

CASE SERIES

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Identification of Antibodies against Neutrophil Surface Antigens in Two Iranian Patients with Autoimmune Neutropenia

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ABSTRACT

Autoimmune neutropenia is a type of immune-mediated neutropenia, caused by antibody-induced neutrophil destruction. Here, we report two cases (a 3-year-old boy and a 9-year-old girl) with suspected autoimmune neutropenia. The presence of neutrophil antibodies in the sera of these two patients was investigated; using standard neutrophil antibody screening tests such as granulocyte immunofluorescence test (GIFT), granulocyte agglutination test (GAT), and lymphocyte immunofluorescence test (LIFT). A positive reactivity with two-panel cells was found in GIFT. No reactivities with panel cells were observed in GAT and LIFT. To the best of our knowledge, this is the first report for detecting the neutrophil reactive antibodies; using genotyped neutrophils in patients with autoimmune neutropenia in Iran. The final diagnosis of our patients was primary autoimmune neutropenia for the boy and autoimmune neutropenia associated with familial Mediterranean fever for the girl.

Keywords: Agglutination test; Autoimmunity; Indirect immunofluorescence; Neutropenia

INTRODUCTION

In the circulation system, neutrophils form the major part of granulocytes.¹ The low number of circulating neutrophils (known as neutropenia) arise from low neutrophil production in bone marrow or increased neutrophil destruction.² The binding of autoantibodies to neutrophils leads to increased neutrophil elimination and consequently neutropenia.³

Since the treatment strategies for neutropenia are based on the etiology, it is important to distinguish the autoantibody-mediated neutropenia (AIN) from other neutropenia-inducing reasons to acquire efficient therapeutic protocols.⁴ In the reference laboratories, granulocyte immunofluorescence test (GIFT) is used to evaluate the reactive autoantibodies against a panel of human neutrophil antigen (HNA)-genotyped neutrophils in patients suspected of having neutropenia.⁵ The bound autoantibodies on neutrophils are then detected; using fluorescence-labeled anti-human antibodies. The fluorescence intensity of patient's serum is evaluated under fluorescence microscopy. In parallel, the serum of healthy

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individuals and the standard serum-containing neutrophil reactive alloantibodies are investigated as negative and positive controls, respectively. The agglutination of neutrophils after binding with patient's serum is known as granulocyte agglutination test (GAT) and is the second standard laboratory technique for detection of neutrophil-reactive antibodies.⁶ To confirm the autoimmune neutropenia, GIFT and GAT are applied in combination as the standard protocol.⁷

To exclude neutrophil-specific antibodies from non-specific antibodies, a lymphocyte immunofluorescence test (LIFT) is performed; using the lymphocytes of donors. Immunology, Asthma and Allergy Research Institute (IAARI) affiliated with Tehran University of Medical Sciences, is a referral center for patients with neutropenia. Here, for the first time in Iran, we have diagnosed two cases with autoimmune neutropenia. One of our patients was diagnosed with primary autoimmune neutropenia and the second one was diagnosed with autoimmune neutropenia associated with familial Mediterranean fever (FMF).

CASE PRESENTATION

Two cases with suspected autoimmune neutropenia were referred to IAARI. This study was approved by the Ethics Committee of IAARI, "IR.TUMS.IAARI.REC.1397.006". Informed consent was obtained from the patients' parents for blood sampling and using patients' data. Bone marrow (BM) evaluation was normal in both cases. Immunologic evaluation including lymphocyte subsets, serum immunoglobulins levels, and nitroblue tetrazolium test (NBT) showed no abnormalities. To investigate the presence of the neutrophil auto-antibodies and diagnose autoimmune neutropenia, 2 mL of blood was drawn from each patient and saved in -70°C .

Serum samples were tested for granulocyte antibodies; using GIFT, GAT, and LIFT assays. Briefly, neutrophils from two HNA-typed healthy donors were isolated from donors' whole blood samples by dextran sedimentation. Neutrophils were separated from the peripheral mononuclear cells (PBMCs) by Ficoll-Hypaque density gradient. Red blood cells (RBCs) were depleted by lysis buffer. Cells were fixed by 4% paraformaldehyde (PFA) solution for GIFT and LIFT assays. For GIFT, neutrophils were incubated for 30 min at 37°C with the patients' serums.

Afterward, the cells were washed and incubated with FITC-labeled rabbit anti-human IgG and then analyzed in a fluorescence microscope. For GAT, isolated neutrophils were incubated with patients' serums in Terasaki plate for 2 h (37°C) and 12 h (30°C), and neutrophil agglutination was then evaluated by inverted microscope. Lymphocytes from whole blood were isolated and a LIFT assay was performed similarly to the GIFT assay with a difference that lymphocytes were assessed; using flow cytometry. Samples were then analyzed by Flow cytometry, (BD FACSCantoII). Positive and negative controls were verified in the reference granulocyte laboratory at the University Hospital of Giessen and Marburg (UKGM) in Giessen (Germany). The medical history of patients and the results of the laboratory investigations are described below.

First Case

A 3-year-old boy was referred to the IAARI for severe persistent neutropenia since he was nineteen months old. His neutrophil count was $140\text{-}534/\mu\text{L}$. White blood cells (WBC) and lymphocytes count were within normal limits. At the age of 8 months old, he had a history of hospitalization for pneumonia and otitis media; while his blood examination was within the normal range (WBC count: $11200/\mu\text{L}$, lymphocytes: 57%, and neutrophils: 40%, monocytes: 2%). During the last year, he has experienced recurrent episodes of low-grade fever and mouth aphthous ulcers. He had mild iron deficiency anemia. Neither thrombocytopenia nor hepatosplenomegaly was noted in the patient's history. There was no evidence of myeloid maturation arrest in BM analysis. Immunological screenings including assessment of the serum levels of immunoglobulins complement, and lymphocyte subsets were within normal limits. His consanguineous parents and older sibling were healthy. Because no reason was found to explain the neutropenia in this case, his serum sample was evaluated for neutrophil auto-antibodies. The results of the GIFT assay revealed neutrophil antibodies against CD16b in variant HNA1a. LIFT and GAT tests were negative, Figure 1.

At the last visit (43-months-old), he had no mouth aphthous ulcers. He had one episode of gastroenteritis that did not require hospitalization. His WBC count was 7260 (neutrophils: 9%, lymphocyte: 71% and monocytes: 11%). Because of no recurrent infection,

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Granulocyte colony-stimulating factor (G-CSF) was not prescribed.

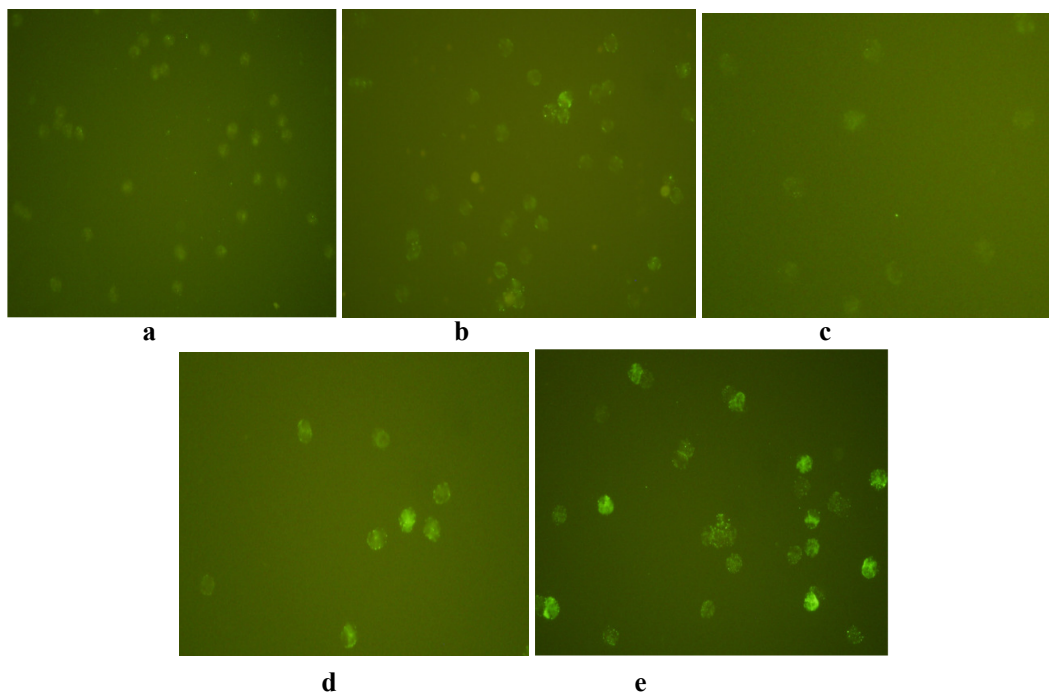
Second Case

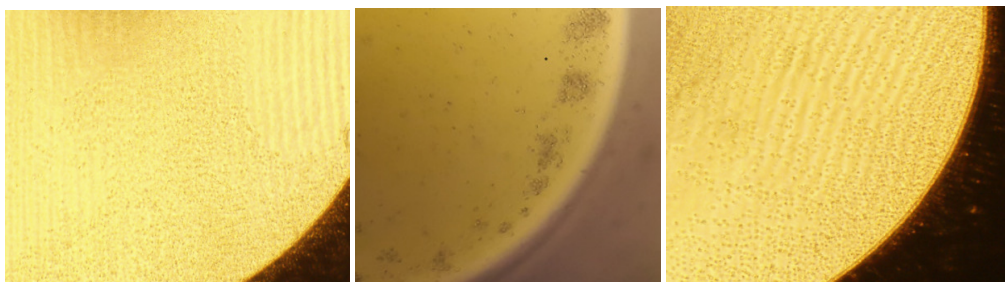
A 9-year-old girl with a history of chronic idiopathic neutropenia was referred to IAARI for evaluation of the possible immune deficiency disorder. Her parents were second cousins and she has two healthy siblings. Until 2 years of age, she was healthy and well developed. Then, teeth decay and gingival bleeding were noticed. Due to the poor appetite and recurrent mucosal bleeding and superficial bruising in the trunk and limbs, haematologic evaluation was requested. The results revealed mild hypochromic, microcytic anemia, and thrombocytopenia. Neither organomegaly nor lymphadenopathy was found. Prednisolone (Disopred, Iran Hormone Pharmaceutical Co, 5 mg Tab) and ferrous sulfate supplementation were administered and her thrombocytopenia and anemia improved (platelets count began to increase to more than $150 \times 10^3/\mu\text{L}$). She was well but after two years, at the age of 4 till 7 years old, she was frequently hospitalized for recurrent episodes of fever, loss of appetite, protracted vomiting, abdominal pain, and non-bloody diarrhea. Chronic leukopenia and neutropenia (WBC: $2.8\text{-}4.6 \times 10^3/\mu\text{L}$, neutrophils: $288\text{-}1000/\mu\text{L}$, lymphocytes: $1.8\text{-}2.9 \times 10^3/\mu\text{L}$) were developed and

continued for 3 years. BM aspiration was performed and the result showed no abnormalities. Immunophenotyping was normal. Immunological screenings including assessment of the serum levels of immunoglobulins complement, and lymphocyte subsets were within normal limits.

She had chronic severe gingivitis and recurrent episodes of otitis media, pneumonia, and hospitalizations that were almost controlled by prescribing Filgrastim (Pooyesh Darou Biopharmaceuticals Co, 300 μg Amp) at a dose of 3 $\mu\text{g}/\text{kg}$ every second day. Filgrastim injection intervals have been adjusted based on her absolute neutrophil count and injection-induced severe bone pain. Considering her recurrent episodes of fever, abdominal pain, and gastrointestinal complaints, a genetic evaluation was done for FMF that showed E148Q heterozygous mutation in the *MEFV* gene. The genetic study for Fanconi anemia was negative.

The normal result of BM aspiration suggested immune neutropenia and thrombocytopenia. To screen for autoimmunity, anti-neutrophil antibodies were tested. The result of the GIFT assay showed a positive reaction with both HNA-1a and HNA-1b positive cells. However, no reactivity was detected in GAT and LIFT (Figure1).

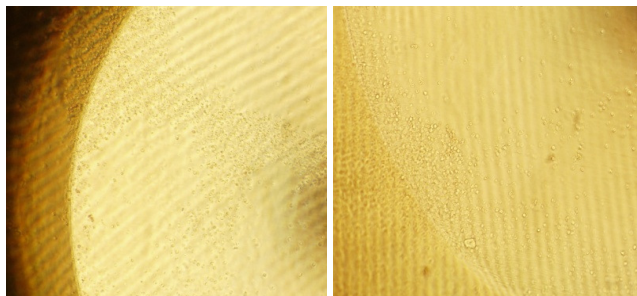




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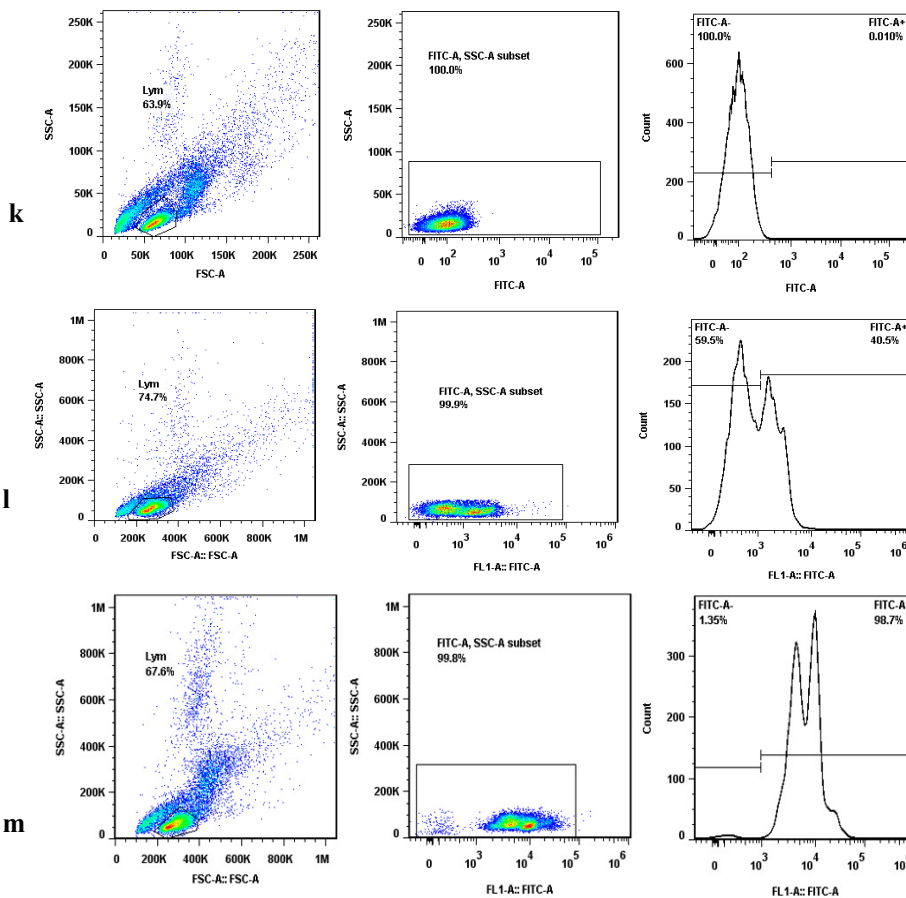
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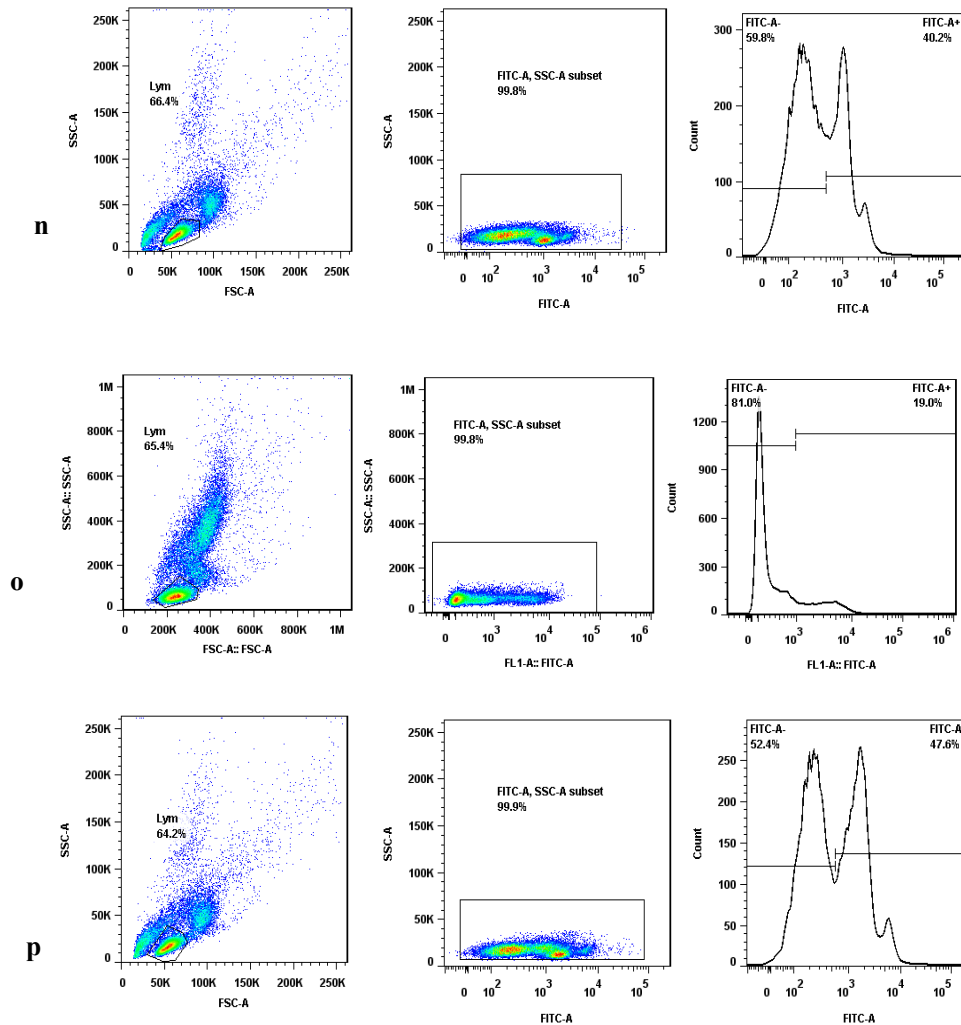


Figure 1. Identification of the neutrophil antibodies in the patients' sera: Neutrophil antibodies in the sera of the patient were evaluated; using granulocyte immunofluorescence test (GIFT), granulocyte agglutination test (GAT), and lymphocyte immunofluorescence test (LIFT) assays. For GIFT, paraformaldehyde-fixed neutrophils were incubated with the serum controls and patients' sera and after washing, the cells were incubated with FITC-labeled rabbit anti-human IgG. The results were evaluated by a fluorescence microscope. a: negative serum control (verified in the reference granulocyte laboratory, UKGM), b: positive serum control (verified in the reference granulocyte laboratory, UKGM) c: human AB serum, d: clear positive result for the first case, e: Strong positive result for the second case. For GAT, unfixed neutrophils were incubated with the sera of controls and patients, and agglutination reaction was evaluated. f: negative control, g: positive control, h: human AB serum, i: negative result for the first case, j: negative result for the second case. For LIFT, paraformaldehyde-fixed lymphocytes were incubated with the sera of controls and patients and after washing, the cells were incubated with FITC-labeled rabbit anti-human IgG. The fluorescence intensity on the surface of lymphocytes was evaluated by Flow cytometry. k-p are representative FACS analyses for detection of the lymphocyte antibodies. Histograms show the percentage of the reactive lymphocyte with FITC-labeled secondary antibody. k: representative FACS plots for unstained cells. l: negative control (Anti-HLA negative control, inno-train), m: positive control (Anti-HLA positive control, inno-train), n: human AB serum, o: negative result for the first case, p: negative result for the second case.

DISCUSSION

HNAs consist of five antigenic systems (HNA1-5) that constitute fourteen HNA alleles.⁸ HNA1 and HNA2 are restricted to the neutrophil surface.⁹ HNA3, 4, and 5 are expressed on the neutrophils as well as other immune cells.⁹ Antibodies against HNAs are involved in multiple pathological conditions.¹⁰

HNAs incompatibilities during pregnancy between mother and child or in a case of transfusion/transplantation between donors and recipients induce alloimmunization and consequent HNAs-alloantibodies production. Autoantibodies reactive with HNAs have been described as the cause for neutropenia.¹¹ The neutrophils' reactive autoantibodies in serum bind to neutrophils and may lead to neutrophil elimination and antibody-mediated neutropenia.² Myelopoiesis arrest in BM is responsible for the remaining cases of neutropenia.¹² Since both conditions are manifested by neutropenia, differentiation between these two neutropenia-inducing mechanisms assists the physician in conducting a proper therapeutic strategy.

Autoimmune-mediated neutropenia occurs both in adults and in children.³ In children, AIN (known as primary autoimmune neutropenia) is normally produced in the second month of life and in most cases lasts for two years.^{13,14} Primary AIN is not associated with other immune-mediated disorders¹² and patients manifest a mild infection in the skin and upper respiratory tract. Severe infection only occurs in 12-20% of patients.^{13,15} Neutropenia in childhood with mild or no infection is in favor of the diagnosis of autoimmune neutropenia. Nevertheless, autoimmune neutropenia in adults occurs with more severe clinical symptoms and a lower probability of spontaneous recovery.¹² However, asymptomatic patients may be seen despite low neutrophil counts.¹⁶ Detection of neutrophil antibodies confirms the diagnosis.^{13,17}

Here, we reported two cases with autoimmune neutropenia. The first case did not take any medications and had no associated disorders. Based on the serologic results and clinical findings, he was diagnosed as primary AIN. For the second case, clinical examination and results of the serologic and genetic tests revealed autoimmune neutropenia secondary to FMF. FMF is an autosomal-recessive, auto-inflammatory disorder. The mutated gene is *MEFV* that codes the pyrin/marenostrin protein. Pyrin is expressed in neutrophils, eosinophils,

and monocytes and has a role in inflammation and response to infection.¹⁸ FMF clinical features comprise recurrent episodes of fever, abdominal pain, lower limb rash, and renal amyloidosis.¹⁹ A previous study has shown an increased rate of spontaneous and induced apoptosis of neutrophils in patients with FMF.²⁰

Auto-antibodies detection against neutrophil antigens are the standard methods to confirm autoimmune neutropenia and should be considered before BM examination.⁴ However, negative results do not rule out the disease.⁷

In the first diagnostic line, GIFT and GAT are performed to evaluate the presence of anti-neutrophil antibodies.⁵ To screen the presence of neutrophil reactive autoantibodies, the International Society of Blood Transfusion (ISBT) committee has considered these two tests as standard tests.²¹

Neutrophil antibodies screening is technically difficult and only a few centers can conduct such tests.²¹ In the Iranian population, the prevalence of neutropenia has been estimated to be as high as 3.1%.²² Since 2007, IAARI is the referral center for patients with neutropenia therefore genetic diagnosis and following-up of patients are performed in IAARI.²³

Since the treatment of referred patients is highly dependent on a proper diagnostic test, our center has recently established optimized diagnostic tests (GIFT, GAT and LIFT) for the detection of neutrophil reactive antibodies in collaboration with the ISBT reference laboratory (Giessen, Germany). As the next step, we plan to establish the monoclonal antibody-specific immobilization of granulocyte antigens (MAIGA) to identify HNA-specificities for GIFT-positive samples.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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