The Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disorder originally described as a clinical triad of thrombocytopenia with small platelets, eczema, and immunodeficiency. Impaired CD43 glycoprotein expression on lymphocytes is a typical hallmark of this disorder. The CD43 gene is located on chromosome 16, and the WAS gene, WASP, was recently isolated from the chromosome X p11.22-p11.23. This gene, mutated in WAS patients, encodes a protein that is likely to play a role in controlling the expression of CD43. However, the molecular mechanism(s) causing WAS are not yet known. Herein, we describe a three-generation family in which clinical and laboratory WAS features were expressed in six of nine subjects available for study. At variance with classic X-linked WAS, this disorder was characterized by the presence of thrombocytopenia with a broad spectrum of platelet size, including giant platelets, and was inherited as an autosomal dominant trait. This last finding led us to hypothesize a mutation of the CD43 gene. However, Southern blot analysis failed to detect structural abnormalities of this gene, and genotype analysis ruled out the possibility that a CD43 allele might be shared by the affected individuals. These findings indicate that an alteration(s) of an autosomal gene distinct from the CD43 gene is responsible for the disease. Thus, results from this family, providing the first observation of an autosomally transmitted WAS variant, indicate that genetic mechanism(s) leading to WAS are more complex than previously recognized.

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SUBJECTS AND METHODS

Case report and family history. The propositus, an 18-year-old boy (IV-2 in Fig 1), came to our observation for asymptomatic thrombocytopenia (60 to 80 × 10^9/L platelets). His medical history is mainly characterized by recurrent episodes of bacterial infections since early childhood. In particular, he has experienced bilateral recurrent otitis since the second month of life (four to five episodes per year), and from the sixth month of life, he has had recurrent episodes of sinusitis, tonsillitis, and cystitis of bacterial etiology. One episode of pneumonia occurred at the age of 10 months and one episode of hematuria at the age of 3 years. Measurements of Ig serum levels performed at ages 3, 6, and 17 years consistently showed low IgM titers (<30 mg/dL), normal IgG and IgA, and slightly increased IgE (130 UI/mL; normal, <100). Serum isoagglutinin titer against B was decreased (1:8). Since the age of 6, the patient has developed severe allergic diathesis. At the time of our observation, he still had recurrent episodes of sinusitis, otitis, and allergic manifestations, and he has recently suffered from relapsing episodes of myositis.

A review of the family history showed that WAS-related symptoms were highly variable among family members on the paternal side (Fig 1). The propositus’ sister (IV-3), aged 17, has suffered from eczema and severe allergic asthma since childhood. From the age of 16, she has suffered from relapsing herpes zoster episodes resistant to acyclovir treatment, and a recrudescence of eczema was concomitant to these relapsing episodes. Polyarthritides episodes of the major joints were observed at the age of 17. Serum Ig concentrations were normal except for IgE levels, which were increased (254 UI/mL; normal, <100). Serum isoagglutinins against B were slightly increased (1:16). A review of the family history showed that WAS-related symptoms were highly variable among family members on the paternal side (Fig 1). The propositus’ sister (IV-3), aged 17, has suffered from eczema and severe allergic asthma since childhood. From the age of 16, she has suffered from relapsing herpes zoster episodes resistant to acyclovir treatment, and a recrudescence of eczema was concomitant to these relapsing episodes. Polyarthritides episodes of the major joints were observed at the age of 17. Serum Ig concentrations were normal except for IgE levels, which were increased (254 UI/mL; normal, <100). Serum isoagglutinins against B were slightly increased (1:16).
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PLATELET STUDIES. Platelet counts were performed on EDTA-anticoagulated venous blood in an electronic particle counter (System 9900; Baker Diagnostic, Bethlehem, PA) and by contrast-phase microscopy. For measuring mean platelet volume (MPV), blood samples were collected in tubes containing a solution of sodium citrate (0.38% final concentration) and PGE_1 (1 μmol/L final concentration). Normal values for MPV of citrated platelets are 6.8 ± 0.16 fl (mean ± SD), and giant platelets were defined as having a MPV more than 10 fl. Platelet aggregation studies were performed on platelet-rich plasma obtained by centrifuging citrated (0.38% final concentration) blood samples. For ultrastructural studies, platelets were prefixed in suspension with 2% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, for 5 minutes and then washed twice with phosphate-buffered saline (PBS) plus 3% sucrose. Platelet diameters were measured by electron microscopy analysis, and on the basis of this method, giant platelets were defined as having a diameter more than 5 μm. For the immunogold labeling procedure, the cells were washed in PBS containing 0.1% bovine serum albumin (BSA) and treated with anti-CD43 murine monoclonal antibody (MoAb) (DAKOPATTS AB, Alvsjö, Sweden) for 30 minutes. The murine antibody was revealed using goat antimouse gold-conjugated antibody (BioCell Laboratory, Rancho-Domingues, CA) at a 1/50 ratio. After washing, cells were fixed in 2% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.4, and processed as described earlier. After labeling, cells were postfixed in 1% osmium tetroxide for 30 minutes, dehydrated in graded ethanol, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM 400 transmission electron microscope (Philips Corp, Eindhoven, The Netherlands).

LYMPHOCYTE STUDIES. For lymphocyte surface antigens, small aliquots of whole blood were incubated under standard conditions with the following fluorescein isothiocyanate- or phycoerythrin-conjugated antibodies: CD3, CD4, CD8, CD16, CD56, CD19, and HLA-DR (Simultest; Becton Dickinson, Mountain View, CA). CD3 antigen expression was analyzed by incubating the cells with anti-CD3 MoAb (DAKOPATTS). Cells were labeled with fluorescein-conjugated goat antimouse IgGs (Ortho Diagnostics Systems Inc, Raritan, NJ) and lysed using a commercially available ammonium chloride lysing reagent (Ortho Diagnostics Systems Inc). After washing in PBS-BSA, samples were analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). Compensation was set using Calibrate beads (Becton Dickinson Immunocytometry Systems) before each set of determinations. A minimum of 3,000 events were acquired in the lymphocyte gate in list mode. Data were analyzed using LYSYS II software (Becton Dickinson Immunocytometry Systems). For standard mitogenic stimulation, 250 μL whole blood was incubated in a 5% CO_2 atmosphere with phytohemagglutinin (PHA) for 72 hours. Following red blood cell lysis, the sample was incubated with a 50-μg/mL propidium iodide solution containing RNAse at 1 mg/mL and 0.2% Nonidet P-40. Samples were analyzed using CellFIT software (Becton Dickinson Immunocytometry Systems) to determine the S-phase dimension. Results are expressed as the stimulation index (% stimulated S-phase cells/% unstimulated S-phase cells).

DNA EXTRACTION AND SOUTHERN BLOT ANALYSIS. Nucleated cells from peripheral blood were used for DNA preparation. Genomic DNA isolation was performed with a salt-chloroform extraction method. DNA samples were digested with EcoRI, BamHI, TaqI, and MspI (New England Biolabs, Beverly, MA) following the manufacturer's recommendations, electrophoresed in 0.7% agarose gels, transferred to nylon membrane (Hybond N; Amersham, Little Chalfont, UK), and hybridized under high-stringency conditions to a 1.2-kb labeled CD43 CDNA probe. After a high-stringency wash, the membrane was exposed to X-ray film (X-omat AR; Eastman Kodak, Rochester, NY).

GENOTYPING BY POLYMERASE CHAIN REACTION. Polymerase chain reaction (PCR) was performed to analyze the microsatellite marker, SPN, located in the 3′ untranslated region of the CD43 gene. The following primers were used: SPN forward, 5′-TCCATTTCTGCA-GTACACTACGTA-3′; and SPN reverse, 5′-AGTCCCCGACG-CGAGGCAAA-3′. The forward primer was labeled at the 5′ end with 32P-ATP (Amersham) and T4 oligonucleotide kinase (New England Biolabs). PCR amplifications were performed in a volume of 25 μL and contained 200 ng genomic DNA, 0.4 μmol/L of each primer (for the forward primer, the ratio of labeled to cold primer was 1:4), 1 U Taq polymerase (Perkin Elmer, Foster City, CA), 0.2 mmol/L of each of the dNTPs, 10 mmol/L Tris hydrochloride, pH 8.4, 50 mmol/L KCl, 1 mmol/L MgCl_2, and 1.3 g/L BSA. After an initial denaturation at 95°C for 4 minutes, the cycle times were 30 seconds of denaturation at 94°C, 30 seconds of annealing at 55°C, and 1 minute of polymerase chain reaction (PCR) was performed to analyze the microsatellite marker, SPN, located in the 3′ untranslated region of the CD43 gene. The following primers were used: SPN forward, 5′-TCCATTTCTGCA-GTACACTACGTA-3′; and SPN reverse, 5′-AGTCCCCGACG-CGAGGCAAA-3′. The forward primer was labeled at the 5′ end with 32P-ATP (Amersham) and T4 oligonucleotide kinase (New England Biolabs). PCR amplifications were performed in a volume of 25 μL and contained 200 ng genomic DNA, 0.4 μmol/L of each primer (for the forward primer, the ratio of labeled to cold primer was 1:4), 1 U Taq polymerase (Perkin Elmer, Foster City, CA), 0.2 mmol/L of each of the dNTPs, 10 mmol/L Tris hydrochloride, pH 8.4, 50 mmol/L KCl, 1 mmol/L MgCl_2, and 1.3 g/L BSA. After an initial denaturation at 95°C for 4 minutes, the cycle times were 30 seconds of denaturation at 94°C, 30 seconds of annealing at 55°C, and 1
minute of extension at 72°C, followed by a final extension at 72°C for 4 minutes. The reactions were performed for 25 cycles on a Delphi thermal cycler (MJ Research, Watertown, MA). PCR products were analyzed on a 6% polyacrylamide/8.3-mol/L urea gel. After electrophoresis, the gel was dried and exposed to x-ray film.

RESULTS

Platelet Studies

Platelet counts and morphology. The characterization of the platelet population in affected family members is detailed in Table 1. Unaffected family members showed normal platelet count and size, whereas in affected subjects platelet count ranged from 60 to 140 × 10^9/L and MPV from 10.5 to 12.5 fl. Peripheral smears and electron microscope analyses showed a heterogeneous platelet population with the presence of both small and giant platelets of diameters ranging from 1.8 to 12 μm (Fig 2). The ultrastructural morphology of platelets was otherwise normal. No leukocyte inclusions, typical of May-Hegglin or Fechtner syndromes, were detected in thrombocytopenic subjects. The percentage of giant platelets was found to comprise 25% to 30% of the whole platelet population.

Platelet aggregation. Platelet aggregation induced by ADP and collagen and ATP release induced by collagen were normal in all tested family members (111:3, 111:4, 111:5, 111:6, and IV:2). In two subjects (111:3 and IV:2), there was a decreased platelet aggregation induced by ristocetin. This finding may suggest a defective expression of platelet glycoprotein Iib, in agreement with other observations in WAS patients.

Lymphocyte Studies

Results of immunologic phenotyping of the lymphocytes are shown in Table 1. In agreement with the most common lymphocyte abnormalities described in WAS, a decreased proportion of CD3, CD4, and CD19 cells, a low CD4/CD8 ratio, and an increased CD16 cell population were variously detected in this family. In addition, a reduced response to PHA mitogen was detected in all tested family members (IV:2, 111:3, and 111:4). We also examined CD43 expression in most subjects by labeling CD3⁺ peripheral blood lymphocytes with an anti-CD43 MoAb. The fluorescence intensity was markedly reduced in family member 111:3, and to a lesser extent in other tested family members (Table 1). CD43-CD3 double-labeling of peripheral blood lymphocytes is shown in Fig 3. This finding was confirmed by electron microscope analysis performed after staining of CD43 with an immunogold-labeled MoAb (Fig 4).

Southern Blot Analysis

Southern blot analysis using four different restriction enzymes and a CD43 cDNA probe failed to detect gross abnormalities of the CD43 gene, ie, rearrangement or altered copy number, in affected family members. Unaffected relatives and control placenta DNA were used for comparison (data not shown).
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Cytometer: FACSCAN
Parameters: FL1=CD43 FL2=CD3 Gated events: 5000 Total events: 5000

Fig 3. Two-color analysis of peripheral blood lymphocytes. (A) Control; (B) family member III:3.

Microsatellite Analysis

To rule out the possibility that CD43 point mutations undetectable by Southern blot analysis could be associated with the disease, we tried to determine whether a CD43 allele was shared by the affected family members. This analysis took advantage of the highly polymorphic microsatellite marker, SPN, which is located within the CD43 gene. SPN genotype analysis showed that the affected family members do not share a common CD43 allele (Fig 5). These findings indicate that the CD43 gene is not linked to the disease observed in this family.

DISCUSSION

The family described in this report displays an autosomal dominant disorder phenotypically indistinguishable from classic X-linked WAS. Clinical and laboratory features reminiscent of WAS were present with different expressivity among affected family members. Thrombocytopenia, the only consistent laboratory finding, was variously associated with other clinical manifestations typical of WAS such as eczema, recurrent infections, nephropathy, nonseptic polyarthritis, severe allergic manifestations, autoimmune and inflammatory diseases. Furthermore, low IgM titers, high IgE values, low isoagglutinin titers, lymphocyte abnormalities, and defective CD43 were also detected in affected family members. The association of several manifestations in affected members and some peculiar characteristics of these manifestations (ie, short-term relapse of herpes zoster despite antiviral therapy, temporal association between infections, and eczema recurrence) strongly support the diagnosis of WAS-like syndrome. In addition, the variable expression of immune abnormalities in this kindred appears to be in agreement with the findings of a recent survey of WAS, which showed that typical manifestations of the disease vary from one patient to another and within the same kindred and are unpredictable during the clinical course of the disorder.

The most interesting feature of the family reported herein was the autosomal dominant transmission. The possible existence of autosomally transmitted WAS variants had only been hypothesized on the basis of reports of women presenting features typical of WAS without either structural alterations or nonrandom inactivation patterns of the X chromosome. However, these reports described sporadic cases that could not prove the existence of an autosomal segregation, which, in contrast, is evident in this family.

Because of the impaired expression of CD43 on lymphocytes as detected by both cytofluorimetry and immunoelectron microscopy, a mutation of the CD43 gene was first hypothesized as the possible cause of the autosomal disorder segregating in this family. However, Southern blot analysis failed to detect structural abnormalities of this gene, and
genotype analysis ruled out the possibility that a CD43 allele might be shared by the affected individuals. These findings indicate that an autosomal gene distinct from CD43 is responsible for the disease in this family.

This opens a new scenario for the mechanism(s) controlling the expression of CD43 in blood cells and thus the transmission of WAS. In fact, our findings support the hypothesis that the recently cloned WASP might not be the only factor involved in CD43 expression. The putative gene product defective in our family, in fact, might cooperate with WASP in controlling CD43. An alternate possibility is that the two gene products responsible for typical WAS and this variant of WAS might be involved in a protein cascade in which the product of one gene acts downstream of the other in a pathway leading to CD43 expression.

Another relevant feature of the disorder segregating in this family is the presence of asymptomatic thrombocytopenia, characterized by the presence of a broad spectrum of platelet size, including giant platelets. Typical WAS is characterized by small platelets, but in this family platelet size was highly heterogeneous and the presence of giant platelets was detected in all the affected members. In a number of inherited thrombocytopenic disorders such as Alport's, Bernard-Soulier, Chediak-Higashi, Epstein, and Fechtner syndromes, von Willebrand variants, and grey-platelet syndrome, thrombocytopenia is associated with giant platelets. We were able to rule out the coexistence of these diseases, since we could not detect in any family member the functional, morphologic, or biochemical platelet defects and/or leukocyte inclusions typical of those disorders. However, the difference between the WASP gene and the one mutated in our family could account for the observed phenotypic differences.

The mechanism(s) underlying the abnormal platelet volume in WAS is still poorly understood. The observation that platelet size increases slightly after splenectomy led to the hypothesis that in WAS patients the platelet abnormality may be secondary to increased cell destruction. However, more convincing findings argue in favor of a primary platelet defect related to altered thrombopoiesis. In fact, several findings suggest that the molecular defect in WAS could influence the cytoskeletal architecture and/or organization, and may lead to ineffective thrombopoiesis with abnormal platelet size. In particular, it has been shown that CD43 can associate with actin filaments, which are known to be involved in megakaryocyte fragmentation. In addition, abnormal levels of platelet calpain, a Ca²⁺-dependent neutral protease interacting with cytoskeletal proteins, have been detected both in WAS patients and in patients with Montreal giant-platelet syndrome. Finally, sequence analysis of the WASP protein predicts that it might bind via its prolinerich motifs with SH3 domain-containing proteins.
ALLY, SH3 domains have been described in several cytoskeletal proteins.\(^{32}\)

Altogether, these findings indicate a possible association between WAS biochemical defect(s) and the mechanism of platelet production. This might also provide a framework for explaining the peculiar finding of giant platelets in our WAS family. It should also be remarked that an isolated thrombocytopenia with giant platelets had been previously described in a Saudi Arabian family.\(^{33}\) The gene responsible for this disease mapped close to or at the same locus as classic WAS, thus raising the possibility that this disorder was a mild allelic variant of WAS. In addition, an atypical form of WAS has been recently described\(^{2}\) in a woman with random X-chromosome inactivation characterized by thrombocytopenia with platelets varying in size and MPV of 13.9 fl.\

In conclusion, we have described the first family affected by an autosomally transmitted variant of WAS, which may contribute considerably to the understanding of the molecular basis of classic X-linked WAS, and this new disorder.

REFERENCES

Wiskott-Aldrich syndrome: report of an autosomal dominant variant

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