Detection of Anti-Neutrophil Antibodies in Autoimmune Neutropenia of Infancy: A Multicenter Study

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ABSTRACT: Background: Autoimmune neutropenia of infancy is caused by neutrophil-specific autoantibodies. Primary AIN is characterized by neutrophil count < 500 ml and a benign self-limiting course. Detecting specific antibodies against the polymorphic human neutrophil antigen usually confirms the diagnosis. Current available tests, however, are expensive and inapplicable in many laboratories as they require the use of isolated and fixed granulocytes obtained from donors pretyped for their distinct HNA alloform. Objectives: To assess the performance of a modified test to identify by FACS-analysis granulocyte-specific antibodies in the sera of neutropenic children. Methods: We evaluated 120 children with a clinical suspicion of AIN, whose sera were analyzed by flow cytometry for the presence of autoantibodies using the indirect granulocyte immunofluorescence test. In contrast to the traditional tests, the sera were tested against randomly selected untyped neutrophils derived from a batch of 10 anonymous healthy subjects, presumably including the common HNA alloforms. Control sera samples were from patients with chemotherapy-induced, familial or congenital neutropenias. To further assure the quality of the new test, we retested six samples previously tested by the gold standard method. All medical files were screened and clinical outcomes were recorded. Results: Our method showed specificity of 85%, sensitivity of 62.5%, and a positive predictive value of 91.8%, values quite similar to those obtained by more traditional methods. Conclusions: The new method showed high specificity for detection of anti-neutrophil antibodies in the appropriate clinical setting and could be an effective tool for clinical decision making.

KEY WORDS: autoimmune neutropenia, anti-neutrophil antibodies, human neutrophil antigen, indirect granulocyte immunofluorescence test

T he causes of neutropenias in infancy and childhood are traditionally classified as a) severe chronic, which includes congenital syndromes; b) cyclical and idiopathic neutropenias; c) acquired or secondary, resulting from drugs, iatrogenic insults (e.g., radiation), or infections; d) primary hematological disorders; and e) immunological diseases, such as autoimmune neutropenia [1]. Primary autoimmune neutropenia results from peripheral destruction of granulocytes by specific anti-neutrophil autoantibodies in the patient’s blood. The origin of these antibodies remains unknown; it is widely accepted, however, that the mechanism involves molecular mimicry of microbial antigens. It has also been hypothesized that the antibodies are formed as a result of modification of endogenous antigens due to drug exposure, abnormal human leukocyte antigen expression, or loss of suppressor activity against lymphocyte clones that are self-reactive [2]. AIN is most often seen in children, with greater prevalence among females (female to male ratio 6:4). Its precise prevalence is unknown, and is usually cited as 1:100,000 in children from infancy to 10 years of age [3]. The prevalence may be higher, however, as many cases remain undiagnosed due to the benign course of the disease [4]. The disease is most prevalent among infants aged 5–15 months and is usually characterized by severe neutropenia with an absolute neutrophil count < 500 ml and only minor intercurrent infections. The remission is spontaneous and resolution occurs in 95% of patients within 7–24 months [4]. When the clinical suspicion cannot establish the diagnosis, detection of specific anti-neutrophil autoantibodies can verify it, obviating the need for bone marrow aspiration and biopsy that are sometimes indicated to rule out chronic neutropenias of different etiologies [2]. Many methods to detect granulocyte-specific autoantibodies exist, but accurate information regarding their sensitivity and specificity is lacking [5-14]. Nevertheless, AIN is more often diagnosed today as the credibility and accessibility of these tests are gradually improving [15].
The methods in use today include the granulocyte direct immunofluorescence test, the granulocyte indirect immunofluorescence test, the granulocyte agglutination test, the enzyme-linked immunosorbent assay, and monoclonal antibody-specific immobilization of granulocyte antigens [16]. In the indirect immunofluorescence test, the patient’s serum is incubated with normal neutrophils and the autoantibodies are later detected using fluorophore-conjugated secondary anti-human immunoglobulin antibodies. As summarized at the Second International Granulocyte Serology Workshop, the combination of several diagnostic techniques for granulocyte-specific autoantibodies detection, and especially the GIFT and GAT, is most beneficial today for the diagnosis of AIN [15,17].

In all the aforementioned methods the detection of autoantibodies is complex, because the antibodies are present in low titers and bind to their target antigens with low avidity. Sometimes it is necessary to test a sample time and again before the presence of the autoantibodies in the serum can be reliably detected [2,8,18].

The specificity of the antibodies to their target neutrophil antigens has been extensively investigated. We know today that in most patients antibodies to the polymorphic human neutrophil antigen system can be found on the Fcy receptor type IIIb (FcRIIIb, CD16b) expressed only on neutrophils [6]. The gene frequency found in the white population is ~33% for NA1 and ~66% for NA2 [19]. The methods currently available for the detection of autoantibodies necessitate purified and fixed granulocytes with a known HNA alloform as typed by anti-NA1 or NA2-specific monoclonal antibodies. Such techniques are both expensive and inapplicable in many laboratories. Since previous trials in Israel to establish a credible and reliable method to detect antibodies using GIFT/fluorescence-activated cell sorting-based methods failed, blood samples are still being sent to a laboratory in Germany, where they are tested using the combination of the GIFT and GAT, at considerable expense. A more efficient, cost-beneficial method is therefore needed.

Since the clinical significance of identifying the specific target antigen has not been proven, we used a modified GIFT method to detect the presence of autoantibodies. Thus, tested sera were reacted against untyped neutrophils obtained by premixing 10 blood samples from randomly selected anonymous healthy subjects. Our working hypothesis was that by using a donor mix the cells would very likely contain neutrophils expressing both HNA1 and HNA2 alloforms. In this study we evaluated 120 children with a clinical picture suggestive of AIN and show that with respect to its performance our test was comparable to more traditional methods to detect anti-neutrophil antibodies.

**PATIENTS AND METHODS**

In this multicenter cohort study conducted during the period 2006–2009, we evaluated 120 patients ranging in age from a few days old to 10 years. These patients presented to the emergency room or to the physician’s clinic with signs of infection and were later diagnosed as neutropenic (ANC < 1500/µl), or were asymptomatic and diagnosed as neutropenic during a routine blood count. In all cases a high clinical suspicion of AIN was the rule.

A blood sample for the anti-neutrophil antibodies test was obtained together with the initial laboratory evaluation for children presenting with fever and/or neutropenia; therefore, no venepuncture was needed. Serum was separated from 1–2 ml of blood and immediately frozen at -20°C. The Institutional Ethics Committee at the Sheba Medical Center approved the study.

Patients with ANC > 1500/µl at presentation, age older than 10 years, a probable alternative clinical diagnosis, and patients who were lost to follow-up or had inadequate data were excluded.

Twenty-seven sera samples from patients with chemotherapy-induced, familial or congenital neutropenias were gathered and used as controls. In the latter patients no antibodies were expected, and hence positive antibody results were defined as false positive.

In order to verify the results and reassure the quality of our method, we reassessed six samples, previously tested in Germany using a combination of the direct GIFT and GAT methods. All medical files were later screened and clinical outcome recorded.

**STATISTICAL ANALYSIS**

We used the efficient-score method (corrected for continuity), described by Robert Newcombe, for the calculation of our test performance and 95% confidence interval. We used Pearson’s chi-square to evaluate the ANC and the time to recovery from neutropenia in patients with different antibody test results. P values < 0.05 were considered significant.

**DETECTION OF ANTI-NEUTROPHIL ANTIBODIES USING THE INDIRECT GIFT METHOD**

Sera samples were collected and kept frozen at -20°C until analysis. Samples were analyzed in batches of 5–10 samples at the core FACS laboratory at Sheba Medical Center, for immunoglobulin G and/or M-type anti-neutrophil antibod-

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GIFT = granulocyte direct immunofluorescence test  
GAT = granulocyte agglutination test  
NA = neutrophil antigen  
GIFT = granulocyte indirect immunofluorescence test  
ANC = absolute neutrophil count  
FACS = fluorescence-activated cell sorter
ies by the indirect GIFT method [20]. A mixture of fresh anticoagulated blood samples obtained from 10 healthy subjects (taken as part of a routine medical checkup) was used as a source for neutrophils. The rationale for using multiple donors was to obtain a good representation of the two common HNA alloforms 1 and 2 [9]. The mixed blood samples were treated with ammonium chloride-based red blood cell-lysing reagent (Lyse™; BD Pharmingen, San Diego, CA, USA). This treatment results in good light scatter separation of all white blood cell populations from RBC debris when analyzed by FACS. The cells were thoroughly washed, resuspended in BD Pharmingen Stain Buffer and incubated at room temperature with the patients’ sera for 30 minutes, washed twice and incubated for 15 minutes with FITC-conjugated anti-human Ig and IgM rabbit F(ab')2 antibody-fragments (Dako, Glostrup, Denmark).

Binding of antibodies to the neutrophils was analyzed by a FACS Calibur Instrument (BD Biosciences, San Jose, CA). The neutrophil population was identified by its distinct forward and side scatter parameters. A sample was defined as positive, as compared to a sample treated with control serum from a healthy subject, when > 30% of the neutrophils were positive and/or the mean fluorescence intensity of the whole population was increased by > 30%. Control samples from children without neutropenia were also sent, at random, to the laboratory together with the neutropenic patients’ sera [Figure 1]. It should be noted that the laboratory staff who performed or interpreted the tests was blinded to the diagnosis at the time of testing.

RESULTS
Table 1 summarizes the demographic data, the clinical characteristics at presentation and the antibody test results of all patients clinically suspected of having AIN and included in our study (n=72). Forty-eight patients did not fully meet our inclusion criteria. No statistically significant differences were found between the group with positive versus negative antibody results in terms of age at presentation, gender, time to recovery and absolute neutrophil count, both at presentation and at the time of the test. Positive antibodies were detected in 34 cases (47.2%), weakly positive in 11 (15.3%), and absent in 27 (37.5%). Altogether, antibodies were detected in 62.5% of the cases.

**Figure 1.** FACS analysis by indirect GIFT method. [A] A mixture of fresh anticoagulated blood samples from 10 healthy subjects was treated with ammonium chloride-based RBC-lysing reagent to allow good light scatter separation of leukocytes from RBC debris by FACS analysis. The granulocytes were identified by their distinct forward and side scatter parameters (rectangular gate, marked by G). The arrows marked by L and M identify the lymphocyte and monocyte populations, respectively. [B] The multidonor granulocytes were then incubated with the different patients’ sera or normal serum. The various samples were then washed twice and tested for anti-neutrophil autoantibodies by incubation with FITC-conjugated anti-human IgG and IgM rabbit F(ab')2 fragments. Binding of antibodies to the neutrophils was analyzed by a FACS Calibur Instrument and depicted as overlay histograms for the granulocyte-gate only (normal serum in gray and patient’s serum represented by a thick black line). Color-coded numbers represent the mean fluorescence intensity of FITC-conjugated F(ab')2 staining. The serum of patient #2 tested negative, whereas patient #1 tested positive against almost all the granulocytes. The serum of patient #4 reacted with ~40% of granulocytes and the mean fluorescence intensity of F(ab')2 binding was increased 1.6-fold compared to normal serum F(ab')2 and therefore was also considered positive.

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**RBC = red blood cells**

**Ig = immunoglobulin**
As mentioned earlier, we reassessed six samples, previously tested in a reference laboratory (Institute for clinical immunology and transfusion medicine, Giessen and Marburg GMBH University Medical Centre, Giessen, Germany) using a combination of the direct GIFT and GAT methods. One sample was positive in both labs. Two samples were negative in our lab, yet positive in the reference laboratory. Therefore, if we use the results of the reference laboratory as the “gold standard test” and compare our results with theirs, the calculated specificity of the test is 100% and its sensitivity 25%. Naturally, the small number of tests that were compared makes the comparison statistically insignificant. Nevertheless, the data emphasize our test’s high specificity and positive predictive value, and imply that repeating the test a few times, as done in the reference lab, should improve its sensitivity and negative predictive value.

Regarding the clinical course of the patients, granulocyte colony-stimulating factor was administered to 9 patients (12.5%), and all responded within a week. Ten patients (13.9%) had undergone bone marrow aspiration and biopsy as part of their workup. Sixty-two patients (86.1%) did not require these procedures, and there was no statistically significant difference in the number of procedures between the groups with positive versus negative antibody results. The examination of bone marrow biopsies showed that in 50% (n=5) the bone marrow was normal, in 30% (n=3) it was reactive – which is compatible with the diagnosis of post-viral or immune neutropenia, and in 20% (n=2) there was a decrease in the myeloid lineage. One bone marrow sample, initiated to exclude a possible diagnosis of Kostman’s syndrome in a patient with suspected AIN, showed no meta-myelocyte arrest (thereby excluding this diagnosis).

As mentioned before, patients with ANC > 1500/µl at presentation were excluded from our study. Patients who had higher counts at the time the antibody detection test was done were, however, included. Because disappearance of the antibodies may precede the normalization of the neutrophil count [21], we used a 1500/µl cutoff of ANC. We found no statistically significant differences in antibody test results between the group with neutropenic count and the group with normal values at the time of the test (P = 0.359).

Since AIN is a chronic condition by definition, we analyzed the difference between the group of patients with proven neutropenia for longer than 6 weeks and those with a shorter neutropenic course. Sixty percent of the patients (n=18) who recovered within 6 weeks tested positive, and 40% (n=12) tested negative, as compared to 73.9% (n=17) and 26.1% (n=6) respectively in patients with a prolonged (>6 weeks) neutropenic course. These differences were, however, statistically insignificant (P = 0.384).

Table 2 shows the intercurrent infections throughout the neutropenic period in all the patients. Forty-six percent of the patients (n=33) had no intercurrent infections and in 18% (n=13) fever was the only symptom. Fourteen percent (n=10) had mild upper respiratory tract infection, and the rest had mild infections [Table 2]. Nevertheless, it is important to emphasize that a probable clinical diagnosis of AIN was defined by inclusion criteria. Although AIN patients with a severe infection [4] were included, such a clinical course raises the possibility that the chronic neutropenias actually had a different etiology. Thus, the inclusion of such patients might have

| Table 1. Patients clinically suspected of having AIN (n=72): general characteristics |
|------------------|----------------|
| Male to female ratio | 5:3 |
| Mean age at presentation (months, mean ± SD, range) | 10.2 ± 8.3 (0–42) |
| Time to recovery (mos, mean ± SD)* | 4.4 ± 5.7 (0.5–30) |
| ANC (1/µl) at presentation (mean ± SD, range) | 467.4 ± 279.1 (0–1100) |
| ANC (1/µl) at time of test (mean ± SD, range) | 811.9 ± 633.2 (50–3080) |
| Positive antibody result | 45 (62.5%) |
| Negative antibody result | 27 (37.5%) |

* Data regarding time to recovery were lacking in 19 patients, and were therefore calculated for 53 patients.

** Other: anemia follow-up, failure to thrive, jaundice, planned surgical procedure (n=2 each); gastrointestinal complaints, rash, acute life-threatening event, chronic fatigue, vomiting, ecchymosis, premature rupture of membranes and urinary tract infection in a mother (n=1 each).

ANC = absolute neutrophil count, URTI = upper respiratory tract infection

A result was defined as true positive when a positive antibody result was compatible with the clinical diagnosis of AIN (n=45). It was defined as false positive when antibodies were unexpectedly detected in a control sample (n=4). Therefore, the calculated specificity of the test was 85% (95% confidence interval 65–95%) and its sensitivity was 62.5% (95% confidence interval 50–73%). The positive predictive value of the test was therefore 91.8% (95% confidence interval 79–97%).

As mentioned earlier, we reassessed six samples, previously tested in a reference laboratory (Institute for clinical immunology and transfusion medicine, Giessen and Marburg GMBH University Medical Centre, Giessen, Germany) using a combination of the direct GIFT and GAT methods. One sample was positive in both labs. Two samples were negative in both labs. Three samples were negative in our lab, yet positive in the reference laboratory but only by one of the methods used. None of the sera that tested positive in our lab was negative in the reference laboratory. Therefore, if we use the results of the reference laboratory as the “gold standard test”
introduced a selection bias whereby cases with more severe infections were not appropriately represented in our study.

**DISCUSSION**

In the present research we demonstrated that the clinical diagnosis of AIN can be further supported using a simple modification of the already well-known GIIFT method, with high specificity and positive predictive value. We have shown that in accordance to our working hypothesis, the mixture of untyped neutrophils from a batch of donors provides a good representation of the common HNA alloforms. Thus, while its sensitivity is comparable to that of the more traditional methods for antibodies detection, it is more accessible and simple to implement, obviating the need to obtain granulocytes from pretyped donors for their distinct HNA alloform.

Our results support the existing data regarding the benign self-limiting course of the disease, as 64% of our patients did not show any infection during the neutropenic period or had fever only [Table 2], and the maximum time to recovery was 30 months [Table 1], in agreement with the literature [4]. Ten of our patients (13.9%), however, had undergone bone marrow aspiration or biopsy to rule out chronic neutropenias of a different etiology. It is important to note that the destruction of segmental neutrophils and bands in an immune mechanism may imitate arrest of maturation in the myelopoiesis at the myelocyte/meta-myelocyte level. In addition, 3% of patients with AIN present with hypocellular bone marrow due to antibodies to the more ‘primitive’ hematopoietic cells [22]. We show that in none of our cases did this invasive procedure contribute to the clinical decision making in a manner that justified it, since 50% had a normal marrow and the rest had an inconclusive test result or a result that supported the initial suspicion of AIN. Therefore, a positive autoantibody test result, given its high specificity, prior to the biopsy could have made it redundant in many of these cases by further reassuring the clinician. Hence, it seems logical to postpone bone marrow biopsy until after the antibody test is done.

Although this notion is not new, the test is not routinely done in Israel due to considerations of cost versus benefit.

The sensitivity of our test in this study was, however, limited by several factors: the inclusion of patients with a normal neutrophil count at the time of testing or without prolonged neutropenia that might also fit the diagnosis of post-viral neutropenia; the previously described low avidity and titer of anti-HNA autoantibodies that require repeated testing for detection and was not done in this study; and the theoretical possibility that some patients produced autoantibodies against less prevalent alloantigens not represented in the particular sample of multidonor-derived granulocytes that the patients’ serum was tested against. The latter includes the possible existence of autoantibodies to early myeloid precursors in the sera of patients with suspected immune neutropenia as described by Hartman et al. [22], and the function of antibody-independent immunological effector pathways, as recently reviewed by Ehl et al. [23].

As for the specificity of our test, the analysis of the 4 false positive results from among the 27 negative control samples raises a few possible explanations. One of those patients was eventually diagnosed with a proliferative autoimmune syndrome, a condition in which autoantibodies were found [24]. One was a neonate who presented with a clinical picture of sepsis and necrotic lesions on his buttocks and was therefore suspected to have a severe chronic neutropenia, most probably Kostman syndrome – a diagnosis that was further supported by the infant’s bone marrow biopsy, demonstrating maturation arrest at the promyelocyte level. This patient, however, recovered fully and his neutrophil count normalized, indicating that AIN with a severe clinical course could have been the correct diagnosis. The third patient did not receive a conclusive diagnosis and was thought to have idiopathic chronic neutropenia lasting for more than 5 years. The fourth patient had neutropenia induced by chemotherapy. The true false positive result in the two latter cases might have indeed resulted from the formation of immune complexes, as previously described with the GIIFT method [4].

Many questions have yet to be answered regarding the diagnosis of AIN by our modified test. We cannot predict how the detection of anti-neutrophil antibodies will change the daily clinical management in children with AIN, and how many bone marrow biopsies will actually be spared. Further investigation is also needed to determine how many repetitions of the test are necessary to improve its sensitivity. Yet another important question relates to the application of our test to the diagnosis of adults with AIN. In most cases, AIN in adults is secondary by nature and is associated with lymphoproliferative autoimmune disorders, infections, immunodeficiencies, and/or drugs. It occurs predominantly in women and is sometimes associated with anemia and thrombocytopenia of an autoimmune nature. The clinical course is usually be-

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**Table 2. Patients clinically suspected of having AIN (n=72): intercurrent infections thought the neutropenic period**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number (%)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>33 (46%)</td>
</tr>
<tr>
<td>Fever only</td>
<td>13 (18%)</td>
</tr>
<tr>
<td>Upper respiratory tract infection</td>
<td>10 (14%)</td>
</tr>
<tr>
<td>Aphthostomatitis</td>
<td>4 (6%)</td>
</tr>
<tr>
<td>Acute otitis media</td>
<td>3 (4%)</td>
</tr>
<tr>
<td>Rash</td>
<td>3 (4%)</td>
</tr>
<tr>
<td>Gastrointestinal symptoms</td>
<td>3 (4%)</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>2 (3%)</td>
</tr>
<tr>
<td>Cellulitis</td>
<td>1 (1%)</td>
</tr>
</tbody>
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nign, and spontaneous remission is rare [2]. Autoantibodies do not constitute the main pathogenic mechanism, and only 35% of patients are positive for anti-neutrophil autoantibodies by traditional methods [25]. In most cases of adult-onset secondary AIN, the target of these autoantibodies remains unknown. The different antibody-independent contributing factors include peripheral sequestration, inhibition of bone marrow turnover, and increased apoptosis of neutrophils and/or their precursors that are mostly mediated by pro-inflammatory cytokines [2]. Due to the more complex, multifactorial pathogenesis of secondary AIN, detection of autoimmune antibodies is less likely to help establish a diagnosis. A clinical trial will be needed to evaluate the test’s true performance in adults with clinical suspicion of AIN.

Acknowledgments:
The following Israeli medical centers participated in the study: Safra Children’s Hospital, Sheba Medical Center (Tel Hashomer); Schneider Children’s Medical Center (Petah Tikva); Dana Children’s Hospital, Tel Aviv Sourasky Medical Center (Tel Aviv); Children’s Hospital, Rambam Medical Center (Haifa); Carmel Medical Center (Haifa); Soroka Medical Center (Beer Sheva); Assaf Haroéf Medical Center (Zerifin); Barzilai Medical Center (Ashkelon); and Shaare Zedek Medical Center (Jerusalem). The authors would like to thank all the pediatric hemato-oncologists who participated in the data collection, and Dr. E. Shabad, Head of the Blood Bank, Department of Hematology, Carmel Medical Center, Haifa. We also thank Judith Tsimir for her help in the statistical analysis.

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