



Hematologic and Hepatic Anomalies in Pediatric Acute Rheumatic Fever

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Abstract

Anemia and hepatic transaminitis were seen in a cohort of Acute Rheumatic Fever (ARF) and were studied. An innovative approach to quantifying Anti-Streptolysin O (ASO) using a multiplex assay was utilized to study the anemia in ARF and indirect agglutination to test for possible autoimmune hemolysis. Subjects with mild hepatic transaminitis prior to the start of Aspirin (ASA), worsened when ASA was started, and this medication had to be stopped. A one week course of steroids followed by naproxen therapy worked. In 78% of anemic subjects, a general trend of increased hemoglobin with a concurrent decrease in ASO was found. No agglutination was observed by indirect agglutination. Multiplex assay and traditional ASO methodology revealed a positive correlation, and the data displayed evidence for multiplex assay as an alternative method. The cause of liver dysfunction may be due to inflammatory processes involving the liver and worsens with ASA therapy. Our findings support the treatment of ARF with an alternate NSAID like naproxen instead of ASA due to the possible adverse effects associated with high dose ASA therapy. However, if ASA therapy is used for ARF serial liver enzyme measurements should be done prior to and after starting salicylates. Anemia appears to be related to the disease process and improves with resolution of the disease clinically and a drop of the ASO titer in most subjects. Seventy-six percent of anemic patients also had evidence of carditis, suggesting that anemia may be caused by inflammation and cytokines, such as TNF- α , which may diminish erythropoiesis.

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Introduction

The cause of liver damage in Acute Rheumatic Fever (ARF) patients has not been definitively characterized in patients that have not received Aspirin (ASA) and despite the fact that liver enzymes are often affected in patients with rheumatic disease, there is no established treatment protocol for ARF patients with elevated liver enzymes [1-12]. A study by Gitlin and Grant reported 6 out of 11 ARF patients with elevated liver enzymes, while Hamdan et al., [5,7] found aspirin-induced hepatitis in 15% of patients. Prior studies have linked elevated transaminase levels to high-dose ASA therapy, while another concluded that the observed abnormal liver enzymes may be linked to the disease process, and not caused by ASA therapy [3-7,9]. Studies in ARF patients have briefly described the presence of anemia, however, little is known about the origin or resolution of the anemia, and it has not been extensively studied in four decades [13-16]. Recent studies reported 62% and 26% of subjects with anemia, however neither reported on severity, duration or resolution of the anemia [17,18]. Studies from the 1950's and 60's did not discuss treatment and incidence of anemia, however they attributed this phenomenon to an increase in plasma volume [13,15]. There have been no recent studies investigating anemia in ARF. Strepto-Lysine O (SLO) is a virulence factor of Group A Streptococcus (GAS) and induces the immune system to form Anti-Strepto-Lysine O (ASO) antibody. ASO rises 7 to 10 days after GAS infection, peaks 3 to 6 weeks after GAS infection and declines after 6 to 8 weeks in patients without complications [19,20]. ASO levels are measured by traditional methods, by titration of patient serum to test for agglutination, to establish a previous strep infection and Median Fluorescence Intensity (MFI) values have been previously compared to levels done by traditional commercial methods with success, however it has not been studied in the ARF population in Hawai'i (Table 1) [21-24].

Table 1: List of terms listed in the paper and their abbreviations.

Term	Abbreviation
Aspirin	ASA
Acute Rheumatic Fever	ARF
Strepto-Lysine O	SLO
Group A Streptococcus	GAS
Anti-Streptolysin O	ASO
Median Fluorescence Intensity	MFI
Alanine Aminotransferase	ALT
Aspartate Aminotransferase	AST
Red Blood Cells	RBC
Multiplex Fluorescent Immuno Assay	MFIA

Materials and Methods

Subjects and samples

Fifty subjects were enrolled between June 2001 and December 2008 at Kapiolani Medical Center for Women and Children in Honolulu, Hawai'i. All participants provided informed consent following IRB approval, and subjects were diagnosed with ARF based on the modified Jones criteria [25]. Serum samples were obtained and frozen at -80°C.

Clinical lab tests

Liver enzymes, Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), and hemoglobin levels were deemed abnormal if they were above or below the normal range, as determined by Clinical Laboratories of Hawai'i for subjects' specific age and gender (Table 1). Subjects were evaluated at time of entry into the study and repeatedly over the course of treatment.

Direct and indirect hemagglutination

In order to characterize the type of anemia present in ARF subjects, indirect and direct agglutination tests were performed as previously described [21]. Serum samples from 3 subjects with the 3 lowest values of hemoglobin with available serum were chosen to test for anti-red blood cell antibody by the indirect agglutination test. Unfortunately, there were not enough sera available on all anemic subjects to test for agglutination. A 1:5 dilution of subject serum was made and mixed with O+ Red Blood Cells (RBC) for 30 minutes. A positive control was created using a serum dilution of 1:40 of B+ serum with 2% washed A+ RBCs. The samples were then washed with PBS/1% BSA three times. A 25 µl aliquot of a 1:50 dilution of AffiniPure F (ab')₂ Fragment Goat Anti-Human IgG (H&L) (Sigma Aldrich Corp, St. Louis, MO) was added to washed RBC, incubated for 30 minutes, and washed. The RBC pellets were smeared on a slide, fixed and observed under light microscope for agglutination.

Multiplex Fluorescent Immunoassay (MFIA)

SLO (Sigma Aldrich Corp, St. Louis MO) was coupled to microsphere beads (Luminex Corporation, Austin, TX) using previously described procedures [24]. A seven-point standard curve was created for SLO by making 2-fold dilutions of the antigen ranging from 100 IU/L to 150 IU/L. The seven different antigen-coupled microsphere beads were mixed together at a working concentration of 5000 of each microsphere per reaction. Subject serum samples were diluted 1:100 with PBS/BSA. Reactions were incubated for 1 hour at room temperature on a shaker using a 96-well filter bottom microtiter plate (Millipore Corporation, Bedford, MA) and washed. Goat anti-

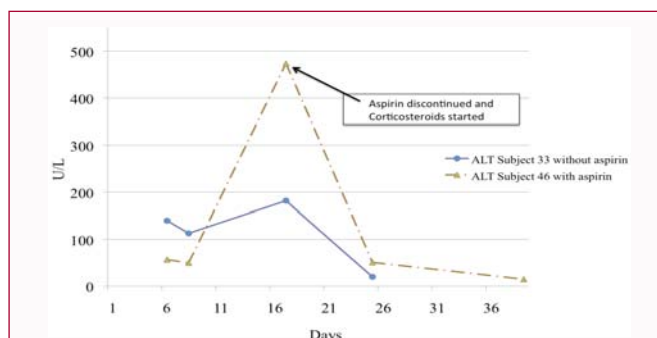


Figure 1: Comparison of liver function abnormalities between two representative ARF subjects: Subject 46 was treated with ASA: The subject had mildly elevated liver enzymes upon presentation at 5 days after the onset of symptoms. At day 17 on high dose ASA, transaminase levels peaked at 535 U/L for ALT. ASA was discontinued and corticosteroid was started at a dosage of 40 mg once a day. At day 2, transaminase levels had returned to the normal range. Subject 33 was not treated with ASA and presented with elevated ALT at 140 U/L at day 5. The subject was treated with corticosteroid (30 mg) and transaminase levels showed a slight decrease the next day before spiking at day 17. Subject was continued on corticosteroid (30 mg) and ALT returned to normal when checked at day 25.

human IgG antibodies conjugated with R-PE were added (Jackson Immuno Research, West Grove, PA). The Median Fluorescent Intensity vs SLO concentrations were plotted on a standard curve using Luminex™ analysis software (version 2.3). Correlations were assessed using Pearson's Product-Moment Correlation coefficient.

Results

Subjects enrolled in the study were classified by major manifestation as set forth by the Jones Criteria [25,26]. Subject manifestations included those with carditis only (n=10), arthritis only (n=13), chorea only (n=1), carditis and arthritis (n=24), and carditis and chorea (n=2).

Elevated liver enzymes

Six subjects of the 50 (12%) had elevated liver enzymes (ALT and/or AST) concurrently with rheumatic fever. Of these six subjects, five had carditis and arthritis, and one had arthritis as the only major manifestation. Four of the six subjects (Subjects 6, 22, 37 and 46) with liver dysfunction received ASA and had an elevation of liver enzymes, after starting ASA. This trend is modeled in Subject 46 (Figure 1). This subject's ALT level was mildly elevated (57 U/L) upon presentation at day 5 after the onset of symptoms, before ASA was administered and worsened once ASA was started. By day 17, ALT levels in Subject 46 reached 474 U/L. ASA were discontinued and prednisone was started at a dosage of 40 mg once a day. ALT levels decreased and returned to normal by day 37. Subject 6 experienced a similar trend. The ALT levels were normal upon admission (19 U/L), but increased upon administration of high dose ASA to 151 U/L when measured 9 days later. ASA was discontinued and replaced with prednisone which resulted in a drop of ALT levels to 40 U/L, 14 days later. Subject 22 had elevated ALT upon first evaluation (ALT 188 U/L) at day 11 and had been in high dose ASA therapy for 5 days. When the subject was switched to prednisone at day 11, the ALT levels dropped to normal range (ALT 23 U/L) when measured 15 days later. The fourth individual, Subject 37, had a similar enzyme pattern as the other three but differed in several respects. Subject 37 was diagnosed with recurrent Rheumatic Fever and took ASA as needed. Upon admission, at day 13 post symptoms, high dose ASA was started and liver enzymes monitored. The initial ALT level was 190 U/L at

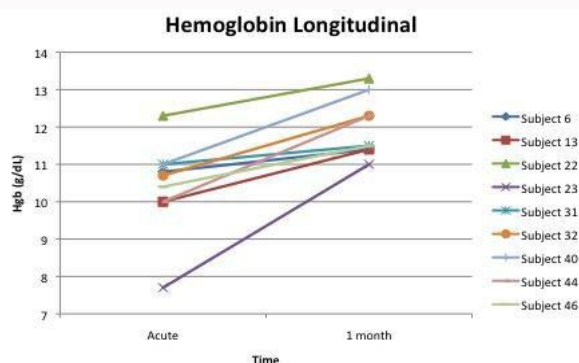


Figure 2a: Hemoglobin levels and ASO levels determined by two different methods in anemic subjects. Nine subjects with hemoglobin levels that were considered below normal by Clinical Laboratories were determined during acute phase (at time of admission) and 1 month later. These subjects were tested for ASO levels determined by commercial methods.

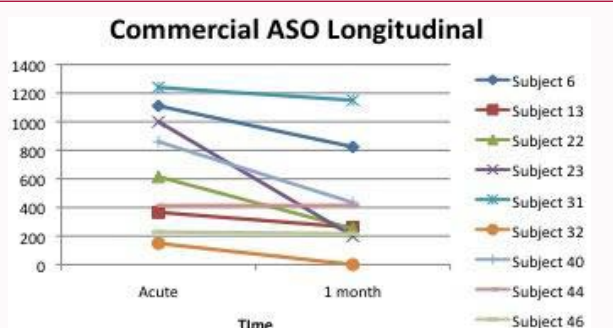


Figure 2b: Hemoglobin levels and ASO levels determined by two different methods in anemic subjects and by multiplex fluorescence immunoassay.

day 13, dropped to 94 U/L by day 19, and continued to drop when checked as an outpatient at day 25 (ALT 33 U/L). Prednisone was not administered. Two subjects (Subject 33 and 43) were not treated with ASA, and were instead treated with corticosteroids with rapid resolution of symptoms, as shown by Subject 33 in Figure 1. Both of these subjects had carditis and arthritis as major manifestations. Subject 33 had initially elevated liver enzymes (ALT 140 U/L), and was given a corticosteroid instead of ASA daily resulting in normal ALT levels by day 19. Similarly, Subject 43 presented with elevated liver enzymes (ALT 175 U/L) taken 14 days after onset of symptoms and treated with prednisone. Liver enzymes gradually decreased over the course of the hospital stay (ALT 129 U/L at day 18 and ALT 95 U/L at day 25). None of the six subjects tested positive for viral hepatitis antibodies. At the highest values for ALT and AST taken on the same date, the ratio of AST to ALT was near or below 1 for all subjects with elevated enzyme levels, which is indicative of hepatocellular damage.

Anemia and ASO levels

Twenty-one of the 50 total subjects enrolled had a hemoglobin value below the normal value. Of these 21 subjects, 5 had arthritis, 2 had carditis, and 14 had both arthritis and carditis, which revealed 76% of anemic patients with carditis. Nine of the 21 anemic subjects had available values for hemoglobin and ASO by both methods over a one-month period. When these nine patients were tracked longitudinally, low hemoglobin levels were shown to return to normal in all nine subjects with the improvement of their condition over the course of a month, without specific treatment for anemia (Figure 2a). This trend was inversely mirrored by a decrease in ASO, measured by

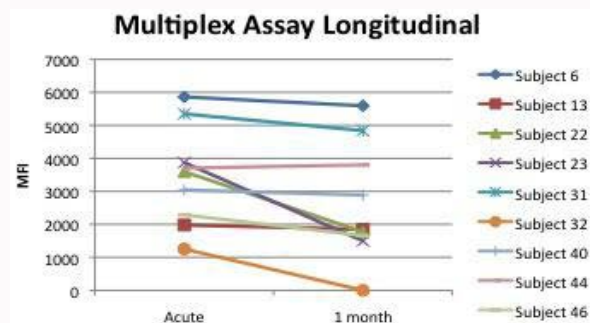


Figure 2c: Hemoglobin levels and ASO levels determined by two different methods in anemic subjects during acute phase and 1 month later.

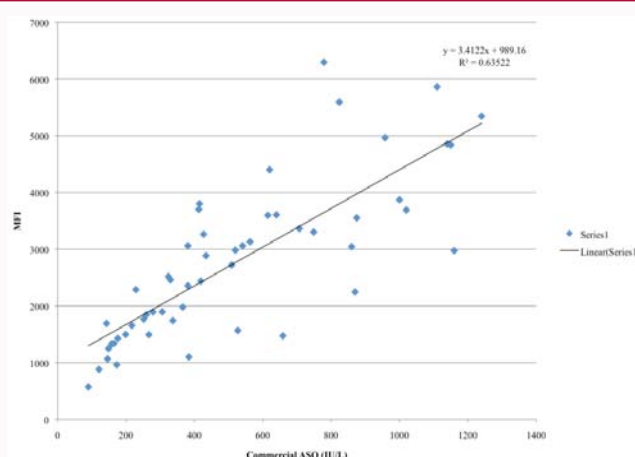


Figure 3: ASO titers compared by Mean Fluorescence Index and by commercial tests. Sera from ARF subjects who had ASO values from both a commercial test and MFIA were included in an X-Y scatter plot. The best fit line yielded a R^2 of 0.64 with a $p < 0.001$ and a correlation coefficient of 0.8.

both methods (Figure 2b) and by MFIA (Figure 2c) in 7 of 9 subjects. The majority of subjects had a decrease in ASO, while 1 subject did not show a significant change, and 1 subject had a slight increase in ASO by both methods. The values that were acquired through MFIA and commercially through Clinical Laboratories of Hawai'i were plotted in Figure 3. The correlation between ASO titer from MFIA significantly correlated with commercial methods ($r^2 = 0.64$, $p < 0.001$). Most of the scatter shown in Figure 3 is above a value of 800 IU/L, and data revealed a better correlation for values below 800 IU/L ($r^2 = 0.78$, $p < 0.001$, data not shown). There appeared to be higher scatter at higher levels of ASO. Indirect agglutination test with three samples of ARF subject serum in 2% washed O+ RBCs did not show agglutination or antibody to RBCs when observed under light microscope. These three samples were chosen as they were the subjects with the lowest hemoglobin levels and an adequate amount of serum was available for testing. We found that four subjects had both anemia and liver dysfunction, and three of the four had carditis and arthritis as major manifestations, and one with arthritis only. This finding supported the idea that this clinical phenomenon may be related to the severity of the inflammatory reaction in these individuals.

Discussion

The incidence of anemia and liver dysfunction is significant in our patient population. The varied onset of liver dysfunction in ARF subjects emphasizes the importance of monitoring liver enzymes throughout the course of the disease, beginning at first presentation.

We recommend checking liver enzymes upon diagnosis of ARF and if ASA is given, then repeating the test within 2 days of starting treatment, and weekly thereafter until discontinued. Our group recommends the use of naproxen in lieu of ASA due to the observed adverse effects of ASA on liver function. Previous studies have attributed the presence of elevated liver enzymes in ARF subjects to treatment with ASA and concurrent viral hepatitis [2,5-7,27]. Russell et al., [8] suggested that a prolonged exposure (2 weeks) to the drug was necessary to induce liver damage in rabbits. In our study, the subjects that received ASA developed elevated liver enzymes after a mean value of 9.8 days with ASA treatment. There was no known viral hepatitis or previous alcohol ingestion in any of these subjects. Two subjects did not receive ASA treatment at all, but presented with elevated liver enzymes. The etiology of this liver damage includes several possible pathologies. A previous study implied that there may be an autoimmune process against the liver that is similar to the heart for ARF subjects, however this has not been formally proven. ALT is a more specific indicator of liver injury and was higher than AST in all subjects except two [9,28]. AST/ALT ratio can be helpful as a diagnostic tool, with a ratio above 1 usually corresponding to alcoholic liver disease, and below 1 suggesting acute hepatocellular injury [28]. This ratio was found to be below or just above 1 in all of our subjects, with a mean of 0.78. Although ASA is commonly used to treat ARF, it may not be appropriate treatment for all subjects, as patients with mildly elevated enzymes were shown to worsen with this therapy. Hashkes et al., concluded that naproxen is a safer alternative to ASA for the treatment of arthritis in ARF. Current recommendations for the treatment of arthritis and carditis in ARF endorse ASA at doses of 80 mg/kg/day to 100 mg/kg/day in children. However, the basis for this recommendation is from 1955, therefore the guidelines are in need of updating. Also, with the correlation between ASA use in children and Reye's syndrome, it can be argued that ASA in high doses should not be the mainstay of treatment for ARF. The dose of ASA recommended is a point of contention as the patient population with ARF in Hawai'i tends to be larger, leading to doses up to the recommended maximum 4 grams, which may increase the risk for liver toxicity. We have discussed with our colleagues in the Pacific Islands a case of a child who had fatal Reye's syndrome after being treated with 100 mg/kg/day of ASA for rheumatic fever (personal communication). Therefore, ASA use in rheumatic fever may be more dangerous than previously reported. Our group reported 42% of our ARF subjects with anemia during the acute stage of the disease. This incidence is within the range of previous publications, which report incidences of 26% to 62% [17,18]. However, previous studies did not report on severity or duration of anemia, nor did they attempt to elucidate the mechanism of anemia. In our study, the anemia was transient and improved without specific treatment. Seventy-eight percent of anemic subjects showed a decrease in ASO titers while hemoglobin increased (Figure 2a,b and c). This trend is seen in both ASO and hemoglobin levels while subjects clinically improved, and the hemoglobin increased in the absence of treatment aimed at improving their anemia. Only two subjects did not follow this inverse relationship of decreased ASO to increasing hemoglobin, however the time between sera tests was quite short and only a very small increase occurred between ASO titers. Our data supports the utility of the multiplex assay as a reliable alternative to commercial laboratory methods, depicted in the longitudinal correlation of ASO obtained commercially vs. MFIA in Figure 3. The benefits of utilizing MFIA in detecting Anti-Strepto-Lysine antibodies include high-content data output with a 1000th the sample size used in

current commercial methods. This may make this assay more useful in developing countries as only a capillary tube of blood is needed for the assay. Anemia has been observed in ARF in previous studies, but never definitively characterized. Aplastic anemia, hemolytic anemia, diminished erythropoiesis and a rise in plasma volume have all been attributed to the reduction in hemoglobin seen during the acute phase of ARF [13,15,16]. In our study, the anemia observed was only present in the acute phase of the disease and resolved without treatment. As 76% of anemic subjects were found to have carditis as a major manifestation, it is possible that the anemia in ARF is related to inflammation. The cause of the anemia may be related to the body's own inflammatory protein response to GAS infection, such as TNF- α , which could also play a role in decreasing erythropoiesis [29-32]. Drug-induced hemolysis was less likely because the subjects' anemia corrected despite them receiving penicillin prophylaxis. Our study did not find evidence of autoimmune hemolytic anemia in a limited number of our subjects, using the indirect agglutination test. Since only previously frozen serum was available, RBCs could not be used for a direct Coombs test. Total bilirubin levels were normal in all anemic subjects, which also provides support that the anemia is most likely not hemolytic in origin (data not shown). In this study, we found an increased incidence of anemia and liver dysfunction in ARF. The elevated liver enzymes worsening in ARF with ASA, highlights the importance of regular monitoring of these tests during treatment or supports using other NSAID's in the treatment of ARF. As a group we have found good outcomes with the use of naproxen, similar to Hashkes et al., stated that the anemia improved with time, and correlated with clinical improvement and a drop in ASO titers, which provides evidence that the anemia may be linked to the disease process. This study supports evaluating and following blood counts and liver enzymes in the work up and treatment of ARF.

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