Analysis of T-cell repertoire in hepatitis-associated aplastic anemia

Jun Lu, Atanu Basu, J. Joseph Melenhorst, Neal S. Young, and Kevin E. Brown

Hepatitis-associated aplastic anemia (HAA) is a syndrome of bone marrow failure following an acute attack of seronegative hepatitis. Clinical features and liver histology suggest a central role for an immune-mediated mechanism. To characterize the immune response, we investigated the T-cell repertoire (T-cell receptor [TCR] \( \gamma \delta \) chain subfamily) of intrahepatic lymphocytes in HAA patients by TCR spectratyping. In 6 of 7 HAA liver samples, a broad skewing pattern in the 21 \( \gamma \delta \) subfamilies tested was observed. In total, 62% ± 18% of HAA spectratypes showed a skewed pattern, similar to 68% ± 18% skewed spectratype patterns in 3 of 4 patients with confirmed viral hepatitis. Additionally, the T-cell repertoire had similarly low levels of complexity. In the peripheral blood lymphocytes (PBLs) of a separate group of HAA patients prior to treatment, 60% ± 15% skewed spectratypes were detected, compared with only 18% ± 8% skewed spectratypes in healthy controls. After successful immunosuppressive treatment, an apparent reversion to a normal T-cell repertoire with a corresponding significant increase in T-cell repertoire complexity was observed in the HAA samples. In conclusion, our data suggest an antigen-driven T-cell expansion in HAA and achievement of a normal T-cell repertoire during recovery from HAA. (Blood. 2004; 103:4588-4593)

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Introduction

Hepatitis-associated aplastic anemia (HAA), the development of hematopoietic failure with bone marrow hypocellularity within 6 months of an episode of hepatitis, is not uncommon, with hepatitis preceding the onset of bone marrow failure in 2% to 5% of aplastic anemia (AA) cases in Europe and the United States. Aplastic anemia is also frequent following orthotopic liver transplantation for non-A, non-B, non-C hepatitis in young patients: 23% to 8% of non-A, non-B, non-C hepatitis patients receiving transplants developed aplastic anemia, compared with fewer than 1% of all liver transplant patients. The hepatitis/aplastic anemia syndrome shows a stereotypical pattern; most often affecting young males, the hepatitis generally follows a benign course, but the onset of aplastic anemia 2 to 3 months later can be explosive and is usually fatal if untreated. The presumed infectious cause of the hepatitis is unknown, but most cases are seronegative for known hepatitis viruses, including hepatitis A, B, C, and G (GB virus C [GBV-C]). We previously reported 10 cases of HAA seen at the National Institutes of Health (NIH) that had evidence of lymphocyte activation; 70% responded to immunosuppression with antithymocyte globulin and cyclosporine. Apart from case reports, the presence of lymphocyte activation, and the clinical response to either immunosuppression or bone marrow transplantation, little is known of the immunopathogenesis of this syndrome.

The time interval between the occurrence of hepatitis and the onset of bone marrow failure suggests that the initial target organ of the immune response is the liver. For both hepatitis B and hepatitis C infection, large numbers of lymphocytes infiltrate the liver at the time of maximal abnormal liver function, and T cells are the predominant inflammatory cells infiltrating the liver parenchyma. Analysis of the T-cell repertoire in these cases has demonstrated clonal expansions, and conserved features of antigen specificity in many of these expansions are linked to the immunopathogenesis of viral hepatitis. Corresponding information concerning the T-cell immune response in HAA in liver or blood is unreported.

T-cell receptor (TCR) analysis by spectratyping is a powerful tool to assess the clonal composition of the T-cell repertoire in both infectious and immune-mediated diseases. The technique depends on the tissue-specific expression of certain TCRs (on T cells) and on the diversity, somatic rearrangements, and insertions at the nucleotide level that create TCR diversity. The majority of mature T cells express an \( \alpha \beta \) TCR, of which the \( \beta \) chain has 3 different regions that correspond to the 3 complementary determined regions (CDRs) of immunoglobulin. The first 2 CDRs are germ line–encoded, but the third CDR is the product of somatic rearrangements. During T-cell development, the multiple TCR genes for the variable (V), diversity (D), and joining (J) segments rearrange with concomitant nucleotide excision and addition at the VD and DJ junctions, creating the third hypervariable CDR. Depending on the combination of segments and transferase activity, CDR3 sequences of varying lengths are produced, and it is these different CDR3 lengths that the spectratype analysis detects.

We have assessed the T-cell repertoire in the liver and peripheral blood lymphocytes in HAA patients and compared the pattern of \( V_\beta \) subfamily expansion in HAA patients and individuals with hepatitis B and/or C infection by spectratyping. Our results show that many different T-lymphocyte clones accumulate in the liver of HAA patients, with a similar broad skewing pattern of the T-cell repertoire observed in the liver of patients with HAA as well as known viral hepatitis disease.
addition, we determined the T-cell repertoire in peripheral blood lymphocytes before and after immunosuppressive therapies and showed an apparent reversion to a more normal T-cell repertoire in HAA patients after successful treatment.

Patients, materials, and methods

Patients and control population

HAA was defined as severe bone marrow aplasia within 6 months of an episode of documented seronegative (non-A, non-B, non-C) hepatitis. Severity was defined as pancytopenia with at least 2 of the following abnormalities: absolute neutrophil count below 0.5 × 10/L (below 500/μL), platelet count below 20 × 10/L (below 20 000/μL), and reticulocyte count below 60 × 10/L (below 60 000/μL), in association with bone marrow cellularity less than 30%. Hepatitis was defined as an increase in serum transaminases to at least 3 times the upper limit of normal (normal alanine transaminase, 6 to 41 IU/L; normal aspartate transaminase, 9 to 34 IU/L). Anonymized liver samples were provided by the University of Minnesota Liver Tissue and Distribution System (LTTPDS) NIH contract no. N01-DK-9-2310 after Institutional Review Board (IRB) approval. Additional liver samples were obtained from Cleveland, OH; Columbus, OH; Denver, CO; and Miami, FL. All tissues were obtained after informed consent and following the human experimentation guidelines of the US Department of Health and Human Services and NIH. Liver samples were obtained at the time of liver transplantation, and diagnoses included 7 HAAs, 4 cases of confirmed hepatitis A virus (HAV), hepatitis B virus (HBV), and hepatitis C virus (HCV) infection. All patients with HAA were negative for hepatitis A virus or hepatitis B and/or hepatitis C infection, and 4 cases of biliary atresia as a complication of extrahepatic biliary atresia. The remaining 2 patients with HAA presented with fever, jaundice, and obstructive liver disease at a mean age of 6.4 months. One patient was born with the diagnosis of HAA, and the other patient had a history of biliary atresia, the spectrum of which included acute liver failure and biliary atresia. The remaining 2 patients with HAA presented with fever, jaundice, and obstructive liver disease at a mean age of 6.4 months. One patient was born with the diagnosis of HAA, and the other patient had a history of biliary atresia, the spectrum of which included acute liver failure and biliary atresia.

DNA was extracted from ground frozen liver samples or PBLs (10⁶) by direct suspension in 0.1 g/mL RNAStat 60 (TelTest, Friendswood, TX). DNA was precipitated by centrifugation according to the manufacturer’s instructions and resuspended in approximately 20 μL RNase-free distilled water, and the concentration was calculated by optical density measurement. First-strand cDNA was synthesized with the use of 1 μg total RNA, reverse transcriptase, and oligo-deoxynucleotide (oligo-dT) at 42°C for 50 minutes and in a final volume of 50 μL following manufacturer’s instruction (Invitrogen Life Technologies, Gaithersburg, MD).

CDR3 length analysis was conducted by using a modification of the polymerase chain reaction (PCR) followed by the run-off reaction method previously described. First, PCR amplification of cDNA was performed with the use of 22 specific Vβ primers (21 Vβ primers in the liver samples [Vβ18 was not included for technical reasons]; 0.5 μL cDNA per reaction) for the TCR Vβ-gene families (excluding Vβ10 and Vβ19, which are pseudogenes) and the common Cγ primer (ggg-gct-gct-cct-tga-ggg-gtc-gc) end-labeled with a fluorescence tag (6-FAM). Amplifications were performed with Ex Taq buffer (Takara, Tokyo, Japan), 0.2 μM mixed deoxynucleoside triphosphate (dNTP), 0.5 mM each primer, and 0.5 U Ex Taq polymerase (Takara) in a 20 μL vol, on a DNA engine machine (PTC-200, MJ Research, Waltham, MA). The amplification profile was denaturation at 94°C for 1 minute, annealing of primers at 60°C for 1 minute, and extension at 72°C for 4 minutes for 40 cycles with a final extension step at 72°C for 10 minutes. Amplified products were electrophoresed on 1.5% agarose gels and detected by ethidium bromide staining. For the run-off reaction, a second round of amplification (3 cycles) was performed with a single 6-hexachlorofluorescein (HEX)–labeled fluorescent constant primer internal to the Cγ primer (0.1 mM) in a volume of 10 μL. A standard size marker was added (500 ROX; Applied Biosystems, Foster City, CA), and first-round and run-off products were analyzed on a 310 DNA sequencer by means of 310 GeneScan Software (Applied Biosystems).

Analysis of spectratype

Owing to the recombination events that occur during TCR generation, the length of the ampiclon varies, and in a normal population of T cells, CDR3 length analysis produces approximately 5 to 10 identifiable peaks spaced by 3 nucleotides, with fluorescence intensity following a quasi-Gaussian distribution. Spectratypes were analyzed in 3 ways. First, the spectratype pattern was visually assessed. A normal spectratype profile was defined as showing an approximate Gaussian bell-shaped distribution, with discrete peaks spaced by 3 nucleotides. If discrete peaks were observed but did not have the Gaussian profile, the spectratype was classified as skewed; if discrete peaks were not present, it was scored as absent. To obtain an indication of the magnitude of skewing, each spectratype was assessed as either normal, skewed, or absent by 3 different observers in a blinded fashion. Second, spectratypes were scored mathematically, as previously described. Evidence of oligoclonal expansion or skewing was assessed by calculating the relative fluorescence intensity (RI) of each peak (RI [%] = 100 × clonal peak area / total peak area). A skewed profile was determined if either (1) a single peak was observed and the RI of the dominant peak was greater than 35% of total peak area; (2) 2 dominant peaks were present and each peak’s RI was greater than 25% of total peak area; or (3) there were multiple peaks with the dominant peaks differing from a Gaussian pattern and the RI of the peaks was greater than 25% of total peak area. Finally, overall complexity within a Vβ subfamily was determined by counting the number of discrete peaks per Vβ subfamily, with each subfamily graded on a score of 0 to 5. Spectratypes containing more than 5 peaks were given a score of 5, and a score of 0 was assigned if no spectratype signal was obtained; spectratypes with 1, 2, 3, or 4 peaks were given a score of 1, 2, 3, or 4, respectively. The overall spectratype complexity score per sample was calculated as the sum of the scores for each subfamily, with a maximum complexity score for any one patient of 110 (22 Vβ × 5).
Statistical analysis

The Student t test was used to assess the differences in $\text{V}_{\beta}$ skewing or complexity scores in the different groups of patients. The paired Wilcoxon test (normalizing transformation by log) was used to determine the significance before and after immunosuppressive treatment. All statistical analysis was performed by using GraphPad InStat software (GraphPad Software, San Diego, CA).

Results

Sensitivity of T-cell repertoire spectratyping

A classic Gaussian spectratype was reproducibly obtained with $10^2$ $\text{V}_{\beta}2$ or $\text{V}_{\beta}14$ lymphocytes per $10^6$ cells, and with $10^3$ $\text{V}_{\beta}22$ lymphocytes per $10^6$ cells (Figure 1). With lower proportions of cells, no amplicon was detected by ethidium bromide staining, and no products of the correct size were seen by GeneScan analysis. Thus, the sensitivity of our spectratype analysis for each $\text{V}_{\beta}$ was between 100 and 1000 cells in a 1 $\mu$g total RNA sample.

T-cell repertoire of intrahepatic lymphocytes in HAA patients

Liver histology was not available on 2 of the HAA livers, but in the other 5 samples hepatic necrosis with a lymphocytic or mononuclear infiltrate was universally reported. CD3 staining was not performed on any of the livers, although one liver was stained for CD43. Despite this, in one of the HAA patients we were unable to detect any $\text{V}_{\beta}$ signal with any primers, despite the presence of RNA as indicated by reverse transcriptase–PCR (RT-PCR) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Histology of this liver was reported as having a mononuclear infiltrate with neutrophils and occasional eosinophils, and from our sensitivity data, we would conclude that the T-cell infiltrate was below our detection limit. Similarly we did not detect any $\text{V}_{\beta}$ amplicons in any of the 4 biliary atresia samples. In 6 of 7 HAA livers, 15 to 21 of the 21 $\text{V}_{\beta}$ subfamilies analyzed had detectable PCR products by ethidium bromide staining and production of a spectratype profile on GeneScan analysis, indicating the presence of a T-lymphocyte infiltrate in the livers.

A typical spectratype pattern in HAA is shown in Figure 2: many of the $\text{V}_{\beta}$’s have a highly skewed pattern. In 70% of the spectratypes, there was 100% concordance among all 3 observers. As a measure of the skewing in the different patient groups, the number of skewed $\text{V}_{\beta}$ spectratypes as a percentage of all $\text{V}_{\beta}$ spectratypes detected were calculated. There were 59.5% $\pm$ 16.7% (mean $\pm$ standard deviation [SD]) skewed $\text{V}_{\beta}$’s in the 21 tested $\text{V}_{\beta}$ subfamilies, with 4.8% $\pm$ 11.7% giving no detectable spectratype in HAA livers (Table 1). Similar results were obtained if the relative fluorescence intensity (RI) index was used to determine skewed spectratypes (Table 1). As a measure of the polyclonality of the T-cell repertoire, the complexity of each $\text{V}_{\beta}$ spectratype was assessed and summed to give a total complexity score. The complexity score of the spectratypes varied between 56 and 80, with a mean of 68 (Table 1).

Individual $\text{V}_{\beta}$ spectratypes were also analyzed to determine if there were certain TCR families skewed in all HAA patients. Although $\text{V}_{\beta}1$, $\text{V}_{\beta}7$, $\text{V}_{\beta}11$, $\text{V}_{\beta}12$, $\text{V}_{\beta}15$, $\text{V}_{\beta}16$, $\text{V}_{\beta}20$, and $\text{V}_{\beta}24$ were skewed in more than 4 of 6 of the patients, when we further analyzed the length of the oligoclonal expansions, no peaks of the same size were found in more than half of the patients. Thus, although oligoclonal expansions affected many $\text{V}_{\beta}$ families, there did not appear to be any shared $\text{V}_{\beta}$ subfamily expansions for all cases, against a common $\text{V}_{\beta}$ CDR3 sequence for all HAA.

The spectratype profiles obtained in the livers of patients with HAA were compared with profiles obtained from 4 patients with viral hepatitis of known etiology (hepatitis B and/or hepatitis C) (Figure 3). In one liver sample of a viral hepatitis patient, no signal could be observed for any of the PCR products, despite the presence of GAPDH product, suggesting a very low number of infiltrating lymphocytes. In the other 3 samples, there were 67% skewed $\text{V}_{\beta}$’s of the 21 $\text{V}_{\beta}$ subfamilies tested, with no signal in 11% $\pm$ 13% of reactions. No significant difference between HAA and hepatitis patients was shown statistically in the number of skewed $\text{V}_{\beta}$ spectratypes ($P > .05$); similarly, the complexity score was not significantly different between these patients.

T-cell repertoire of peripheral blood lymphocytes (PBLs) in HAA patients before and after immunosuppressive treatment

Spectratype analysis was performed on PBLs from 3 HAA patients and compared with the PBL spectratype analysis of healthy volunteers. The 10 healthy controls showed predominantly normal or Gaussian spectratype profiles, with only 17% $\pm$ 12% having a skewed $\text{V}_{\beta}$ pattern; the complexity score was 92 ($\pm$ 11). In contrast, the HAA patients showed a highly skewed $\text{V}_{\beta}$ pattern (60% $\pm$ 17%) ($P < .001$), similar to the $\text{V}_{\beta}$ skewing seen in the liver-infiltrating lymphocytes. However, for many of the spectratypes, no signal was observed, and the complexity score was markedly reduced to 37 $\pm$ 13 ($P < .001$) (Figure 4).
All 3 patients were treated by immunosuppressive therapy, with good hematologic response. Follow-up samples were available on 2 patients 1 year after treatment, and in the third at 2.5 years after treatment. After immunosuppressive therapy, many skewed TCR Vβ's reversed to a normal Gaussian distribution (Figure 5), and many of the Vβ's that were previously not detected now gave a spectratype signal. The number of both skewed Vβ's and absent Vβ's before and after treatment decreased. Similarly, there was a significant increase in mean complexity scores ($P < .01$), with a mean score after treatment of 94 ($\pm 8$), not different from the complexity scores of the healthy donors (mean score, 92 $\pm 11$) (Figure 4).

Discussion

Although the clinical characteristics and response to immuno-therapy indicate that HAA is immune-mediated, there have been no studies of the T-cell repertoire in this syndrome. However, a number of reports have studied the Vβ T-cell repertoire in hepatitis B infection, hepatitis C infection, and autoimmune hepatitis. Pham et al. studied the Vβ composition of liver-infiltrating lymphocytes and showed that, in both hepatitis B and C infections, there was a preponderance of certain Vβ families; the overrepresented Vβ families differed in the 2 viral etiologies. Spectratype data have confirmed these observations. Sing et al. compared the clonality of Vβ T-cell receptor-bearing population in the liver and peripheral blood of patients with hepatitis B and found clonotypic expansions in 4 to 9 TCR Vβ subfamilies, indicating a high restriction in the T-cell composition of liver-infiltrating lymphocytes. Similar experiments in hepatitis C and autoimmune hepatitis, showed many skewed spectratype patterns in lymphocytes in the liver.

Our results from the 4 cases of chronic hepatitis B and C for TCR-spectratyping analysis found a similarly highly skewed Vβ pattern consistent with these published results (and validating our use of frozen tissue). In addition, we demonstrated that we could detect a similar skewed spectratype pattern in the livers of patients with HAA. This was in contrast to livers from patients with biliary atresia, as is the case in healthy livers, should not have an inflammatory or T-cell infiltrate. When we also compared the complexity of the T-cell repertoire in HAA and viral hepatitis, similar complexity scores were obtained in both diseases. Although we cannot conclude whether the same or different antigens are involved in this broad stimulation, our data are suggestive of a similar pathogenic mechanism, perhaps triggered by an antigen-specific immune response as in viral hepatitis and supporting the hypothesis, based on epidemiologic and clinical investigations, that an unknown pathogen may be involved in this disease.

Some studies using peptides as antigens have suggested a limited T-cell response, with different clones having the same or related CDR3 sequences. Shared CDR3 sequences have been harder to identify in hepatitis infections, although putative shared motifs have been suggested. When we analyzed the specific CDR3 lengths in the TCR Vβ repertoire in these patients, we did not find any common peaks shared by 3 or more samples. However, our samples were not human leukocyte antigen (HLA) matched. The current negative finding nevertheless does not support the role of a superantigen or shared CDR3 sequences in the pathogenesis of HAA.

One of the major limitations of our study was that we were unable to obtain paired liver and peripheral blood samples from the same patients. However, when PBLs from different patients with HAA were examined, we observed a similar broadly skewed T-cell repertoire pattern (greater than 50% of Vβ families), as found in the liver samples, with a statistically significant increase in the complexity score of the spectratypes compared with controls, suggesting an antigen-driven TCR repertoire and limited usage of the TCR. In addition, evaluation of the TCR Vβ spectratypes before and after successful immunosuppressive therapy showed reversion to a normal spectratyping profile after treatment. This strongly suggests that pathogenic T cells had been eliminated or decreased so as not to be detectable, and a relationship between Vβ changes and an autoimmune pathophysiology.

Our results in the HAA patients can be compared and contrasted with similar data in idiopathic aplastic anemia. Manz et al. found a restrictive T-cell expansion in both bone marrow and PBLs of patients with severe AA, with 1 or 2 oligoclonal Vβ patterns per patient, and suggested that the T-cell repertoire expansion was random with respect to the Vβ chain. Data from a Japanese study showed that patients with cyclosporine-dependent AA had highly skewed Vβ spectratypes, with patients who responded to immunosuppressive therapy still showing

### Table 1. Comparison of the Vβ spectratype analysis by different methods in liver-infiltrating lymphocytes in HAA and viral hepatitis patients

<table>
<thead>
<tr>
<th>Observation type</th>
<th>Skewed by R1 measurement, %</th>
<th>Total complexity score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HAA</strong></td>
<td>Absent, %</td>
<td>Skewed, %</td>
</tr>
<tr>
<td>6</td>
<td>4.8 ± 11.7</td>
<td>59.5 ± 16.7</td>
</tr>
<tr>
<td><strong>Viral hepatitis</strong></td>
<td>3</td>
<td>11.1 ± 12.5</td>
</tr>
</tbody>
</table>

*Mean ± SD.

### Figure 3. Marked T-cell skewing in liver-infiltrating lymphocytes of HAA and hepatitis patients.

Skewing was observed in HAA patients ($n = 7$) and hepatitis patients ($n = 4$). Indicates the skewing Vβ profile by observation and RI; III indicates the normal Vβ profile; and [] indicates no detectable signal. Overall, there were 60% ± 17% skewed spectratypes, with 5% ± 12% absent spectratypes in the HAA patients, compared with 67% ± 0% skewed Vβ's, with no signal in 11% ± 13% of reactions, in the viral hepatitis patients. Thus, no significant difference was shown in the number of skewed Vβ spectratypes in each group ($P > .5$).
after immunosuppressive therapy, the degree of expansion of individual fi
immunosuppressive therapy, no signi
in other immune-mediated diseases, such as multiple sclerosis,38,39
HAA patients. Differences in T-cell repertoire have been observed
restriction patterns may differ (J.L. et al, manuscript in prepara-
AA patients, Zeng et al 37 reported that most CD4 clones displayed V
strated that although the abnormal V
rheumatoid arthritis, 40,41 and autoimmune hepatitis, 42 where a
often associated with AA.35 In a study of CD4 and CD8 lymphocyte
seen in paroxysmal nocturnal hemoglobinuria (PNH), a syndrome
V
sequences in HLA-DR2 patients.

Although HAA is usually considered a subset of AA, HLA restriction patterns may differ (J.L. et al, manuscript in prepara-
We have observed no increased association of HLA-DR2 in HAA patients. Differences in T-cell repertoire have been observed
in other immune-mediated diseases, such as multiple sclerosis,38,39
rheumatoid arthritis,40,41 and autoimmune hepatitis,42 where a
limited number of T cells using a restricted diversity of the V
subfamilies to proliferate dominantly is revealed in different
patients,21 but the TCR repertoire pattern is different among these
diseases owing to the different antigenic triggers.39,43,44

In summary, we show that in HAA there is an infiltration of both
clonal and many nonclonal T cells, giving rise to a markedly skewed
T-cell repertoire, as seen in both viral and autoimmune hepatitis. This
highly skewed T-cell repertoire is also evident in the blood at the time
of presentation of bone marrow failure. The expanded T-cell clones are
replaced by a normal Gaussian distribution of T-cell repertoire after
immunosuppressive treatment, possibly associated with an antigen
clearance and/or loss of T cells due to therapy. Further study on the
disease-specific T-cell clones (CD8+ or CD4+) and their roles in the
immunopathology of HAA is ongoing.

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References


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