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] Vβ 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 Vβ 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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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 × 10⁹/L (below 500/μL), platelet count below 20 × 10⁹/L (below 20 000/μL), and reticulocyte count below 60 × 10⁹/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).³ Anonymized liver samples were provided by the University of Minnesota Liver Tissue and Distribution System (LTTPDS) NHI 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 B and/or hepatitis C infection, and 4 cases of biliary atresia as controls. All patients with HAA were negative for hepatitis A virus (HAV), hepatitis B virus (HBV), and hepatitis C virus (HCV) infection. All liver samples were immediately frozen and stored at −80°C or in the gaseous phase of liquid nitrogen until RNA was extracted.

Peripheral blood samples or follow-up samples were not available from the HAA liver patients. However, peripheral blood was obtained from 3 additional HAA patients at the time of presentation and after immunosuppressive treatment. All patients were enrolled on National Heart, Lung and Blood Institute (NHBLI) IRB-approved protocols, and information on one of these patients, had been previously reported.⁴ In addition, lymphocytes were obtained from 10 healthy donors (younger than 40 years old) who served as healthy controls and from 3 additional healthy volunteers to assess the sensitivity of spectratype analysis. Lymphocytes were isolated from heparinized peripheral blood by Ficol-Hypaque density centrifugation (ICN Pharmaceutical, Costa Mesa, CA) and used immediately or stored at −80°C until RNA was extracted.

**Cell sorting by flow cytometry (FACS) for V₅ subfamily spectratyping sensitivity analysis**

To confirm that we could detect specific V₅ subtypes in tissue RNA samples, we first examined the sensitivity of our spectratype assay. Three representative V₅ subfamilies, V₅₂, V₅₁₄, and V₅₂₂, were randomly selected. Purified peripheral blood lymphocytes (PBLs) (10⁷) were suspended in 50 μL fluorescence-activated cell sorter (FACS) buffer (phosphate-buffered saline plus 0.4% bovine serum albumin [BSA]) and incubated on ice with phycoerythrin-conjugated antibody specific for either TCR V₅₂, V₅₁₄, or V₅₂₂ (7 μL, 17 μL, or 7 μL, respectively) (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 occurs 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.²⁰²² 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.¹⁷,²³ 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 multipeaks 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 V₅ subfamily was determined by counting the number of discrete peaks per V₅ subfamily, with each subfamily graded on a score of 0 to 5.²⁴ 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 V₅ × 5).
Statistical analysis

The Student t test was used to assess the differences in \( V_\beta \) 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 \( 10^2 \) \( V_\beta \)2 or \( V_\beta \)14 lymphocytes per 10\(^6\) cells, and with \( 10^3 \) \( V_\beta \)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 \( V_\beta \) 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 \( V_\beta \) 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 \( V_\beta \) amplicons in any of the 4 biliary atresia samples. In 6 of 7 HAA livers, 15 to 21 of the 21 \( V_\beta \) 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 \( V_\beta \)'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 \( V_\beta \) spectratypes as a percentage of all \( V_\beta \) spectratypes detected were calculated. There were 59.5% \( \pm \) 16.7% (mean \( \pm \) standard deviation [SD]) skewed \( V_\beta \)'s in the 21 tested \( V_\beta \) 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 \( V_\beta \) 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 \( V_\beta \) spectratypes were also analyzed to determine if there were certain \( V_\beta \) families skewed in all HAA patients. Although \( V_\beta \)1, \( V_\beta \)7, \( V_\beta \)11, \( V_\beta \)12, \( V_\beta \)15, \( V_\beta \)16, \( V_\beta \)20, and \( V_\beta \)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 \( V_\beta \) families, there did not appear to be any shared \( V_\beta \) subfamily expansions for all cases, against a common \( V_\beta \) 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, the \( V_\beta \) spectratype profile was assessed and summed to give a total complexity score.24

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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 \( V_\beta \) pattern; the complexity score was 92 (\( \pm \) 11). In contrast, the HAA patients showed a highly skewed \( V_\beta \) pattern (60% \( \pm \) 17%) (\( P < .001 \)), similar to the \( V_\beta \) 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 ± 8, not different from the complexity scores of the healthy donors (mean score, 92 ± 11) (Figure 4).

Discussion

Although the clinical characteristics and response to immunotherapy indicate that HAA is immune-mediated,8 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 al16 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 al25 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,17,18,26 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 that, 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.27

Some studies using peptides as antigens have suggested a limited T-cell response, with different clones having the same or related CDR3 sequences.28,29 Shared CDR3 sequences have been harder to identify in hepatitis infections, although putative shared motifs have been suggested.17,25,26 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.30

Our results in the HAA patients can be compared and contrasted with similar data in idiopathic aplastic anemia. Manz et al31 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 study32 showed that patients with cyclosporine-dependent AA had highly skewed Vβ spectratypes, with patients who responded to immunosuppressive therapy still showing
skewing, but in fewer $V_\beta$ families (fewer than 20% had abnormal patterns). In an earlier study comparing TCR repertoire at initial presentation and after therapy in AA patients, we found much broader spectratype skewing (44% ± 33%), with oligoclonal expansions of $V_\beta$15, $V_\beta$21, and $V_\beta$24 in more than 70% of AA patients with HLA-DR2.33 In contrast to the HAA patients reported here, after immunosuppressive therapy, no significant change was found in the degree of $V_\beta$ skewing, with patients treated with cyclophosphamide even showing more oligoclonality. Skewed T-cell repertoires34 were also seen in paroxysmal nocturnal hemoglobinuria (PNH), a syndrome often associated with AA.35 In a study of CD4 and CD8 lymphocyte subpopulations in patients with AA and PNH, Risitano et al36 demonstrated that although the abnormal $V_\beta$-distribution pattern was retained after immunosuppressive therapy, the degree of expansion of individual $V_\beta$’s was lower. For transformed CD4 and CD8 clones obtained from AA patients, Zeng et al37 reported that most CD4 clones displayed $V_\beta$5 and CD8 clones displayed $V_\beta$13, and that ATG and cyclosporine treatment led to marked decrease in clones bearing the dominant CDR3 $V_\beta$5 sequence in HLA-DR2 patients.

Although HAA is usually considered a subset of AA, HLA restriction patterns may differ (J.L. et al, manuscript in preparation). 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 $V_\beta$ 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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Analysis of T-cell repertoire in hepatitis-associated aplastic anemia

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