PREVALENCE OF AUTOANTIBODIES IN A TUNISIAN COHORT OF HEPATITIS C VIRUS INFECTED DIALYSIS PATIENTS

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RESUME

Le virus de l'hépatite C (VHC) est l'agent principal des hépatites chroniques virales dont les complications majeures sont la cirrhose et le carcinome hépato-cellulaire. Chez les hémodialysés, l'infection par le VHC reste un problème préoccupant. Plusieurs manifestations autoimmune ont été décrites au cours de cette infection, avec l'expression d'autoanticorps de spécificité variable. Deux cent patients hémodialysés, tous anti-VHC (+), ont été colligés dans ce travail, en vue de déterminer la prévalence des autoanticorps ant-nucléaires (AAN), anti-mitochondrie (AAM), anti-muscle lisse (AML), anti-cardiolipine (ACL), anti-thyroïdiens (ATPO) et des facteurs rhumatoïdes (FR) au cours de l'infection virale C comparativement aux sujets sains. Soixante huit sérums (34%) de malades anti-VHC (+) se sont révélés positifs à un ou plusieurs auto-anticorps. Cette fréquence est statistiquement plus élevée que celle retrouvée chez les contrôles sains. Ces marqueurs sont dominés par les FR d'isotype IgM et les ACL d'isotype IgG. Toutefois la fréquence des AAN, AAM, ATPO, et des AML n'est pas statistiquement différente par rapport au groupe contrôle. Par ailleurs, une association entre la positivité de ces autoanticorps et la réplication virale a été constatée, suggérant que l'infection par ce virus serait responsable de l'induction d'un état d'auto-immunisation.

Mots clés: *Hépatite virale C, autoanticorps, hémodialyse.*

SUMMARY

The hepatitis C virus (HCV) is the principal agent of viral chronic hepatitis. Cirrhosis and hepatocellular carcinoma are the major complications of this chronic infection. In haemodialysis, HCV infection remains a very frequent problem. Several autoimmune phenomena have been described during this infection. Two hundred haemodialysis patients, all of them anti-HCV (+), were included in this study to evaluate the frequency of Anti-Nuclear Autoantibodies (ANA), anti-cardiolipine antibodies (ACL), anti-smooth muscle antibodies (ASMA), anti-mitochondria antibodies (AMA), anti-thyroperoxydase antbodies (ATPO) and Rheumatoid Factor (RF) comparing them to healthy controls. Sixty eight serums (34%) patients were positive to at least one of the auto-antibodies tested. The difference between patients and controls was statistically significant. These markers were dominated by RF of the IgM isotype and ACL of the IgG isotype. Nevertheless, the positivity of ANA, ASMA, AMA and ATPO was not statistically different comparing to the controls. In addition, an association between the presence of the auto-antibodies and the viral replication was found suggesting that HCV is responsible for inducing these autoimmune phenomena.

Key words: *Hepatitis C virus, auto-antibodies, haemodialysis.*

INTRODUCTION

It is widely established that autoimmune phenomena are the result of the breakdown of tolerance against self antigens. However, the presence of potentially auto-reactive lymphocytes is a necessary condition but generally not sufficient to induce autoimmunity. Thus, the clinical or biological expression of autoimmune phenomena may depend on environmental factors such as microbial pathogens, and more precisely viral infections. The latter condition may induce an aberrant activation of autoreactive lymphocytes and contribute to the onset or the exacerbation and the relapse of autoimmune diseases [1, 2, 3]. Among these viral infections, Hepatitis C Virus (HCV) infection is particularly known to be associated to autoimmune disorder. In fact, in addition to hepatic manifestations, these disorders are common in chronic HCV infection and many studies report the occurrence of a wide-ranging of organ and non-organ-specific auto-antibodies during HCV infections [4, 5, 6, 7, 8].

Because autoimmune diseases could represent one cause of morbidity in patients infected with HCV, we aimed in this study to evaluate the frequency of Anti-Nuclear Auto-antibodies (ANA), anti-cardiolipin antibodies (ACL), anti-smooth muscle antibodies (ASMA), anti-mitochondria antibodies (AMA), anti-thyroperoxydase antibodies (ATPO) and Rheumatoid Factor (RF) in a Tunisian cohort of dialysis patients comparing them to healthy controls, and to investigate their possible association to clinical manifestation and viral replication. Besides their presence may cause some diagnosis and therapeutic difficulties especially with auto-immune hepatitis type I, which is characterized with the presence of ASMA. In addition, several clinical or biological autoimmune disorders may be exacerbated by alpha interferon (IFN), such as autoimmune thyroid dysfunction and also autoimmune hepatitis [9, 10].

MATERIEL AND METHODS

PATIENTS

A total of 200 sera from HCV infected Tunisian dialysis subjects followed up at different dialysis unities in Tunisia, were included in this study between January at December 2002. None of these patients were under alpha IFN treatment. All the samples were collected before dialysis. They were centrifuged and the serum was stored in small aliquots at -80°C until the time of use. All the sera tested were positive for anti-HCV antibodies. This serologic diagnosis of HCV was performed by the detection of antibodies to HCV using a commercial Enzyme Linked Immuno Sorbent Assay (ELISA) (Kit INNOGENTEICS Innotest® HCV Ab IV).

We also included in this study a group of 100 age and gender matched healthy controls recruited from the blood donors of the same area than patients at the same period (year 2002). Medical examination and a questionnaire were performed for all controls. All the donors were negative for HCV, B Hepatitis Virus (HBV), Human immunodeficiency Virus and Syphilis infections. The study was approved by the local ethics committee and informed consent was obtained from all subjects.

METHODS

Molecular diagnosis of HCV replication and genotype

The molecular diagnosis of HCV was performed using a qualitative reverse transcription-PCR technique (Kit Amplicor® Hepatitis C Virus HCV Test Version 2.0). HCV genotypes were determined by the Line Probe Assay (LIPA) (Kit INNGENTEICS InnoLIPA® HCV II) of the 5' noncoding genome region that characterizes the different genotypes. The two techniques were performed according to the manufacturer's instructions.

Autoantibody screening

Sera of all patients and controls were screened for the presence of ANA, ACL, ASMA, AMA, ATPO and RF.

ANA, ASMA AMA antibodies were determined by indirect immunofluorescence (IFI) on homemade rat liver, stomach and kidney cryostat sections respectively. A fluoresceine isothiocyanate (FITC)-conjugate anti-human IgG (Biorad) was used as secondary antibody. The Cut-offs was 1/50 for ANA, ASMA and AMA. The detection of anti-DNA was performed only for the ANA positive sera using, as previously described, an IFI on home prepared Crithidia Luciliae with a cut-off at 1/10. Anti-Extractible Nuclear Antigens (ENA) were also performed in the case of positive AAN using a commercial immunoblot technique (Kit Innogenetics) according to the manufacturer's instructions. The characterisation of the AMA of anti-M2 specificity was performed using a commercial ELISA (Biorad) according to the manufacturer's instructions.

ATPO were detected using the same IFI technique

performed on primate thyroid cryostat sections. Significant titres were considered ≥ 1/5.

The ACL were detected by an in-house ELISA. The coated antigens used were bovine cardiolipin (SIGMA) diluted in absolute ethanol. The coating was made using 30 µl of the antigen solution for each well. The micro plates (NUNC polysorb 96) were then incubated at room temperature (RT) for 24 hours. Because fetal calf serum (FCS) contains the co-factor Beta 2 Glycoprotein 1 (ß2-GP1), the coated well, after washing with Phosphate buffer saline (PBS)-Tween 0.1%, were saturated, at RT, using PBS-Bovine serum albumin (BSA) 1% FCS 10%. 100 µl of diluted samples (dilution at 1/100 in PBS-BSA 1%-FCS 10%) were then incubated for 1 hour at RT in the coated wells. Non coated wells were also used for each sample to determine the background of non specific reactivity. After washing, the immune complexes were revealed using 100 µl of peroxydase conjugated human anti-IgG (Biorad) and 100 µl of the Tetramethylbenzidine (TMB) substrate. The reaction was stopped by the addition of the 100 µl of the stop solution (3M NaOH) to all wells and the optical absorbance (OA) of each well was read at 450-630 nm. A positive value is defined as an OA > 3 standard deviations (SD) above the mean normal control value of the 100 normal subjects.

RF were also detected with an in-house ELISA. As coated antigen we used aggregated human IgG. The coated wells were incubated 2 hours at 37°C then at 4°C over the night. The rest of the steps are similar to the ACL ELISA previously described. For this ELISA we used a peroxydase conjugated human anti-IgM (Biorad) as secondary antibody.

The patients' RF concentrations were determined according to a standard curve. A positive value is defined as ≥ 16 International Unit/ml.

RESULTS

EPIDEMIOLOGICAL RESULTS

The Collected data included age, gender, mode of dialysis, frequency of transfusion, viral replication and VHC genotype distribution. The group of HCV infected patients was composed of 90 men and 110 women, sex ratio = 1.22. The mean age of the subjects was 49 years (13 to 80 years). The number of patients under haemodialysis was 197/200 (98.5%) and only 3 patients were under peritoneal dialysis. In our cohort, 55% of the patients have required blood transfusion on one occasion at least and 10% were under erythropoietin. An active viral replication was detected in 154/200 (77%) and the genotype 1b was the most frequent in our cohort (80%).

GLOBAL RESULTS

Among our patient, 34% (68/200) were positive for at least one of the auto-antibodies tested, while in healthy controls, auto-antibodies were detected in only 7% (7/100) of them. The difference between patients and controls was statistically significant (p= 0.00001). The main results are summarized in (Table I). In the group of patients positive for the auto-antibodies tested, IgM RF and IgG ACL were the most frequent auto-antibodies found in the sera of the haemodialysis patients (38.3% and 33.8% respectively), and were statistically more prevalent in these patients than in controls (p=0.0003 and p=0.003 respectively) (Table II). 7/68 (10.3%) patients had an association of 2 auto-antibodies, ACL and RF in all the cases.

ASMA were positive in four sera all of them belonging to haemodialysis patients. However, the difference was not statistically significant comparing to the controls. The titres were low (1/100) in three case. In one case, ASMA were positive at 1/200.

Table I : Prevalence of the auto antibodies among the groups of patients and controls.

AUTOANTIBODIES	PATIENTS	CONTROLS	P
AAN only	2/200 (1%)	2/200 (2%)	NS
AMA only	1/200 (0.5%)	1/100 (1%)	NS
ASMA only	4/200 (2%)	1/100 (1%)	NS
ATPO only	5/200 (2.5%)	1/100 (1%)	NS
ACL only	23/200 (11.5%)	1/100 (1%)	0.003 (S)
RF only	26/200 (13%)	1/100 (1%)	0;0003 (S)
Association of 2 or more autoantibodies	7/200 (1%)	0/100 (0%)	NS
Total	**68/200 (34%)**	**7/100 (7%)**	**0.00001 (S)**

Table II : Distribution of the auto antibodies according to the viral replication and the genotype.

Autoantibodies	Virale replication (+)	Genotype
ACL	17/23	72% genotype 1b
ASMA	3/4	100% genotype 1b
AMA	1/1	75% gentype 1b
ANA	2/2	100% gentype 1b
ATPO	3/5	70% gentype 1b
Association of 2 or more autoantibodies	7/7	100% gentype 1b

ATPO were positive in 5/200 (2.5%) patients and in only one healthy control without any significant difference. ANA were present in only 2 patients and 2 healthy controls at a low titre and without any particular antigenic specificity.

Among all the sera tested only one patient and one healthy control were positive for the presence of AMA with a low titre at 1/100 but the AMA specificities were not identified.

Analytic results

In our cohort, there were no significant differences related to gender for the presence of the auto-antibodies tested, however women showed more frequently positivity (64.5%) than men (35.5%).

Although not statistically significant, the detection of auto-antibodies was frequently associated to HCV replication. In fact 72% (49/68) of patients showing positivity to the auto antibodies tested had a detectable viral replication. However, taking alone, RF were the only auto-antibodies significantly associated to the viral replication ($p<0.01$). Besides, all the hemodialysis patients with positive RF and detectable viral replication had the 1b genotype of the HCV. The distribution of the other auto-antibodies investigated in this study according to the viral replication and the genotype is illustrated in the table II. Finally, in patients and controls groups, the presence of auto-antibodies was isolated and not associated to any clinical auto-immune symptoms.

DISCUSSION

HCV infection remains a problem in haemodialysis patients [11]. The parenteral way is the principal mode of HCV transmission, thus haemodialysis subjects are exposed to a high risk of HCV contamination since they are often transfused because of their anaemia. This risk increases with the duration of the haemodialysis. Actually, in our cohort, 55% of the patients were transfused, and the mean duration of dialyses was 5 years. Viral replication was detected in 77% of the patients and the genotype 1b was the most frequent in our cohort.

In this study we evaluated the prevalence of organ specific auto-antibodies such as ATPO and non organ specific auto-antibodies such as ANA, AMA, ASMA, ACL and RF. Our results corroborate previous study showing that HCV is associated with an increased incidence of auto-antibodies and autoimmune disorders comparing to healthy subjects [4, 5, 6, 7, 8]. In fact, we found that 34% (68/200) of these HCV infected dialysis patients were positive for at least one of the auto-antibodies investigated. This frequency was significantly higher comparing to the healthy controls ($p=0.00001$).

RF was the most frequent auto-antibodies found in our cohort (38.3%). Similar results are reported in previous study [4, 8].

Commonly, RF detected in routine diagnosis are of IgM isotype. However, patients' sera can also contain RF of IgG, IgA or even IgE isotype [12]. Generally, these antibodies are able to recognise the Fc determinants of the IgG witch correspond to their classical antigen. However RF are also known to be able to cross-react with non classical antigens [13]. This poly-reactivity of RF could explain their higher frequency in our cohort of patients comparing to the other auto-antibodies tested. Besides, the absence of clinical features related to the presence of RF and the positive association between the HCV replication and RF suggest that HCV infection is responsible for the production of RF probably through the high level of inflammation and tissues damage that help the release and the presentation of self cryptic antigens. Other mechanisms, such as molecular mimicry, can also be involved [14, 15].

ACL were also significantly more frequent in our cohort of HCV infected patients than in healthy

controls. Our findings support results of previous studies [15]. Anti-cardiolipine antibodies belongs to the heterogeneous family of antibodies that bind serum proteins such as b2-glycoprotein I (b2GPI) or prothrombin, or protein/phospholipid complexes, and they are detected in a variety of autoimmune disorders, most commonly systemic lupus erythematosus, primary and secondary anti-phospholipid, but also in the course of many infections diseases such as syphilis, hepatitis C and HIV [16, 17, 18, 19, 20].

In the present study, there were no thrombotic manifestation associated to ACL, thus we can suggest that HCV infection is itself responsible for the production of ACL. Again, the high level of inflammation and tissues damage could explain this autoreactivity.

Although ASMA are the serologic markers of autoimmune hepatitis, these auto-antibodies are also present in the course of some viral infections [21, 22]. HCV infections are known to be associated to the presence of ASMA [22]. In our cohort ASMA were not as frequent as reported in the literature (2%). Nevertheless more frequent than in healthy controls (0.3%). Besides, since ASMA are the serologic markers of autoimmune hepatitis type I, their presence in sera of HCV infected patient may cause some diagnosis and therapeutic difficulties. In fact, in front of the presence of ASMA in HCV infected subject, it is not simple to discriminate between autoimmune hepatitis type I infected with HCV and an HCV infection associated to the presence of ASMA which is crucial for the therapeutic attitude: in the case of autoimmune hepatitis infected with HCV, steroid therapy is not allowed. In the contrary, in the case of a chronic HCV infection associated to autoimmune hepatitis, the latter can be exacerbated by the interferon treatment [10]. Similarly, the interferon treatment can also exacerbate or induce de novo an autoimmune thyroid dysfunction [9]. Thus, although thyroid disease is not a contraindication for the alpha IFN therapy, the screening for anti-thyroid auto antibodies such as ATPO is very important to identify predisposed patient for the development of autoimmune thyroid dysfunction and to establish rapidly the appropriate treatment [9]. In fact, 2.5% of our patients are positive for ATPO. Although, clinically and biologically, these patients are not in hypothyroidism, they should be closely followed up to detect at time this thyroid dysfunction.

Some studies have reported the presence of a raised level of ANA in HCV infected patients. However, in our study we found only two patients (1%) that were positive for AAN with a low titer. These contradictory results may be due to differences in characteristic of the cohorts studied and to differences in the techniques used for the detection of AAN [23].

AMA are considered to be the serological hallmark of Primary Biliary Cirrhosis (PBC). However these auto antibodies can also be detected in other hepatic disorders such as chronic HCV [24]. In our study, AMA were detected in only 0.5 % of the patients with a low titer and were not associated to any clinical manifestation related to their presence. Thus, the presence of AMA in HCV infected patients could be just epiphenomenal. Nevertheless chronic hepatitis C viral infection can co-exist with PBC [25]. Although this association is uncommon, these patients should be followed up and screened for the presence of other clinical or biological signs of PBC.

CONCLUSION

Similarly to other studies, our study demonstrated a high prevalence of serological markers of autoimmunity in HVC chronically infected patients. The relationship between viral infection and autoimmunity remains not yet clarified. Many overlapping mechanism such as chronic inflammation, persisting viruses and molecular mimicry are involved in auto-immunization induction and may contribute to the development of auto-immune diseases. Their early detection may be useful, especially in the case of HCV infection because of the therapeutic attitudes.

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