HV) as hypervariable as the hypervariable regions of HIV. Direct sequencing of PCR products allows for rapid characterization of viral genomes.

One of the most difficult diagnostic problems is the detection of perinatal transmission of HCV. The usefulness of anti-HCV for that purpose is limited because the immature immune system of the newborn may be unable to bring about stable anti-

HCV production. Weiner reported on the detection of HCV RNA by PCR in some children of anti-HCV-positive mothers. Currently, it is not clear how frequently transmission occurs, nor is it known whether chronic disease will develop from that infection. Weiner reported that the virus variant of an infected baby differed from the predominant virus variant in the mother, suggesting an individual adaption of the virus to its host. C. Stringhi from the group of A. Zanetti (Milano) reported that 18 asymptomatic HCV RNA-positive mothers *without* HIV did not transmit HCV to the baby, but 7 of 14 *HIV- and* HCV-positive mothers infected their baby with HCV. Possibly, HIV-infected mothers have higher HCV titers in the blood plasma and/or in leukocytes.

Assay of HCV RNA by PCR is probably advisable for all anti-HCV positive persons. G. Taliani examined persons with antibodies against the core protein of HCV as the only HCV-reactive antibody. Such persons may be either HCV RNA-negative and healthy, or HCV RNA-positive, and in this case they suffer from liver disease.

Interferon therapy

Assay of viral genomes has become particularly important in monitoring interferon therapy. Z. Yun from Stockholm found that disappearance of HCV RNA was a prognostic marker of a permanent response after interferon therapy, but unfortunately only 2 of 17 treated patients showed such a response. Similar results were obtained by M. Artini from Rome. C. Cammà and colleagues from Palermo had also only in 17% of 361 patients a long-term response after interferon therapy. Best markers of such a favorable response were short duration of HCV infection and the absence of cirrhosis. F. Piccinino and co-workers from Naples observed a rather good short-term response (50%) to interferon in 310 HCV patients, without evaluating the long-term response. Altered dosage or type of interferon in non-responders could sometimes improve the result of therapy. Similar results were reported by several other investigators, e.g., by G. Budillon et al. (Napoli) who used lymphoblastoid interferon. Lymphoblastoid interferon has the additional advantage that it does not induce anti-interferon antibodies so often. G. Antonelli and F. Dianzani (Rome) found such antibodies in 17% of patients treated with recombinant interferon alpha 2a, in 6% treated with recombinant interferon alpha 2b, but in only 1% of patients treated with lymphoblastoid interferon. Moreover, the antibodies against interferon 2a did not neutralize lymphoblastoid interferon.

Given the side effects of interferon, decision for therapy has to be made very carefully. M. Sata and colleagues found that T-lymphocytes of responders to interferon contained more of a novel surface antigen named Le^Y, but the mechanism of how this carbohydrate antigen interacts with HCV or interferon is not yet known. G. De Sanctis et al. (Rome) found severe side effects in 8 of 401 patients treated for 6–18 months: autoimmune-thyroiditis in two, thrombocytopenia with anti-platelet antibodies in one, psychosis in two, and severe hepatitis in three patients. No untreated control group with chronic hepatitis C was studied.

Non-responders are also a major problem in chronic hepatitis B. G. Fattovich et al. reported that combination of interferon with the immune-stimulatory drugs thymopentin or levamisol did not improve the response.

A. Alberti (Padova) summarized the problems of non-responders to interferon. He defined non-responsiveness as persistence of viremia at essentially unchanged levels and persistence of elevated transaminases. Such a complete non-responsiveness

occurs in 30–60% of cases with only minor differences between HBV, HCV and HDV. In agreement with the data of H.C. Thomas (see above), he found that high HBV titers in serum prevented a response. In contrast to other groups he did not observe an influence of HBeAg-negative variants (see below) on responsiveness. With HCV, the genotype of HCV and the level of viremia but not the transaminase level may have an influence. Better responsiveness was found at low patients' age, short duration of the disease and absence of cirrhosis.

Hepatitis B variants

In chronic hepatitis B, treatment with interferon often leads to selection of virus variants which are no longer able to produce HBeAg (T. Santantonio, Bari). These variants may impair the long-term response to interferon. HBe-negative variants have been implicated in more severe courses of acute or chronic hepatitis B. However, W. Carman discussed the evolution of virus variants and pointed out that two important questions remain: 'Firstly, are these variants the end of the road as far as evolution is concerned and are they transmitted to other patients? Secondly, are they appearing in response to severe disease or are they causing severe disease?'

H.J. Schlicht (Ulm) analysed the structural features which determine the antigenic reactivity of HBeAg and HBcAg by directed mutations. A cystein and three hydrophobic amino acids in the proximal preC sequence of HBe protein prevent assembly to core particles and folding of the HBeAg epitopes. H. Will and collaborators (Hamburg) identified several variants lacking either HBe protein and/or the middle-sized HBs protein which are able to replicate in cell culture and – most likely – independently of the wild-type virus in the infected host.

M.A. Petit (Paris) reported also on the generation of HBe-negative and preS1-defective variants in chronically infected patients. However, those epitopes of preS1 and preS2 which have been found to participate in viral attachment were conserved. In particular, preS1 epitopes around amino acids 30–50 seem to be essential for *viability*. Thus, wild-type HBV expresses at least two seemingly non-essential proteins. Currently, it appears that HBe protein, and possibly middle-sized HBs protein may contribute to the development of persistent viremia, because the variants lacking those proteins are found after severe acute or long-lasting chronic hepatitis B.

Infectivity in cell culture

Studies on the viability of HBV have been hampered by the fact that infection of cell cultures has not yet led to unequivocal results. While M.A. Petit could infect the human hepatoma cell line HepG2, others were not able to reproduce these findings. X. Lu and W. Gerlich (Giessen) were able to infect HepG2 cells with HBV particles which were treated with V8 protease. V8 protease cleaves off the preS domains and exposes a hydrophobic sequence of the particles which probably causes fusion of the viral envelope with the cellular membrane. Although the putative fusion sequence resides at the amino end of the S domain, it is only accessible in proteolytically-cleaved large or middle-sized HBs proteins. Given the fact that the major attachment site of HBV is in the preS1 domain of the large HBs protein, it becomes apparent that the middle-sized protein may be dispensible.

The non-essentiality of MHBs became also apparent in studies on the infectivity of HDV for primary hepatocytes. C. Sureau (San Antonio, Texas) reported that he and his colleague R. Lanford had generated primary hepatocyte cultures from chimpanzee livers. These cultures were susceptible for infection by HDV and HCV, but

not by HBV. Both the large and the small but not the middle-sized HBs protein were necessary for infectivity of the HDV particles. Antibodies against preS1 or S epitopes were able to neutralize that infectivity.

HCV seems to replicate in primary hepatocyte culture. Minus-strand HCV RNA was detectable in the infected cells, and virus-like particles of *toga-* or *flavivirus*-like appearance were detectable in the electronmicroscope. G. Carloni (Rome) reported infection of primary human hepatocytes and detection of HCV antigen by immune fluorescence. He was able to passage the virus at least three times.

Liver transplantation

For spread of HDV in vivo, the HBs envelope is obviously not essential. M. Rizzetto, Torino, and A. Zignego, Firenze, reported that HDV- and HBV-positive recipients of liver transplants often experienced HDV reinfection without concomittant HBV infection. Such HDV reinfections remained asymptomatic until HBV also became reactivated. As M. Rizzetto pointed out, reinfection of the graft is frequent in chronic carriers of hepatitis viruses. Reinfection by HBV can and should be prevented in low-level carriers of HBV by passive immunization, because the prognosis for reactivated HBV infection is very bad. Reinfection by HCV leads to liver disease of variable severity, but is usually more benign.

The source of the reactivating hepatitis viruses seems to be cells of the bone marrow and the cell lineages derived from them. Replication of HCV in white blood cells has been reported recently by several groups and was described at this meeting by M. Artini et al. (Rome), and by A. Zignego et al. (Florence). Detection of minus-strand HCV RNA proved that HCV particles not only entered the cell, but also induced the early steps of viral replication.

Prevention

In view of the dim perspectives of therapy of chronic viral hepatitis, prevention of infection is of the highest importance. While transmission of the parenterally-transmitted hepatitis viruses seems to be decreasing in European countries, specific vaccines are still highly desirable. Hepatitis B vaccine has now been successfully introduced in many high risk groups and in Italy even for all children. However, the appearance of escape mutants may become a problem. W. Carman described various mutants with amino acid exchanges in the a-epitope of HBs protein. It appears that these mutants are infectious and may possibly be able to spread within the population in the future. If this becomes true, future hepatitis B vaccines must either contain the epitopes of the escape mutants as well, or should contain the protective epitopes of the preS1 and preS2 domain. S. Chassot, Lyon, showed that even in the duck hepatitis virus the preS region carries a major neutralizing epitope, which she has mapped accurately. Unfortunately, experimental preS1 containing vaccines produced in yeast failed to induce satisfactory antibody levels, as was reported by F. André (Rixensart, Belgium). It is not clear whether subtype-specific antibodies contribute to the protection by hepatitis B vaccine. Experience with other viruses strongly suggests this. In that respect, a thorough study on the molecular nature of HBsAg subtypes, presented by H. Norder et al. (Stockholm), may prove very valuable not only for phylogenetic and epidemiological studies.

HCV vaccine?

M. Houghton (Chiron Corporation) presented his first attempts to protect chimpanzees against HCV infection by immunization with various types of recombinant HCV envelope proteins. These included life vaccinia virus vectors, envelope proteins with conformational epitopes expressed in animal cells, and fusion proteins containing the sequential epitopes of HCV envelope proteins. None of the four chimpanzees were protected against a challenge with infectious HCV, but in one chimpanzee a more favorable course of the infection was observed. Given the fact that the sequence of the proteins used for immunization and of the challenge virus were identical, and that the animals had developed antibodies against the envelope proteins, the result must be considered disappointing. In the field, the vaccinated persons would have to deal with all kinds of variants against which they would not have antibodies. At present, hygiene in handling of blood and screening of blood and organ donors seems to remain the only way to prevent transmission of HCV.

Publication

There was a lively discussion of the data. Moreover, approximately 90 posters were presented, the findings of which were mostly not reviewed in this brief report. The abstracts of the meeting have been published as Supplement 1 of Volume 17 of J. Hepatol. and a full progress report on all oral presentations will appear end of 1993 as Supplement of Archives of Virology.