Identification of an Unusual Fcy Receptor IIIa (CD16) on Natural Killer Cells in a Patient With Recurrent Infections

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We found an unusual Fcy receptor IIIa (CD16) phenotype on the natural killer (NK) cells of a 3-year-old boy, who suffered from recurrent viral respiratory tract infections since birth. He also had severe clinical problems after Bacille Calmette-Guérin (BCG) vaccination and following Epstein-Barr virus and Varicella Zoster virus infections. His peripheral blood lymphocytes contained a normal percentage and absolute number of CD3+CD7+ cells, which were positively stained with the CD16 monoclonal antibodies (MoAbs) 3G8 and CLBFCRgran1, but did marginally stain with the CD16 MoAb Leu11c/B73.1. FcyRIIib expression on granulocytes appeared to be normal. NK cell function, analyzed in vitro by direct cytotoxicity on K562 target cells and ADCC-activity on P815 target cells, was normal compared with an age-matched healthy control. Sequence analysis of the FcyRIIia gene, encoding CD16 on NK cells and macrophages, showed a T to A nucleotide substitution at position 230 on both alleles, predicting a leucine (L) to histidine (H) amino acid change at position 48 in the first extracellular Ig-like domain of FcyRIIia, which contains the Leu11c/B73.1 epitope. The combined use of CD16 and CD56 MoAbs labeled with the same fluorescent dye, as often applied in routine immunophenotyping procedures, will leave these homozygotes undiagnosed. The pattern of infections in this patient is in agreement with the postulated function of NK cells in the immunological defense against viruses and other intracellular microorganisms. Further analysis of the NK cell function in vitro and follow-up of the clinical course of FcyRIIia-48H homozygotes is required to ascertain whether this genotype is causally related to an NK cell immunodeficiency.

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NATURAL KILLER (NK) cells are generally defined as large granulocytes that express CD16 and/or CD56 and are negative for pan-T- and B-cell markers. They are able to lyse sensitized target cells in vitro through the mechanism of antibody-dependent cell-mediated cytotoxicity (ADCC) and to kill K562 target cells spontaneously. Their function in vivo is not fully known. NK cells probably play a role in the destruction of tumor cells and in the resistance to infections caused by certain viruses and other intracellular pathogens.1

The Fcy receptor IIIa (CD16) is involved in the triggering of ADCC1,2 and spontaneous cytotoxicity.3,4 Recently, an expression polymorphism of CD16 on NK cells derived from healthy individuals, based on a difference in the number of binding sites for monomeric IgG per NK cell, has been described.5 Until recently, no genetic polymorphism of FcyRIIia had been reported.6

Here, we describe a 3-year-old boy who suffered from recurrent viral respiratory tract infections since birth. He also had severe problems with Bacille Calmette-Guérin (BCG) vaccination, and Epstein-Barr virus and Varicella Zoster virus infections. This clinical pattern might be compatible with an in vivo dysfunction of NK cells. Immunophenotyping of peripheral blood mononuclear cells (PBMC) indicated that NK cells, defined as CD3+CD7+ lymphocytes, are normally present, but that the CD16 molecule expressed by these cells lacks the epitope recognized by the MoAb Leu11c/B73.1. The in vitro NK cell function was normal compared with an age-matched healthy control. Sequence analysis of the FcyRIIia gene showed a T to A nucleotide substitution at position 230 on both alleles, predicting a leucine change at amino acid position 48 in the first extracellular domain of FcyRIIia.

MATERIALS AND METHODS

Patient. The patient was born at term after an uneventful pregnancy and delivery. He was the first-born child of nonconsanguineous parents of Turkish and Dutch-Norwegian descent. Recently, a second son was born in this family. From 3 months of age onward, the patient suffered from recurrent, mainly viral, upper respiratory tract infections. These were accompanied by wheezing and nocturnal dyspnea, which reacted well to inhaled salbutamol and, later on, to inhaled steroids. BCG vaccination at 6 months of age resulted in local abscess formation, fever, and malaise, only cured 1 year later by excision of the abscess and 2 months of isoniazid therapy. At 18 months of age, he developed a prolonged Epstein-Barr virus infection with fever and malaise, which lasted about 10 months. He mounted a normal antibody response to Epstein-Barr virus during this period. At 30 months of age, he had chickenpox, which was progressive during 2 weeks, and was finally cured with acyclovir therapy. Despite all this, his growth and development were normal.

The absolute number and function of his peripheral blood granulocytes, T, and B cells were normal, as were the levels of serum Ig isotypes and specific antibodies following diphtheria toxoid and tetanus toxoid-inactivated poliovirus type I, II, and III (DT-IPV) vaccination. However, the CD3+CD7+ NK cells were not stained by the CD16 MoAb Leu11c/B73.1. NK cell function was tested by spontaneous cytotoxicity of K562 target cells and by ADCC of P815 targets, and further investigation of the FcyRIIia (CD16) expression on the NK cells and of the FcyRIIia gene was undertaken.

Isolation of cells. Fresh anticoagulated blood was diluted 1:2 in phosphate-buffered saline (PBS) and centrifuged over a Percoll gradient with a specific gravity of 1.076 g/mL (Pharmacia, Uppsala, Sweden). Mononuclear cells (PBMC) were harvested from the

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Fig 1. Staining pattern of lymphocytes with CD3, CD7, and CD56 MoAbs. The lymphocyte gated mononuclear cells of the patient contained 9% CD3-CD7+ lymphocytes and 7% CD3-CD56+ lymphocytes, indicating that NK cells are present in a relative number, which is comparable to that of the healthy, unrelated, age-matched control.

MoAbs. The following MoAbs were used: pan-FcγRIII CD16 MoAbs: CLBFcRgran1 (mlgG2a), 3G8 (mlgG1), Leu11a (mlgG1), D1130c (mlgG1), BW209/2 (mlgG2a); NAl-FcγRIIIb plus FcγRIIIα CD16 MoAbs: B73.1 (mlgG1), phycoerythrin (PE)-labeled Leu11c (mlgG1); NA2-FcγRIIIb plus FcγRIIIa CD16 MoAb: GRM1 (mlgG2a); less-glycosylated NAZFcyRIIIb plus FcyRIIIa CD16 MoAb: PEN1 (mlgG2a). The MoAbs Leu11c/B73.1, GRM1, PEN1, and BW209/2 recognize epitopes located at the first extracellular Ig-like domain of CD16, whereas the epitopes recognized by the MoAbs CLBFcRgran1, 3G8, and Leu11a reside at the membrane-proximal Ig-like domain. BW209/2 was a gift from Dr R. Kurrle, Behring Werke, Marburg, Germany. B73.1 was kindly provided by Dr B. Perussia, Thomas Jefferson University, Philadelphia, PA. PE-labeled Leu11c (clone B73.1) was obtained from Becton Dickinson, San Jose, CA. The other CD16 MoAbs were obtained via the Vth Leukocyte Typing Workshop. PE- and fluorescein isothiocyanate (FITC)-labeled Leu4 (CD3; mlgG1) and PE-labeled Leu19 (CD56; mlgG1) were obtained from Becton Dickinson. Biotin-labeled CD7 (mlgG2a), FITC-labeled goat antimouse Ig, and murine control MoAbs of the IgG1 and IgG2a subclass with irrelevant specificities (mlgG1 and mlgG2a, respectively) were from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands.

Flow cytometry. PBMC and neutrophils were incubated with CD16 MoAbs for 25 minutes at room temperature. After washing with PBS containing 0.2% bovine serum albumin (BSA) (wt/vol), the cells were incubated with FITC-labeled goat antimouse Ig for 25 minutes at room temperature. In triple color experiments, the free F(ab')2 parts of FITC-labeled goat antimouse Ig were first blocked with a mixture of control mlgG1 and mlgG2a. Thereafter, PE-labeled Leu4 (CD3) and biotin-labeled CD7 MoAbs were added. Binding of CD7-biotin was detected with Cy-Chrome-labeled streptavidin (Pharmingen, San Diego, CA). Immunofluorescent staining was assessed by flow cytometry (FACScan, Becton Dickinson). A gate was set around the lymphocyte population on the basis of forward and orthogonal light scatter characteristics and staining patterns with the combination of CD45 and CD14 MoAbs.

Cytoxicity assays. Cytolytic activities of PBMC were determined in standard 3-hour 51Cr-release assays. Briefly, varying numbers of lymphocytes were seeded in triplicate in 96-well, round-bottomed microtiter plates (150 µL/well). A fixed number of target cells labeled with 51Cr (200 µCi/106 cells) was added in a volume of 100 µL/well. At the end of the incubation period (37°C and 5% CO2), release of 51Cr into the supernatants was measured. The following target cells were used: for the assessment of NK activity, the K562 erythromyeloid leukemia line, and for ADCC, the P815 mouse mastocytoma cell line coated with rabbit anti-P815 IgG. The cytotoxicity assays were performed at four different effector to target (ET) ratios, i.e., 50:1, 25:1, 12.5:1, and 6.25:1. The percentage of specific lysis (SL) was calculated according to the formula:

\[
\%SL = \frac{\text{Exp. cpm} - \text{Spon. cpm}}{\text{Max. cpm} - \text{Spon. cpm}} \times 100
\]
where Exp. is the experimental number of counts obtained from target cells incubated with effector cells; Spon. is the spontaneously released counts obtained from targets incubated in medium alone; and Max. is the maximal counts obtained from targets lysed with a 1% Triton X-100 solution.

Sequence analysis. Messenger RNA was isolated from mononuclear cells and reversely transcribed into cDNA. Primers used to amplify the entire coding region of FcyRIIIA-encoding cDNA were: Tfl (sense; nt 5-21) 5' CGC AAG CTT TGG TGA CTG GTC CAC TC-3' and Tf2 (antisense; nt 963-988) 5' CGC TCT AGA TCA TGG GCT TTT CCC 7T-3'. The polymerase chain reaction (PCR) amplified fragment was ligated into a pGEM-T vector, according to the manufacturer’s instructions (Promega, Madison, WI). After cloning into E. coli, the insert was amplified and the nucleotide sequence was determined by cycle sequencing with 32P end-labeled primers (Amersham, Amersham, UK), using the BRL cycle sequencing kit according to the manufacturer’s instructions (BRL, Gaithersburg, MD). The following primers were used to sequence the entire coding region: sense direction: Tfl (see above), NA-L (nt 106-125), P306 (nt 322-342) and Sn (nt 658-679); anti-sense direction: Tf2 (see above), P664 (nt 658-679) and NA-R (nt 329-348).

FcyRIIIA gene-specific fragment amplification and FcyRIIIA genotyping. Amplification of an FcyRIIIA gene-specific fragment containing the site under investigation was achieved by means of an allele-specific primer annealing (ASPA) assay. The sense primer IIA1 (5' CAC AGT GGT TTC ACA ATG AGA G-3') was compatible with NA2-FcyRIIB and FcyRIIIA. The antisense primer IIA2 (5' CTG TAC TCT CCA CTG TGG TC-3') annealed completely to NA1-FcyRIIB and FcyRIIIA. The PCR assay was performed with 1 μg of genomic DNA, 150 ng of each primer, 200 μmol/L of each dNTP and 2 U of Taq-DNA polymerase (Promega), diluted in a buffer recommended by the manufacturer in a total volume of 50 μL in a Perkin-Elmer Cetus Cycler (Norwalk, CT). The first cycle consisted of 5 minutes of denaturation at 95°C, 1 minute of primer annealing at 64°C and 1 minute of extension at 72°C. This was followed by 35 of these cycles in which the denaturation time was decreased to 1 minute. The final cycle was followed by 9 minutes at 72°C to complete extension.

The 91-bp product was electrophoresed in 10% polyacrylamide gels, stained with ethidium bromide and visualized with ultraviolet (UV) light. Cycle sequencing of the amplified fragments was performed with primer IIA2 as described above.

RESULTS

Flow cytometry. The PBMC of the patient contained 9% CD3⁺CD7⁺ lymphocytes and 7% CD3⁺CD56⁺ lymphocytes, respectively, indicating that NK cells are present in normal relative numbers (Fig 1). As shown in Fig 2, the CD3⁺CD7⁺ lymphocytes stained minimally with the CD16 MoAb B73.1. Therefore, we tested several other CD16 MoAbs. CD3⁺CD7⁺ lymphocytes were positively stained with CLBFcRgran1, indicating that FcyRIIIa was expressed on the cell membrane. The results of the complete flow cytometric analysis of CD3⁺CD7⁺ lymphocytes of the patient, his family members, and a healthy, unrelated, control are summarized in Table 1. Except for Leu1lc/B73.1, the CD3⁺CD7⁺ lymphocytes of the patient and his newborn brother stained positively with the applied CD16 MoAbs. NK cells of both parents stained positively with B73.1, albeit
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Fig 3. NK and ADCC cytolytic activities of lymphocytes. Spontaneous cytotoxicity of K562 target cells and ADCC of P815 targets were measured in a 51Cr release assay using different effector:target ratios. There was no difference between the patient ( ), his brother ( ), and a healthy, unrelated, age-matched control (*).

with a somewhat lower fluorescence intensity compared with the control. Flow cytometric analysis of the patient’s granulocytes showed a normal reactivity pattern with CD16 MoAbs and an NA(1+2+) phenotype, which was confirmed by genotyping. Granulocytes of his mother and brother were also NA(1+2+), and his father had an NA(1-2+) phenotype.

Cytotoxicity assays. Spontaneous cytotoxicity of K562 target cells and ADCC of P815 targets measured by a 51Cr release assay were normal for the patient and his younger brother, as compared with an age-matched healthy control (Fig 3).

Table 1. Immunofluorescent Staining of CD3+CD7+ Cells With CD16 MoAbs

<table>
<thead>
<tr>
<th>FcyRIIIA Genotype</th>
<th>48-H/H (patient)</th>
<th>48-L/H (father)</th>
<th>48-L/H (mother)</th>
<th>48-H/H (brother)</th>
<th>48-L/L (control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD16 MoAb</td>
<td>% Pos</td>
<td>MFI</td>
<td>% Pos</td>
<td>MFI</td>
<td>% Pos</td>
</tr>
<tr>
<td>B73.1</td>
<td>9</td>
<td>136</td>
<td>71</td>
<td>251</td>
<td>63</td>
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<tr>
<td>CLBFegran1</td>
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<td>92</td>
<td>1,703</td>
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<td>3G8</td>
<td>65</td>
<td>989</td>
<td>91</td>
<td>981</td>
<td>72</td>
</tr>
<tr>
<td>Leu11a</td>
<td>55</td>
<td>432</td>
<td>87</td>
<td>374</td>
<td>66</td>
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<tr>
<td>DJ130c3</td>
<td>54</td>
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<td>75</td>
<td>360</td>
<td>65</td>
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<tr>
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<td>PEN1</td>
<td>57</td>
<td>691</td>
<td>88</td>
<td>956</td>
<td>56</td>
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</table>

Percentage of CD3+CD7+ cells stained positively with the indicated CD16 MoAbs and mean intensity of fluorescence (channel values, MFI: 4 log decade, relative linear units) of the patient, his family, and a healthy, unrelated control. Staining of CD3+CD7+ cells with MoAbs of irrelevant specificities was less than 1.0% for the IgG1 and less than 4.0% for the IgG2a control MoAbs.

Sequence analysis. Sequencing of the entire coding region of FcyRIIIA-encoding cDNA of the patient showed a T to A nucleotide substitution at position 230 in both alleles. This substitution predicts a leucine to histidine change at amino acid position 48 in the first extracellular domain of FcyRIIIA. As shown in Fig 4, cycle sequencing of the FcyRIIIA-specific PCR products of the parents showed that they both were heterozygous for this nucleotide substitution (FcyRIIIA-48L/H). Like the patient, the second son also had an FcyRIIIA-48H/H genotype (data not shown).

DISCUSSION

Only one patient with an absolute NK cell deficiency has been described until now. This was an adolescent with recurrent life-threatening herpes-virus infections, who completely lacked CD16 and/or CD56 positive cells in vivo and NK-activity in vitro. Although NK cell function is impaired in patients with Chediak-Higashi syndrome and Leucocyte Adhesion Deficiency disease, this impairment does not dominate their clinical course.

Here, we describe a patient with an as yet unknown FcyRIIIA-48H/H genotype, caused by a T to A nucleotide substitution at position 230 on both alleles. This particular genotype leads to an unusual phenotype, with loss of the Leu11c/CD16.1 epitope of the Fcy Receptor IIIa (CD16) on NK cells (and, presumably, on macrophages). At nucleotide position 230 of the FcyRIIIA gene a biallelic polymorphism (T or G) has recently been found, and further analysis, prompted by the findings in this patient, has shown that, in fact, a triallelic polymorphism (T, G or A) exists at this position, with gene frequencies in Caucasians of 86, 6, and 8%, respectively. With this frequency of the 230A allele, it can be predicted that 6.4 FcyRIIIA-48WH homozygotes per thousand individuals should be present in the Caucasian population. It is important to realize that this Leu11c/CD16.1 negative phenotype will not be detected in most routine immunophenotyping procedures. NK cells are mostly defined as the CD16 and/or CD56 positive and CD3 negative lymphocyte population. A CD16 (most frequently Leu11c) and a CD56 MoAb labeled with the same fluorescent dye are then used simultaneously. The lack of reactivity with
Leu11c/B73.1 will remain unnoticed, as a large part of the NK cell population is CD56 positive, as well. This can explain why this phenotype was not described before.

The question of whether the unusual Fcγ Receptor IIIa and the clinical problems in this patient are causally related cannot be answered on the basis of the available data. Although the infectious problems of the patient described here fit into the postulated role of NK cells in vivo immune surveillance, the NK cell activity as measured in vitro was not affected. In addition, the binding site of FcγRIIIa for IgG was described to be located at the second, membrane-proximal, extracellular Ig-like domain, whereas the Leu11c/ B73.1 epitope is located at the first extracellular Ig-like domain of FcγRIIIa, albeit not at amino acid position 48. The epitope recognized by 3G8 and CLBFcRgran1 is located at the second extracellular Ig-like domain. On the other hand, it has also been described that B73.1 inhibits IgG binding to FcγRIIIa.11

The FcγRIIIA-48H/H genotype with loss of the Leu11c/ B73.1 epitope of the FcγRIIIa (CD16) on NK cells was also present in the younger brother of the patient. He suffers from recurrent, mainly viral, upper respiratory tract infections as well. Also, he has food allergy and eczema. He has not yet come into contact with either Epstein-Barr or Varicella Zoster virus. Very recently, a 2-year old boy of Caucasian descent, not related to the patient described here, was born prematurely as one of a triplet and had suffered from food allergy and eczema, like the younger brother of the patient described here. This boy was tested after an unusually severe and prolonged infection that looked like chickenpox. Varicella Zoster virus was not isolated, and he did not mount an antibody response to this virus. The identification of this unrelated boy with the same unusual Fcγ Receptor IIIa and problems with combating an infection could make a causal relationship between the FcγRIIIA genotype and the clinical presentation described here more likely. Bearing in mind the relatively high allelic frequency of 8% in the Caucasian population, it could be that other intrinsic factors, besides FcγRIIIA-48H/H homozygosity, need to be present before infectious problems occur. More elaborate analysis of the NK cell function in vitro, and follow-up of the clinical course of newly traced FcγRIIIA-48H/H homozygotes in the population is required to ascertain whether this genotype is really the cause of an NK cell immunodeficiency.

NOTE ADDED IN PROOF

While our report was being reviewed, another patient with FcγRIIIA-48H/H homozygosity was published by Jawahar et al.13

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