MAJOR ARTICLE

Non–Organ-Specific Autoantibodies in Children with Chronic Hepatitis C: Clinical Significance and Impact on Interferon Treatment

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We evaluated the prevalence and clinical significance of non–organ-specific autoantibodies (NOSAs) in 47 hepatitis C virus (HCV)–positive children with abnormal alanine transaminase levels and analyzed the association between NOSAs and virus level, genotype, human leukocyte antigen status, and interferon (IFN) response. Forty-two hepatitis B virus (HBV)–positive children and 25 age- and sex-matched healthy children served as control subjects. NOSAs were found in 34% of the HCV-positive children, 12% of the HBV-positive controls, and none of the healthy control subjects. Liver-kidney microsomal antibody type 1 (LKM1) was detected in 11% of the HCV-positive children but in none of the controls. The HCV load was significantly higher in NOSA-negative than in NOSA-positive children. HCV genotype distribution and human leukocyte antigen alleles were similar, irrespective of NOSA status. Long-term response to IFN therapy was achieved by 18% of the NOSA-positive and 55% of the NOSA-negative subjects. Two LKM1-positive children developed acute, self-limited hepatocellular necrosis while receiving IFN therapy. NOSAs are frequently present in children with hepatitis C, who are less likely to benefit from IFN therapy.

Non–organ-specific autoantibodies (NOSAs) are commonly found in adult patients with chronic hepatitis C virus (HCV) infection, and it is generally accepted that the global NOSA prevalence among individuals with HCV-associated chronic hepatitis is ~25% [1–5]. Recent experimental studies suggest hypothetical pathogenic explanations for the appearance of NOSAs in patients with HCV infection. The E2 envelope protein of HCV binds to CD81 on the surface of B lymphocytes, and this interaction promotes not only B cell proliferation and clonal expansion, but it also lowers the B cell activation threshold, thus favoring antibody production [6, 7]. In addition, the identification of several significant homology motifs between HCV polyprotein and autoantigens [8] indicates that "molecular mimicry" mechanisms may also play a role in developing particular autoantibodies in patients with chronic HCV infection.

However, the clinical significance of NOSA in the course of HCV-associated chronic hepatitis is still a matter of debate. In adult patients, a strong correlation has been shown between NOSAs and the biochemical and histological activity of liver disease [5], but it is unclear whether NOSAs are directly involved in the progression of the liver damage or whether they simply represent a physiological response to autoantigens released from dying hepatocytes [9]. The prevalence of autoantibodies in children with HCV infection has been investigated in 2 different retrospective studies [10, 11] that showed that autoimmune phenomena—often with fluctuating behavior—may be present in up to two-

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thirds of such patients and that the presence of NOSAs represents part of the natural course of the disease [11].

The aim of the present study was to evaluate the clinical significance of NOSAs in HCV-infected children. In particular, we investigated whether the presence of NOSAs characterizes a particular clinical, biochemical, and histological subset of HCV-associated liver disease, is dependent on a specific HLA background, and may affect the response to antiviral treatment.

PATIENTS AND METHODS

Patients. We evaluated a retrospective study of 47 consecutive white children (24 of whom were boys) with HCV-associated chronic hepatitis who were treated at the same institution by G.V. and L.A. All 47 HCV-infected children had tested positive for anti-HCV antibody and HCV RNA. Twenty-three had received plasma or blood transfusions, and the mothers of 20 were HCV positive when the children were born. One HCV-infected child had undergone major surgery, and, for the 3 who were adopted, the route of HCV infection was unknown. The diagnosis of HCV-associated chronic hepatitis was based on the detection of abnormal alanine transaminase (ALT) levels at least twice during a 6-month period and/or results of histological analysis of liver biopsy specimens that revealed chronic inflammation. Liver biopsy specimens were obtained from 28 HCV-infected children, and the histological activity was scored according to the modification of the Knodell index by Ishak et al. [12]. One HCV-infected child had serological and virological evidence of coinfection with hepatitis B virus (HBV) in replicating phase (i.e., test results were positive for hepatitis B e antigen and HBV DNA).

Forty-two children with chronic HBV infection, 33 of whom tested positive for HBV DNA, constituted the HBV control group. Two of the HBV-infected controls had received blood transfusions, and 25 were born in families with at least 1 carrier of hepatitis B surface antigen (HBsAg; the mothers of 15 had tested positive for HBsAg). For the 15 HBV controls who were adopted, the route of HBV infection was unknown. The baseline demographic, clinical, and biochemical features of HCV-infected children and HBV-infected controls are summarized in table 1. Twenty-five age- and sex-matched healthy children (median age, 8.5 years; age range, 2–16 years; female sex, 52%) who had normal ALT levels and were negative for HCV and HBV markers constituted the group of healthy controls.

Twenty-two HCV-infected children with chronic hepatitis and 19 HBV-infected controls with chronic hepatitis (all 19 of whom had tested positive for HBV DNA) were treated with recombinant IFN-α2a for 12 months (5 MU/m^2 3 times per week). Three serum samples (obtained at baseline and at the middle and end of treatment) obtained from all 41 children were available for autoantibody testing. Response to IFN treatment was defined as HCV RNA loads and ALT levels that remained undetectable and normal, respectively, throughout the treatment and follow-up periods. Patients were classified as "nonresponders" if HCV RNA was still detectable in serum after 6 months of therapy, as "relapsers" if reactivation of HCV RNA replication was observed during the posttreatment follow-up period, and as "long-term responders" if the HCV RNA load was undetectable 6 months after completing the treatment schedule.

NOSA testing. Testing for detection of anti–nuclear antibody (ANA), anti–smooth muscle antibody (SMA), liver-kidney microsomal antibody type 1 (LKM1), and liver cytosol antibody type 1 (LC1) was performed using indirect immunofluorescence (IFL) on cryostat sections of rat liver and kidney specimens at a serum dilution of 1:10 [13]. Each serum sample was tested at the time of diagnosis. For children treated with IFN, 2 additional serum samples (obtained during and at the end of treatment) were also evaluated. The presence of NOSAs was assessed blindly by 2 different investigators, both of whom were unaware of the child's HCV infection status. Serum samples positive for NOSAs were titered by double dilution up to extinction. ANA-positive serum samples were subsequently tested by IFL at a dilution of 1:10 on HEp-2 cells (Kallestad). ANA IFL patterns were defined in accordance with established criteria [14]. Testing for detection of anti–double-stranded DNA was conducted using the *Crithidia luciliae* IFL assay (Binding Site Limited). Anti-extractable nuclear antigen (anti-ENA) reactivities and the XR1 precipitin system were tested using counterimmunoelectrophoresis (CIE) with rabbit thymus extract (Pel-Freez).

The SMA pattern was assessed by IFL on kidney sections,

Table 1. Clinical, biochemical, histological, and epidemiological data for children infected with hepatitis C virus (HCV) or hepatitis B virus (HBV).

Variable	HCV group (n = 47)	HBV group (n = 42)
Male/female sex, no. of patients	24/23	23/19
Age, years	8.9 (1.5–16)	9.4 (2.5–12.11)
Follow-up duration, months	53 (11–167)	37 (6–167)
Knodell index	4 (3–10)	5 (2–12)
Albumin level, g/L	43 (36–55)	42 (35–54)
γ-Globulin level, g/L	12 (5–19)	11 (8–23)
Route of infection, no. (%) of patients		
Parenteral	24 (51)	2 (5)
Vertical	20 (43)	25 (60)
Unknown	3 (6)	15 (38)

NOTE. Data are median values (ranges), unless otherwise indicated.

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and results were defined as SMA-V (isolated staining of the vessel walls), SMA-G (staining of the vessel walls plus glomerular mesangium), or SMA-T (presence of SMA-V and SMA-G plus staining of the peritubular structures) patterns, in accordance with the criteria of Bottazzo et al. [15]. The actin-associated positivity for the XR1 precipitin system was evaluated by CIE with rabbit thymus extract (Pel-Freez), as reported in detail elsewhere [16]. In addition to IFL, the presence of LC1 was also evaluated by double immunodiffusion and counter-immunoelectrophoresis using human liver cytosol as a source of antigen. LKM1 reactivity was also evaluated by immunoblotting with rat and human liver microsomes [17].

Virus testing. Testing for detection of anti-HCV was performed using a third-generation ELISA assay (Ortho HCV Version 3.0 ELISA; Ortho-Clinical Diagnostics). The HCV RNA load was tested using nested PCR with primers derived from the 5′ noncoding region. HCV genotyping was performed by PCR amplification of the core region with specific antisense primers (HCV Gen-Eti-K DEIA; Sorin Biomedica), and results were available for 42 of 47 HCV-positive children. Tests for detection of HBsAgs and hepatitis B surface antibodies were performed using commercial RIA kits (Sorin Biomedica). The HBV DNA load was measured using quantitative hybridization (Abbot Laboratories). HCV levels were available for 34 children (Roche Diagnostics Systems).

HLA typing. The standard microcytotoxicity assay was performed for detection of HLA class I and II antigens in 31 children with HCV- and in 20 with HBV-associated chronic hepatitis using a panel of antisera from the 11th Histocompatibility Workshop and several commercial antisera (Onelamba).

Statistical analysis. Univariate analysis was performed using the χ^2 test, with or without Yates' correction, or Fisher's exact test, when dichotomous variables were considered. Comparison of continuous variables was performed by means of the unpaired Student's *t* test. Statistical analysis was performed using GraphPad InStat, version 3.0a for Macintosh (GraphPad Software).

RESULTS

NOSA detection. At baseline, HCV- and HBV-positive children shared similar demographic, clinical, and biochemical features, as summarized in table 1. However, NOSAs were found significantly more often in children with HCV-associated chronic hepatitis (16 [34%] of 47 children) than in those with HBV-associated chronic hepatitis (5 [12%] of 42) or in healthy controls (0 of 25), as summarized in table 2. The median titer of NOSA in HCV- and HBV-positive children was 1:20 (range, 1:10 to 1:320) and 1:10 (range, 1:10 to 1:160), respectively.

SMA was detected in 8 (17%) of 47 HCV-positive children (median titer, 1:20; range, 1:10 to 1:160) and in 3 (7%) of 42 HBV-positive children (median titer, 1:10; range, 1:10 to 1:80), and the immunomorphological pattern always corresponded to SMA-V. None of these serum samples immunoprecipitated XR1.

LKM1 was detected only in HCV-positive children (5 [11%] of 47; $P = .01$, compared with HBV-infected and healthy con-

Table 2. Prevalence and distribution of non–organ-specific autoantibodies (NOSAs) in children infected with hepatitis C virus (HCV) or hepatitis B virus (HBV) and in healthy control subjects.

	Study group, no. (%) of patients		
NOSA type	Chronic HCV ($n = 47$)	Chronic HBV ($n = 42$)	Healthy controls ($n = 25$)
All	16 (34)[b]	5 (12)[b]	0[b]
Anti-nuclear antibody (diffuse)			
Diffuse	0	0	0
Speckled	4 (9)[c]	3 (7)[c]	0
Smooth muscle antibody			
Vessel or glomerular pattern	8 (17)[d]	3 (7)[c]	0[e]
Peritubular pattern	0	0	0
Liver/kidney microsomal antibody type 1	5 (11)[b]	0[b]	0[b]
Liver cytosol antibody type 1	0	0	0

NOTE. See Methods for description of NOSAs.

[a] One child tested positive for both liver/kidney microsomal antibody type 1 and antinuclear antibody.

[b] $P < .01$.

[c] $P =$ not significant.

[d] $P =$ not significant for vessel pattern, and $P < .05$ for glomerular pattern.

[e] $P < .05$.

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trols). The LKM1 titer was 1:10 in 2, 1:40 in 1, and 1:320 in the remaining 2. All 5 LKM1-positive serum samples reacted by immunoblotting with a 50-kDa microsomal protein corresponding to CYP2D6.

ANA was present in 4 HCV-positive children (9%; median IFL titer, 1:10; range, 1:10 to 1:40) and in 3 HBV-positive children (7%; median IFL titer, 1:10; range, 1:10 to 1:20). The ANA pattern on HEp2 cells was "speckled" in all cases, and none of these serum samples had anti–double-stranded DNA or anti-ENA reactivities. In a single case, 2 different NOSA reactivities (to LKM1 and ANA) coexisted.

Response to IFN therapy. Before starting IFN therapy, 8 of 22 HCV-positive children tested positive for NOSAs (3 were LKM1 positive, 3 were SMA positive, and 2 were ANA positive). Two of these children lost the autoantibody by the end of the treatment schedule (one tested positive for ANA and the other for SMA, with a titer of 1:10 in both cases). Of these 8 NOSA-positive children, 3 (38%) cleared serum HCV RNA and achieved normal ALT levels, but only 1 (13%; ANA titer, 1:40) remained a long-term responder, whereas the other 2 (25%; 1 with an SMA titer of 1:80 and 1 with an LKM1 titer of 1:320) relapsed after IFN therapy was discontinued. Among the 5 nonresponders, 2 children with LKM1 (titers, 1:10 and 1:40) experienced a severe ALT flare (ALT level, 7 and 10 times the upper limit of normal after the fourth and fifth months of therapy, respectively). Treatment was discontinued for these patients, and, without receipt of immunosuppressive therapy, ALT levels returned spontaneously to those measured at baseline.

Eight (57%) of the 14 NOSA-negative children treated with IFN cleared serum HCV RNA and achieved normal ALT levels, and 5 (36%) became long-term responders. In 5 (36%) of the

Table 3. Clinical, virological, immunological, biochemical, and genetic features of the 16 children with chronic hepatitis C who tested positive for non–organ-specific autoantibodies (NOSAs).

Patient	Sex	Age, years	ALT level,[a] U/L	HCV genotype	HCV load, MEq/mL	IAIHG score	NOSA, titer	Treatment response	HLA haplotype
1	M	4.8	78	1b	2.64	10	LKM1, 1:40	7-fold increase in ALT level	A2,11; B55,65; DR1,4,53; DQ1,3
2	F	9.6	104	2c	0.2	NA	SMA-V, 1:20	Not treated	A3,68; B7,37; DR10,15,51; DQ1
3	F	3.6	80	2c	1.02	NA	ANA, 1:10	Not treated	A3,28; B7,37; DR10,15,51; DQ1,6
4	M	2.6	67	1a	0.65	7	ANA, 1:10	Nonresponder	NA
5	M	13	75	NA	NA	NA	SMA-V, 1:10	Not treated	A1,24; B13,35; DR7,15,51,53; DQ1,2
6	M	13	45	1b/3a	NA	NA	SMA-V, 1:10	Not treated	A24,32; B8,35; DR3,11,52; DQ2,7
7	M	3	69	NA	0.9	4	SMA-V, 1:20	Not treated	A1,69; B35,57; DR7; DQ3
8	M	16	36	1a	1.52	NA	SMA-V, 1:80	Relapser	A1,24; B35; DR7,15; DQ1,2
9	F	3.5	31	1a	0.2	4	LKM1, 1:10; ANA, 1:20	10-fold increase in ALT level	NA
10	F	8.9	93	1b	0.88	4	SMA-V, 1:80	Nonresponder	A24,33; B35,44; DR7,11,53; DQ2,7
11	M	8.2	60	2c	0.39	NA	LKM1, 1:10	Not treated	A24,32; B8,18; DR3,11,52; DQ2,7
12	M	7.2	82	1a/3a	8.26	NA	LKM1, 1:320	Not treated	A3,24; B8; DR3,4; DQ2,3
13	F	6	87	1b	1.4	10	LKM1, 1:320	Relapser	A2,30; B44,51; DR1,16,51; DQ1
14	M	9.8	52	1b	NA	4	SMA-V, 1:10	Nonresponder	A2,23; B8,44; DR3,7,52,53; DQ2
15	F	13	56	2c	NA	4	ANA, 1:40	Long-term responder	A1,24; B35,62; DR4,53; DQ3
16	F	2	65	1a/3a	3.89	NA	SMA-V, 1:160	Not treated	A2,3; B13,38; DR4,7,53; DQ2,3

NOTE. ALT, alanine transaminase; ANA, anti-nuclear antibody; HCV, hepatitis C virus; IAIHG, International Autoimmune Hepatitis Group; LKM1, liver/kidney microsomal antibody type 1; NA, not available; SMA-V, smooth muscle antibody with vessel pattern.

[a] Normal range, 0–40 U/L.

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14 NOSA-negative children, the following autoantibodies appeared during IFN treatment: 4 SMAs (1 at a titer of 1:10 and the remaining 3 at titers of 1:80) and 1 ANA (titer, 1:80). A long-term response was more often achieved in persistently NOSA-negative children (5 [55%] of 9) than in NOSA-positive children (1 [8%] of 13, including those who became NOSA-positive during follow-up; $P = .02$).

Only 1 of the 19 HBV-positive children treated with IFN developed SMA-V (titer, 1:20). As far as HCV genotype is concerned, no significant difference was observed between NOSA-positive (table 3) and NOSA-negative children (data not shown). In addition, no association was observed between NOSA and any particular HCV genotype. The analysis of the HLA status of HCV-infected children did not show any association with NOSA occurrence.

Among the diverse clinical, virological, and biochemical parameters for NOSA-positive and -negative children reported in table 4, the only significant difference between the 2 groups was in the HCV load ($\pm$SD), which was significantly higher among NOSA-negative children (mean, 7.84 ± 6.23 vs. 1.74 ± 2.32 MEq/mL; $P = .0027$). To assess the possible contribution of autoimmunity to the pathogenesis of liver disease in NOSA-positive children, we calculated the International Autoimmune Hepatitis Group (IAIHG) score before IFN treatment was initiated [13]. Results of these calculations indicated a diagnosis of "probable" autoimmune hepatitis in 2 LKM1-positive children (IAIHG scores, 10 and 11). Both were treated with IFN, and one developed an ALT flare (ALT level, 7 times the upper limit of normal) after 4 months of therapy; the other child achieved a complete biochemical and virological response during the 12 months of treatment but relapsed shortly afterwards.

DISCUSSION

The course of HCV-associated chronic liver disease in children is usually milder than it is in adults, with lower levels of ALT, lower histological activity, and lower HCV levels, even without differences in HCV genotype distribution [18–21]. In keeping with observations in adult patients, the occurrence of NOSA has been described also in children [10, 11]. In the present series, the global prevalence of NOSA among children with chronic hepatitis C was 34%, a figure similar to that reported among adult patients [2] but significantly higher than that among children with chronic HBV infection and among healthy children. This observation further supports the notion that immune system behavior is altered when challenged by chronic HCV infection but not by HBV infection and, in particular, that autoimmune reactions frequently occur also in the pediatric setting. The physiopathological reasons are, at present, only hypothetical and include HCV-specific mechanisms, such as "molecular mimicry" [22] and the potential interactions between HCV E2 and CD81 [6].

NOSA prevalence observed in this study does not differ from that reported by Bortolotti et al. [10], whereas Gregorio et al. [11] reported a higher global prevalence of NOSA, which was mainly confined to SMA reactivity. Differences in the methodological approach (e.g., the use of homemade vs. commercial kidney sections and different secondary antibodies) probably account for the observed discrepancy.

The most striking difference in the autoantibody profile between HCV-infected and HBV-infected children is represented by LKM1, an autoreactivity detected in 5 (11%) of 47 HCV-positive children but in none of the HBV-positive children and healthy controls ($P = .01$). At variance with reactivity data for ANA and non-T SMA, LKM1 reactivity is therefore peculiar

Table 4. Comparison of children with chronic hepatitis C who tested positive or negative for non–organ-specific autoantibodies (NOSAs).

Variable	NOSA-positive group ($n = 16$)	NOSA-negative group ($n = 31$)
Male/female sex, no. of patients	9/7	15/16
Age, years	8.2 (2–16)	8.4 (1.5–15.8)
ALT level, U/L	68 (31–104)	65 (22–570)
Albumin level, g/L	45 (37–55)	42 (36–55)
γ-Globulin level, g/L	13 (5–19)	12 (5–16)
HCV viremia level,[a] mean MEq/mL $\pm$ SD	1.74 ± 2.32	7.84 ± 6.23
Disease duration, years	4.8 (2–9.4)	4.6 (1.5–8.6)
Knodell index	4 (4–10)	4 (3–7)

NOTE. Data are median values (ranges), unless otherwise indicated. ALT, alanine transaminase; HCV, hepatitis C virus.

[a] The HCV load was available for 12 NOSA-positive and 22 NOSA-negative children ($P = .0027$).

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to HCV infection, with a prevalence similar to that reported in previous pediatric studies [10, 11] and typically and consistently higher than that in studies involving adults [1–5, 23]. It is therefore likely that LKM1 may clear over time in a significant number of adult HCV-positive patients. The NOSAs most frequently detected in autoimmune hepatitis, such as SMA-T, ANA with a "homogeneous/diffuse" pattern, and LC1 [2, 24], have never been observed in the present series of children with chronic viral hepatitis.

From a clinical standpoint, the detection of NOSA reactivity does not appear to modify the clinical, biochemical, and histological expression of the disease (table 4). The only relevant difference between NOSA-positive and NOSA-negative children is represented by the lower viremia levels in the former group. Even if the limited number of children studied does not allow us to draw definite conclusions, it is worth mentioning that we have already reported a similar observation in a series of LKM1/HCV-positive adults who had lower viremia levels than did LKM1-negative controls [25]. It is conceivable that the unbalanced immune response observed in these patients not only favors autoimmune reactions but possibly generates a cytokine environment which hampers virus replication.

In this series of NOSA-positive children with chronic hepatitis C, we did not observe any specific HLA association. The absence of any correlation between NOSAs and HLA antigens is somehow unexpected and conflicts with results of previous studies in adults [26]. In particular, we were unable to confirm in LKM1-positive children the strong association with HLA DQ2, which had already been reported in ~90% of LKM1-positive adults with chronic hepatitis C [27].

IFN is the treatment of choice for chronic hepatitis C, but its immunomodulatory activity may also favor the appearance or amplification of autoimmune reactions [28]. Therefore, it should be used with caution, particularly when autoimmune phenomena are present. In the present pediatric series, more than one-third of the NOSA-negative children tested positive for 1 autoantibody during IFN treatment. Whether the presence of NOSAs will alter the future natural history of the liver disease in these children remains to be established.

The primary response rate to IFN was not significantly different between NOSA-positive and NOSA-negative children. However, it is worth noting that the lower virus levels detected in NOSA-positive children were not predictive of a good response to the antiviral treatment. According to our data, the presence of NOSAs—before or during treatment—appears to reduce significantly the rate of long-term response to IFN monotherapy. It will be interesting to rechallange this observation when more-effective regimens (i.e., IFN plus ribavirin) are widely administered to pediatric patients.

It is of utmost importance to note that 2 of 3 LKM1-positive children treated with IFN had an acute increase in ALT levels, which resolved after therapy was discontinued. Similar ALT flares have been described in LKM1-positive children [10, 11, 29] and in adults receiving IFN [30, 31]. Given the accessibility of the liver enzyme CYP2D6—the LKM1 antigen—on the hepatocyte plasma membrane [17], it could be hypothesized that, in LKM1-positive patients, IFN may amplify the autoimmune response targeting CYP2D6 and thereby trigger acute LKM1-mediated liver damage. Particularly careful clinical and biochemical monitoring for possible adverse reactions in LKM1-positive patients receiving IFN therapy is therefore essential.

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