Human nuclear factor κB essential modulator mutation can result in immunodeficiency without ectodermal dysplasia

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Background: Many receptors rely on the appropriate activation of nuclear factor (NF)-κB to induce cellular function. This process depends critically on the phosphorylation of the inhibitor of NF-κB (IκB) by the IκB kinase. This targets IκB for ubiquitination and degradation, allowing NF-κB to translocate to the nucleus, where it can direct transcription. Hypomorphic human mutations affecting one IκB kinase component, the NF-κB essential modulator (NEMO), result in impaired signaling from receptors required for ectodermal development and immune function. Male subjects with these mutant NEMO molecules have an X-linked syndrome known as ectodermal dysplasia with immunodeficiency, which is characterized by severe infections, with herpesviruses, bacteria, and mycobacterial susceptibility.

Objective: We sought to genetically and biochemically characterize a patient with a mutant NEMO molecule without ectodermal abnormalities.

Methods: We evaluated NEMO in a patient who had immunodeficiency and atypical mycobacterial infection but normal ectoderm.

Results: We identified a novel NEMO mutant causing immunodeficiency without ectodermal dysplasia. The mutation, which altered the exon 9 splice site, was present in cells of ectodermal and hematopoietic origin and resulted in a heterogeneous mixture of mutant and wild-type cDNA species. Immunologic function was variably impaired, with reduced CD40-induced B-cell proliferation, partially reduced NF-κB p65 nuclear translocation, and variable Toll-like receptor–induced TNF production. This variability might be explained by an inconsistent ratio of mutant to wild-type NEMO. The lack of any ectodermal phenotype, however, suggested a separation in the hematopoietic and ectodermal function of NEMO that leads to NF-κB activation.

Conclusion: Mutation of the gene encoding NEMO can result in immunodeficiency without ectodermal dysplasia. (J Allergy Clin Immunol 2004;114:650-6.)

Key words: NF-κB, IκB kinase, immunologic deficiency syndromes, X-chromosome–linked genetic diseases, innate immunity

The syndrome of ectodermal dysplasia (ED) with immunodeficiency (EDID) can be caused by hypomorphic mutations in the IKBKG gene on the X chromosome encoding the nuclear factor (NF)-κB essential modulator (NEMO).1 NEMO is a regulatory protein (also called the inhibitor of NF-κB [IκB] kinase [IKK]-γ) that is central to the formation of a functional IKK complex. Intact IKK can phosphorylate IκB, thus directing its degradation, which allows NF-κB to dimerize, translocate to the nucleus, and induce gene transcription.2 Patients with EDID and IKBKG mutations have ED characterized by conical pointed teeth, fine sparse hair, frontal bossing, and absent eccrine sweat glands.1,3 This phenotype arises because a receptor ligand pair of the TNF/TNFR superfamily (ectodysplasin A and ectodysplasin A receptor) critical for ectodermal development requires an intact NEMO molecule to activate NF-κB.4 At least part of a functioning NEMO molecule is needed for survival because male subjects inheriting large deletions, frameshifts, or other amorphic mutations of IKBKG die in utero.5

The immunodeficiency associated with EDID caused by hypomorphic IKBKG mutation is characterized by hypogammaglobulinemia, impaired specific antibody production, deficient natural killer cell cytotoxicity, and poor proinflammatory cytokine production in response to physiologic stimuli and results in severe bacterial infection early in life, as well as susceptibility to severe
Abbreviations used
ED: Ectodermal dysplasia
EDID: Ectodermal dysplasia with immunodeficiency
IxB: Inhibitor of NF-κB
IKK: IκB kinase
LZ: Leucine zipper
NEMO: NF-κB essential modulator
NF: Nuclear factor
pAb: Polyclonal antibody
TLR: Toll-like receptor
TWC: Total white blood cells
WT: Wild-type
ZF: Zinc finger

herpesviral disease and atypical mycobacterial infections.1,3,6-11 The immunodeficiency and infectious susceptibility presumably results from impaired signaling through several key receptors, including CD40, TNFR, IL-1R, and Toll-like receptors (TLRs), that depend on action of IκB or nuclear translocation of NF-κB. Thus, human hypomorphic IKBKG mutation affects both innate and adaptive immunity.

Importantly, different IKBKG mutations result in distinct clinical and immunologic phenotypes.1,5 For example, the most common NEMO alteration associated with EDID, C417R, completely blocks the generation of specific antibody, whereas some specific antibody can be produced in patients possessing certain other mutant proteins.3,7,8 These features are potentially due to differential use of upstream regulatory proteins by mutant NEMO molecules that bind particular regions of NEMO. In this regard several proteins that bind to or act on NEMO have been described, and some of these expected interactions are interrupted by alterations in IKBKG found in patients with EDID.12,13 Because distinct mutant NEMO molecules can differentially interfere with immune function, we speculated that certain mutations of IKBKG affect the signal transduction essential for immune function without impairing ectodermal development. This would lead to patients with immunodeficiency without ED. Thus, efforts were undertaken to evaluate boys without ED who have immunodeficiency and susceptibility to infection characteristic of EDID for mutations in IKBKG. In the course of studying immunodeficient male patients with mycobacterial infections, we identified one with a novel IKBKG mutation without ED. We report the characterization of this IKBKG mutation and its effect on NEMO expression and signaling through CD40 and TLRs.

METHODS
Patients and control subjects

The patient was 16 to 17 years old when samples were obtained for study. Some of his clinical details, including his longitudinal serum immunoglobulin values, lymphocyte subsets, mitogen-induced lymphocyte proliferation, and natural killer cell function, have been included as part of a clinical review of patients with IKBKG mutations.3 The patient provided assent and his parent’s consent for participation in these studies and the publication of his likeness. All studies were performed with approval of our respective institutional review boards. The patient’s parents and unrelated control subjects were also enrolled for study. Where possible, late adolescent male subjects were recruited to serve as control subjects.

Reagents

TLR ligands included the synthetic triacylated bacterial lipopeptide Pam3-CSSNA corresponding to the N-terminus of a BLP from Escherichia coli B/r (Baychem, Redwood City, Calif), the synthetic macrophage-activating lipopeptide 2 from Mycoplasma fermentans (Alexis Biochemicals, San Diego, Calif), poly dI-dC (Amersham, Piscataway, NJ), ultrapure R595 LPS from Salmonella minnesota (List Biologicals, Campbell, Calif), flagellin from Salmonella typhimurium (Alexis Biochemicals), imiquimod (3M Pharmaceuticals), R-848 (InvivoGen, San Diego, Calif), and oligodinucleotide 2216 (InviroGen). Specificity of individual TLR ligands for their cognate receptors was confirmed by using either TLR- and NF-κB luciferase reporter cotransfected Human Embryonic Kidney 293 cells, a neutralizing mAb to TLR2, or both, as previously described.14

The following antibodies were used in studies of patient cells: NEMO-specific mouse mAb clone 54 (BD Biosciences, San Diego, Calif) raised against a portion of NEMO containing the leucine zipper (LZ); NEMO-specific rabbit polyclonal antibody (pAb) SC8032 (Santa Cruz Biotech, Santa Cruz, Calif) raised against a polypeptide containing amino acids derived from the extreme C-terminus of NEMO comprising part of the zinc finger (ZF); and CD40-specific mouse mAb clone 626 (gift of S. M. Fu, University of Virginia).

Cell isolation

PBMCs or total white blood cells (TWC) were prepared from heparinized peripheral blood by means of centrifugation through Ficoll-Hypaque or 3% Dextran, respectively. When specified, B cells were enriched from PBMCs, as previously described.8 Buccal epithelial cells were isolated by using an oral lavage method.15 Cells for nucleic acid analysis were typically flash-frozen in liquid N2 or dry ice–ethanol before extraction of DNA or RNA. Cells used for NEMO protein analysis were lysed in 1% Nonidet P-40 lysis buffer and cleared of debris by means of centrifugation.

IKBKG and NEMO analysis

Genomic DNA was prepared by means of phenol-chloroform extraction, RNA by means of Trizol reagent (Invitrogen, Carlsbad, Calif), and cDNA by means of Superscript reverse transcriptase PCR system (Invitrogen), all according to manufacturer recommendations. IKBKG-specific primers were used to evaluate the full cDNA with the following primer sets, as previously described2; forward, 5’-CCCTTGCCCTGGTGGAATAGC-3’; reverse, 5’-AGCCGAGAAGGAAAGCGCACTG-3’ and forward, 5’-AACCTGGAAGAGCTGGTGCAGT-3’; too much space reverse, 5’-AGGTGGAACCCATCCCAGGTGG-3’. The patient’s particular mutation was identified in genomic DNA by using the following primers focused on the exon 9 region: forward, 5’-CCCTGACACTAGTCCACGCGAGGAGCTTTACCAGAAAT-3’; reverse, 5’-AGCAACAGGAGAGCGAGACCACACTTGTGAC-3’. Western blotting of NEMO or actin was performed with 4% to 12% bis-Tris NuPage gradient gels, NuPage buffer systems, and polyvinylidene difluoride membranes (Invitrogen). Membranes were blocked for nonspecific protein binding by the use of 1% BSA in PBS with 0.1% Tween-20 for 1 hour at room temperature, followed by overnight incubation with anti-NEMO or anti-actin antibodies.

Basic and clinical immunology
Bound mouse or rabbit antibodies were detected with peroxidase-conjugated goat anti-mouse or anti-rabbit (Sigma, St Louis, Mo), respectively, and the enhanced chemiluminescence detection system (Amersham Pharmacia).

**Functional assays**

CD40-induced proliferation, IgE production, and nuclear translocation of p65 were performed in exactly the same manner as for other patients with *IKBKG* mutations. For evaluation of TLR function, TLR ligands were incubated in citrated whole blood for 5 hours at 37°C with end-over-end rotation. Samples were diluted with 5 volumes of ice-cold RPMI 1640 medium (Invitrogen) and centrifuged at 1000g at 4°C for 5 minutes. The supernatant was recovered and stored at −20°C until assay of TNF-α by means of ELISA, according to the manufacturer’s instructions (R&D Systems, Minneapolis, Minn).

**RESULTS**

We considered *IKBKG* mutation in a 15-year-old-boy because of his specific pattern of infectious susceptibility and immunodeficiency. He did not have ED (Fig 1, A) and had normal dentition, hair pattern, and perspiration. He had pneumonia 4 times between the ages of 3 and 7 years and *Haemophilus influenzae* type b sepsis at age 5 years. He had a normal total IgG level, but IgG3 levels that were never greater than 10 mg/dL and no specific antibody against tetanus toxoid (despite receiving immunization) or *H influenzae* polyribosyl phosphate, and thus he was treated with immunoglobulin replacement. Despite receiving this therapy, a lesion on his abdomen developed at age 13 years (Fig 1, B), which was composed of granulomatous tissue (Fig 1, C and D), contained acid-fast bacilli (Fig 1, E), and grew *Mycobacterium bovis* when cultured. Despite the fact that the only known boys with immunodeficiency caused by *IKBKG* mutation have ED, we evaluated the patient’s *IKBKG* gene because of his lack of specific antibody production and atypical mycobacterial infection.

In contrast to the wild-type (WT) gene sequence, our patient had a G-to-A substitution at position 1056 of the *IKBKG* gene, 1 nucleotide before the exon 9 splice site (Fig 2, A). This was not a polymorphism present in his mother (Fig 2, A) and thus was a mutation that presumably arose de novo. Interestingly, both the WT G and mutant A appeared to be present in the patient’s genomic DNA. Because *IKBKG* is also present in a pseudogene, it was essential to evaluate the patient’s *IKBKG* mRNA. Incorrect diagnoses of an *IKBKG* mutation have been conferred because of confusion with the NEMO pseudogene obtained through genomic analysis alone. The patient’s cDNA obtained from his peripheral blood yielded both WT and mutant messages (Fig 2, B). The mutant message appeared to have a skipped exon 9, as might have been predicted from the 1056(−1) alteration. To evaluate this result at the protein level, we took advantage of a mAb raised against the portion of NEMO containing its LZ and the region encoded by exon 9. We also used a pAb raised against a segment of the NEMO ZF

**FIG 1.** Phenotype of a patient with *IKBKG* mutation and immunodeficiency without ED. A, Facial appearance of a patient with *IKBKG* mutation with normal hair pattern and teeth and without characteristics of ED. B, Abdominal skin lesions that contained granulomatous inflammation at 48× (C) and 750× (D) magnification. E, Lesional detail shown at 1200× magnification contained acid-fast bacilli (arrow) and grew *Mycobacterium bovis* on culture.
encoded by exon 10. Compared with his mother and father, the patient had barely detectable levels of NEMO in his PBMCs as determined with the LZ mAb but normal levels as determined with the ZF pAb (Fig 3, A). Because the IKBKG exon 9 encodes only 19 amino acids, the apparent molecular weight of the mutant protein did not differ substantially from WT. Thus the exon 9 deletion found in the patient’s cDNA translates into an aberrant NEMO protein but does not affect the total level of NEMO protein.

Because both WT and mutant IKBKG sequences were found in our patient’s blood cells, it is possible that only a subset of hematopoietic precursors carry the mutation, whereas ectodermal precursors do not, thus explaining his
Thus our patient’s was contained in cells of presumably ectodermal origin. We and others have previously found that patients with EDID and from exon 9 on immune function. We and others have significances of the presence of a NEMO molecule deleted participate in host defense were evaluated to determine the relevance primarily to the immune system. Mutations have impaired CD40 signaling. All boys with EDID and IKBKG mutation have impaired CD40 signaling. We have found that most boys with EDID and an IKBKG mutation have significantly impaired TNF responses to LPS, as well as to other TLR ligands (Levy, Orange, Roy, and Geha unpublished results). This finding is similar to the response found in patients with a mutation in the IL-1 receptor–associated kinase 4 (IRAK4), which is upstream of NEMO in the TLR-signaling pathway and serves to prevent function of IKK after TLR ligation. Surprisingly, our patient had variable TNF-α responses to 8 different TLR ligands across time (Fig 5). In some assays responses to all TLR ligands tested were well below the mean control responses, but in others the responses were comparable with those of control subjects. The only exceptions were the responses to the TLR5 ligand flagellin and the TLR9 ligand oligonucleotide 2216, which were comparable with those of control subjects. The only exceptions were the responses to the TLR5 ligand flagellin and the TLR9 ligand oligonucleotide 2216, which were consistently decreased relative to control values (P ≤ .01). The response to the highest concentration of LPS was also decreased relative to control values (P ≤ .01). These results suggest that some TLRs require completely intact function of NEMO.

The variability present in our patient’s TLR responses suggested that dominance of the mutant NEMO form (Fig 3, A) might not be static. To evaluate this, we sequenced IKBKG cDNA in different cell populations at separate time points. Although the patient had both mutant and WT forms present in all samples, the sequence signal intensity appeared to vary between PBMC and TWC populations and within these populations on different dates (Fig 2, B). This result was confirmed at the protein level by using the LZ mAb. As an example, in a sample obtained at a time point distinct from the one studied in Fig 3, A, there was noticeably more WT NEMO present in both TWC and PBMC populations isolated from the patient (27% and 50% of the NEMO level of a control donor, respectively; Fig 3, B). This finding demonstrates variability in the ratio of WT to mutant NEMO over time.
DISCUSSION

The presence of heterogeneous IKBKG cDNA species in the hematopoietic cells of our patient with immunodeficiency but without ED might have arisen as a result of one of 3 possibilities: (1) mosaicism present only in his hematopoietic cells caused by isolated hematopoetic stem cell mutation; (2) true germline mosaicism; or (3) homogenous germline mutant IKBKG and leakage through the splice-site mutation (with the normal germline DNA sequence originating from the WT pseudogene). A reversion event in a hematopoietic stem cell is a fourth possibility, but would imply that his somatic cells all contain the mutant IKBKG. Because both WT and mutant sequences were obtained from patient cells of ectodermal origin (Fig 2, A, bottom), the first possibility is excluded. It is also unlikely that the presence of a normal IKBKG gene in some ectodermal cells would be sufficient to completely prevent an ED phenotype. In this regard several boys who have germline mosaic mutations for IKBKG have been described, and all have a partial ectodermal phenotype. Therefore if our patient were to be a true germline mosaic (as suggested from Fig 2, A), he would be expected to have some ectodermal phenotype. Thus, it is possible that exon 9 is dispensable for normal development of the ectoderm.

Importantly, the patient had a variable ratio between WT and mutant NEMO that was reflected at the message level. It follows that if the patient were a germline mosaic, the ratio of cells containing mutant and WT IKBKG could vary over time. Despite this, if exon 9 were critical for ectodermal development and at least some ectodermal cells exhibited the mutant sequence (as shown in Fig 2, A), a partial ectodermal phenotype would be expected, and this was not the case. Alternatively, the patient is not mosaic, and leakage through the mutated splice site creating WT NEMO varies over time. Leakage through splice-site mutations has been described in many human diseases, including X-linked hyper-IgM, and can be an expected feature of these types of mutations. If there was leakage through a splice-site mutation, it is possible that the boy’s ectodermal phenotype could have been rescued by complementation from varying amounts of WT NEMO expressed during ectodermal development. The complete absence of dysplastic ectoderm, however, suggests that this would have had to occur in all cells consistently at the appropriate stages in ectodermal development, and this is unlikely. Thus to explain the discordance between the ectodermal phenotype and immunodeficiency, we favor a mechanism in which exon 9 is dispensable for signaling required in ectodermal development. Alternatively, ectodermal development could occur with lower levels of WT NEMO function than is required for intact immunity. Further studies will distinguish between these 2 models and will require an experimental system in which IKBKG exon 9 is completely absent.

In contrast, the patient’s variability in immune function most probably results from the heterogeneity of NEMO species present at a given time. This suggests that the region encoded by exon 9 is critical for some immunologic functions. Certain receptors, however, could still function.
in the presence of limited WT NEMO. The ligands for TLR1/2, TLR2/6, TLR3, TLR7, and TLR7/8 induced normal function in some experiments, whereas the ligands for TLR4, TLR5, and TLR9 did not (Fig 5). Interestingly, the response to the TLR3 ligand poly dI-dC, which can signal independently of IKK, was never less than the response to the TLR3 ligand poly dI-dC, which can signal independently of IKK.

The specific biochemical role of IKBKG exon 9 is unknown. The NEMO LZ, which is flanked by the region encoded by exon 9, however, is important in NEMO trimerization. It is possible that an absence of exon 9 could affect this process. Presumably, defective homotrimerization would affect immune and ectodermal function equally. Therefore we believe that an immunologically relevant signaling intermediate requires the presence of exon 9, whereas signaling for ectodermal development does not. Certain immune receptor systems are likely to rely more intimately on this intermediate than others. Alternatively, IKBKG exon 9 might be required for the activation of particular NF-κB family members but not others. Because much of the specificity imparted by NF-κB is a feature of the specific NF-κB proteins used, an inability to activate certain NF-κB family members could account for the distinctions between our patient’s ectodermal and immunologic phenotype.

This patient has demonstrated that immune deficiency can be caused by an IKBKG mutation in the absence of ED. This novel presentation of IKBKG mutation suggests that selected patients without ED should be considered for a genetic aberration affecting NEMO. His particular gene mutation also raises questions as to the specific role and function of IKBKG exon 9 in the signaling pathways used by immunologic and ectodermal receptor systems to activate NF-κB.

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