The pattern of hepatitis C virus (HCV) serum markers and liver disease was investigated in 11 leukemic children showing anti-HCV reactivity at least once during long-term observation to define the role of HCV infection and the behavior of HCV serologic markers in this patient cohort. Antibodies to HCV by first- and second-generation enzyme-linked immunosorbent assay (ELISA) and by second-generation (four antigens) recombinant immunoblotting assay (RIBA) and HCV-RNA by nested polymerase chain reaction (PCR) were serially examined in serum. Liver disease was defined according to transaminase levels. Seven of 11 patients were found HCV-RNA positive during chemotherapy and after blood transfusion, 3 of 11 became viremic during follow-up, and 1 of 11 was always HCV-RNA negative. Seroconversion to anti-HCV positivity by second-generation ELISA occurred in all the HCV-RNA positive children either during or after chemotherapy. Alanine aminotransferase (ALT) levels were elevated in all the HCV-RNA positive patients during antileukemic treatment and normalized in seven of them after therapy withdrawal, despite persisting viremia. These results indicate that HCV-RNA testing by polymerase chain reaction is required to correctly identify HCV infection in patients with leukemia while on chemotherapy. Viremia did not correlate with ALT levels and anti-HCV patterns.

BIOCHEMICAL and clinical signs of liver disease occur frequently during chemotherapy in patients with leukemia and correct recognition of etiologic factors is needed to define the most rational treatment.1

Besides the toxic effect of drugs, parenterally acquired viral hepatitis, mainly of the nonA, nonB type, plays a significant role in this clinical setting.2,5 The ability to diagnose the infection as caused by hepatitis C virus, the main blood-borne nonA, nonB agent,3 has dramatically improved in recent years. Indeed, first- and second-generation immunosassays have been introduced to test for the different antiviral antibodies in serum and the polymerase chain reaction (PCR) can also be used to measure viremia directly.5,6 Anti-HCV screening by means of the first-generation test has been available in our institution since January 1990, and soon after its introduction all children receiving chemotherapy for leukemia were studied for serum anti-HCV reactivity. Although more than two thirds of these patients had evidence of ongoing chronic liver disease, according to persistent alanine aminotransferase (ALT) elevation, only 1 of 114 patients (0.7%) had detectable anti-HCV reactivity in serum when tested once during the on-therapy period, usually at the time of significant abnormalities in liver enzyme levels (unpublished data). This figure was far below the prevalence of anti-HCV positivity seen in our transfusion-dependent thalassemic children (76%),5 being comparable with the one observed in Italian volunteer blood donors (0.7% to 0.8%).8

All of the patients treated in our institution are entered in a prospective study of liver disease that is based mainly on periodic clinical and biochemical assessment, including tests for hepatitis virus markers during and after chemotherapy. We observed that some patients did develop anti-HCV positivity during follow-up, a finding that became particularly evident after the second generation enzyme-linked immunosorbent assay (ELISA) test was introduced for screening in May 1991.

Here we investigate the pattern of HCV serum markers, including the anti-HCV reactivities as detected by the first- and second-generation ELISA and by the recombinant immunoblotting assay (RIBA) confirmatory test and serum HCV-RNA measured by PCR, having selected for this study those cases who showed serum anti-HCV reactivity at least once during long-term observation.

The aim is to determine the contribution of HCV infection to biochemical evidence for liver disease in our patients with leukemia and the behavior of HCV diagnostic markers in this particular patient population.

MATERIALS AND METHODS

Patients. Patients with leukemia treated at the Department of Pediatric Hematology, S. Gerardo Hospital, Monza, are prospectively followed for liver disease since presentation. Antileukemic treatment is performed according to the Italian Cooperative Protocols for Acute Lymphoblastic Leukemia (ALL)3 and Acute Myeloid Leukemia (AML).8 Briefly, therapy for ALL, consisting of induction, consolidation, and maintenance phases, for a duration of 2 years, includes the following drugs: vincristine (VCR), daunorubicin (DNR), L-asparaginase, prednisone (PDN), cyclophosphamide (CPM), cytosine arabinoside (Ara-C), 6-mercaptopurine (6-MP), methotrexate (MTX) high (5,000 mg/m2) and standard (20 mg/m2) dose; prophylaxis of central nervous system (CNS) involvement is performed with intrathecal (IT) MTX alone or in combination with Ara-C and PDN, with or without 1,800 rad cranial irradiation. Polychemotherapy for AML is based on anthracyclines, Ara-C standard (200 mg/m2) and high (3,000 mg/m2) dose, etoposide, CPM, and 6-thioguanine for induction and consolidation phases lasting 4 months. CNS prophylaxis is performed with Ara-C IT. In the absence of an HLA-identical donor, patients are subse-
quently randomized to receive autologous bone marrow (BM) transplantation or maintenance therapy with 6-TH, Ara-C, and anthracyclines for a duration of 2 years.

For this study, we selected patients diagnosed from 1988 to 1989, as all of them had sera stored both at -20°C and -70°C and could be observed after chemotherapy withdrawal.

Among 89 children with various forms of leukemia diagnosed in our institution in this 2-year period, 57 (64%) completed chemotherapy treatment in first complete remission. Eleven of 57 (19.2%) were found anti-HCV positive in serum at least at one occasion during follow-up and were thereby included in this study.

There were six boys and five girls, with a mean age at diagnosis of 8.2 years (range: 2.2 to 12.9 years). Ten patients had ALL and one AML, according to the French-American-British (FAB) classification.10

All children were treated with antileukemic drugs for a median period of 24 months (range 4 to 28 months). Median follow-up was 32 months (range: 28 to 51 months) from diagnosis and 10 months (range: 4 to 27 months) from treatment withdrawal. All children had been transfused and the average number of transfusions was 15 per patient (range: 6 to 107). None received intravenous (IV) immune globulin, which may produce passive ELISA and RIBA positivity, during the entire period of observation. None had a positive history of liver disease or had received blood or blood products before the onset of leukemia.

**Assessment of liver disease.** The 11 children included in this study were monitored after the diagnosis of leukemia for clinical and biochemical evidence of liver disease, using the following parameters: serial testing of serum ALT, alkaline phosphatase (AP), bilirubin, albumin, and prothrombin time. Serum samples were regularly stored at -20°C and -70°C.

**Assessment of hepatitis B (HBV) and C (HCV) serum markers.** HBV markers, including HBsAg, anti-HBs, and anti-HBc, were tested in serum by commercially available radioimmunoassay (Abbott Laboratories, North Chicago, IL).

Antibodies to HCV (anti-HCV) were detected by first- and second-generation Ortho-ELISA tests (Ortho Diagnostic Systems, Raritan, NJ) and the methods and evaluation of results were performed according to the manufacturer’s instructions. To define the specificity of the results obtained by ELISA, reactive sera were also investigated by RIBA. For this purpose, sera were analyzed by second-generation (four antigens) RIBA (Chiron Corp, Emeryville, CA and Ortho Diagnostic Systems), following the manufacturer’s instructions.

Table 1. Hepatitis C Virus Serum Markers and Transaminase Profile in 11 Children With Acute Leukemia

<table>
<thead>
<tr>
<th></th>
<th>Within 6 mo From Diagnosis</th>
<th>At The End of Treatment</th>
<th>At Latest Follow-Up</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-HCV positive by first-generation ELISA*</td>
<td>0/11</td>
<td>0/11</td>
<td>2/11</td>
</tr>
<tr>
<td>Anti-HCV positive by second-generation ELISA*</td>
<td>3/11</td>
<td>2/11</td>
<td>9/11</td>
</tr>
<tr>
<td>HCV-RNA positive by PCR</td>
<td>6/11</td>
<td>7/11</td>
<td>10/11</td>
</tr>
<tr>
<td>No. of patients with raised ALT1</td>
<td>11/11</td>
<td>11/11</td>
<td>3/11</td>
</tr>
<tr>
<td>Mean/median ALT value</td>
<td>404/400</td>
<td>212/146</td>
<td>137/134</td>
</tr>
</tbody>
</table>

* Confirmed by RIBA.
† ALT, serum alanine aminotransferase.
‡ Normal value < 40 IU/L.

HCV-RNA was detected in serum using the nested PCR technique. Two sets of primers specific for the 5' untranslated region (5'UTR) were used. External primers: 5'-GCCATGGCGTTAGTATGAGT-3' (sense) and 3'-TCCAGAGCCTGCGACCTA-5' (antisense). Inner primers: 3'-TATCCCACGAAGCTCACGG-5' (antisense) and 5'-GTGCAAGCTCAGAGGCCC-3' (sense). Amplification products were visualized by ethidium bromide staining. Two normal sera and water were used as negative control in each experiment and a known positive reference sample was also included.11

Blood components administered to the patients were obtained from HBsAg-negative volunteer donors, who were also tested for the presence of anti-HCV since January 1990.

This investigation was approved by local institutional human research committees.

**RESULTS**

The results of serum HCV markers and of transaminases during the observation period in the 11 patients with leukemia are summarized in Table 1. Six patients were found HCV-RNA positive in serum early in the course of leukemia, usually 1 to 2 months after diagnosis and blood transfusion. None of them was positive for anti-HCV by first-generation ELISA while 50% (three cases) were positive by second-generation ELISA. All six patients had evidence of chronic hepatitis during chemotherapy with persistent (four cases) or intermittent (two cases) elevation of ALT. Four of them (Table 2, group A) normalized ALT after cessation of chemotherapy, although viremia persisted, whereas two maintained abnormal ALT up to the end of follow-up. Only 1 of the 6 children had jaundice during follow-up. At the end of the chemotherapy period (Table 1), seven patients were HCV-RNA positive. All of them had raised ALT, as the four additional cases who were HCV-RNA negative. Anti-HCV was not found in any of the cases by first-generation ELISA whereas it was positive in two and negative in five HCV-RNA–positive cases by second-generation ELISA. However, all but one HCV-RNA positive patients...
became anti-HCV positive after cessation of chemotherapy. Overall, 10 of 11 patients (90.9%) were HCV-RNA positive in serum at latest follow-up (Table 1).

The patient HCV-RNA negative had a unique false positivity for anti-HCV by second-generation ELISA not confirmed by RIBA.

The behavior of liver disease after chemotherapy withdrawal in the 10 HCV-RNA positive cases was as follows (Table 2): seven patients showed normalization of ALT within 6 months although with persistent viremia, whereas three children continued to have elevated ALT levels.

Among the 7 children with normal biochemical profile at 6 months, 3 had experienced a sharp peak in ALT levels shortly after cessation of treatment, followed by rapid and complete normalization. In the other 4 cases, biochemical improvement was concomitant with chemotherapy withdrawal. The relation between the outcome of liver disease and anti-HCV reactivity by second generation assay was analyzed after chemotherapy withdrawal (Table 3). Of the 3 patients with ongoing hepatitis off-therapy, 2 were persistently anti-HCV positive and 1 showed intermittent reactivity. Among the 7 cases showing ALT normalization, 5 resulted persistently positive whereas in the remaining 2 anti-HCV appeared only transiently.

HBsAg remained negative in serum during follow-up in the 11 children, whereas anti-HBs or anti-HBC and anti-HBs were transiently positive in 5 and 4 patients, respectively.

**DISCUSSION**

Signs of liver damage, mainly represented by elevation of transaminase levels, are frequently detected in patients with leukemia when on chemotherapy and, among the various possible etiologic factors, an important role is attributed to infection with hepatitis viruses, whose expression and replication is certainly favored by immunosuppression. We have previously documented the role of hepatitis B virus, but many patients have chronic liver disease in the absence of a positive hepatitis B serology. Therefore, we have analyzed the possible role of hepatitis C virus using available serologic assays for the detection of anti-HCV and the PCR for the study of serum HCV-RNA. Screening of patients sera by anti-HCV with second-generation assays showed a group of patients who became antibody positive during follow-up. These results were compared with those obtained with first-generation ELISA and all patients were also analyzed for HCV-RNA in serum by the sensitive PCR to assess the prevalence and behavior of viremia. The results obtained during and after the period of chemotherapy show that first-generation assays are insensitive in detection of the infection, either in the early phase or during further follow-up, as most HCV-RNA positive patients were negative by ELISA for anti-C100-3 during the observation period. Other studies have shown that immunosuppression has a more profound influence on the ability of HCV-infected patients to produce anti-C100 compared with production of anti-C22 and our findings are in agreement with the conclusion that anti-C100 testing greatly underestimates HCV infection. Second-generation ELISA was more sensitive in this respect and several patients developed anti-C22 and/or anti-C33 either during chemotherapy or after its withdrawal. Overall, when on chemotherapy, 7 of 11 patients were HCV-RNA positive: none of them were anti-HCV positive by first-generation assay and three were anti-HCV positive by second-generation assay, leaving four patients with viremia but without detectable anti-HCV. All four patients became anti-HCV positive when off therapy. These results clearly confirm that HCV-RNA testing by PCR is required to correctly identify HCV infection in patients under immunosuppression. However, it should be mentioned that there are still limitations on the availability of HCV-RNA testing and that the reliability of HCV-RNA detection by PCR among laboratories has been reported to be poor.

Interestingly, serum HCV-RNA remained detectable after chemotherapy withdrawal in all the patients, whereas transaminases, which were elevated while on antileukemic treatment, normalized in several of them during follow-up. This would suggest that either HCV was not the main cause of liver damage, which could have been related to drug toxicity, or that the cytopathic effect of the virus was potentiated by immunosuppression, then being attenuated after restoration of immunocompetence. Another possibility is that the association of antileukemic drugs and HCV infection could lead to complementary liver-damaging processes, as is well established in alcoholics with severe liver disease.

Efforts in this study to identify factors that might predict progression of liver disease were unrewarding. Neither the severity of the liver disease, defined by the mean ALT levels, nor the pattern of anti-HCV reactivity during and after chemotherapy had a clear-cut profile. In agreement with other studies, the observation that patients with biochemical resolution remained positive for HCV-RNA could suggest that persistent infection in the absence of liver inflammation may occur similarly to that observed in HBsAg carriers. On the other hand, the apparent absence of liver disease among patients with viremia might be caused in part by the strict criteria based on ALT levels we commonly use to define liver disease. Indeed, progressive liver disease has even been documented in patients with normal ALT.

Interestingly, we observed no severe hepatitis reactivation after chemotherapy withdrawal, despite the fact that all the children remained viremic. Three of them showed symptomless ALT peaks shortly after cessation of chemotherapy, followed by enzyme normalization within 6 months. This observation is in agreement with previous studies performed in larger series of children with leukemia followed off-therapy. Indeed, we have never observed a fulminant hepatitis following immune reconstitution after chemother-

<table>
<thead>
<tr>
<th>Anti-HCV Positivity</th>
<th>ALT Normalization (7 cases)</th>
<th>Ongoing Hepatitis (3 cases)</th>
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<tbody>
<tr>
<td>Persistent</td>
<td>5/7</td>
<td>2/3</td>
</tr>
<tr>
<td>Transient</td>
<td>2/7</td>
<td>—</td>
</tr>
<tr>
<td>Intermittent</td>
<td>—</td>
<td>1/3</td>
</tr>
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</table>
apy withdrawal also in children with hepatitis B,\textsuperscript{22} in contrast to other experiences.\textsuperscript{23,24} In regard to anti-HCV profiles, we had serial sera only in a proportion of patients, because in leukemic children it is often very difficult to take blood samples. However, in the cases with serial testing, we observed that a persistent anti-HCV reactivity was present not only in patients with ongoing hepatitis, but also in cases with ALT normalization. This observation is in contrast with a previous study\textsuperscript{25} in which we established a close correlation between persistence of anti-HCV and progression of liver disease.

The need for prolonged follow-up of patients surviving leukemia with chronic HCV infection is emphasized by the fact that the natural history of HCV infection has not been fully clarified and that additional late consequences are likely.\textsuperscript{19} Thus, our observation in this study cohort continues.

REFERENCES

Hepatitis C virus serum markers and liver disease in children with leukemia during and after chemotherapy

A Locasciulli, D Cavalletto, P Pontisso, L Cavalletto, E Scovena, C Uderzo, G Masera and A Alberti