Atypical Presentation of Wiskott-Aldrich Syndrome: Diagnosis in Two Unrelated Males Based on Studies of Maternal T Cell X Chromosome Inactivation

By Jennifer M. Puck, Katherine A. Siminovitch, Mortimer Poncz, Cheryl R. Greenberg, Menochem Rottem, and Mary Ellen Conley

Congenital thrombocytopenia may occur in isolation or accompanied by eczema and immunodeficiency, as part of the X-linked hereditary Wiskott-Aldrich syndrome (WAS). Because the clinical and immunologic picture of WAS is variable, particularly early in life, definitive diagnosis cannot always be made in cases with a negative family history. Two unrelated males with sporadic congenital thrombocytopenia had only questionable immunologic abnormalities as infants, making them clinically indistinguishable from cases of isolated thrombocytopenia, although one developed episodic neutropenia and the other began to manifest a multisystem autoimmune disease at 2 years of age. Evaluation of X chromosome inactivation in the T cells of both patients' mothers showed each of these women to have the same highly skewed X chromosome inactivation pattern seen in carriers of typical familial WAS. A T-cell defect was subsequently directly demonstrated in the second patient, whose lymphocytes failed to proliferate to periodate and anti-CD43. Taken together, these data suggest the presence of T cell immunodeficiency consistent with WAS in these patients. Furthermore, their mothers were found to have a very high likelihood of being carriers, lending support to the diagnosis of a hereditary disease in these boys and making possible genetic prediction in other family members and subsequent pregnancies.

WISKOTT-ALDRICH syndrome (WAS) is an X-linked disease of unknown pathogenesis characterized by developmental defects of platelet, lymphocyte, and possibly other bone marrow-derived cell lineages. Boys with typical WAS present in infancy with bleeding and a history of similarly affected male maternal relatives. They subsequently manifest immunodeficiency with development of eczema, recurrent infections, and, in 10% to 20% of cases, lymphoreticular malignancy. However, as with all X-linked recessive genetic lethal diseases, over one third of cases of WAS are expected to be the first manifestation of a new mutation. In the absence of a positive family history WAS must be distinguished from other causes of thrombocytopenia, including maternally derived or autologous anti-platelet antibodies, congenital cytomegalovirus and human immunodeficiency virus (HIV), and other genetic defects. Decreased mean platelet volume, alterations in lymphocyte glycoprotein CD43 (gpl113), and morphologic abnormalities detected by scanning electron microscopy have been reported in WAS; when present, these abnormalities can aid in diagnosis, but it is not known how they are related to the primary genetic lesion, which has been mapped to the proximal short arm of the X chromosome.

Recent advances in the management of WAS, including bone marrow transplantation and carrier detection, increase the clinical relevance of early diagnosis. Therefore, it is important to clarify whether WAS and simple X-linked thrombocytopenia represent separate entities, or are variant forms of the same disease, as suggested by reports of immune dysfunction in the latter.

We and others have previously studied X chromosome inactivation in female carriers of X-linked immunodeficiency diseases. In the cell lineages primarily targeted by the gene defect, the X chromosome that bears the mutation is consistently inactive, as opposed to the normal pattern of random X inactivation. Thus, the B cells, but not other cell types, of mothers of boys with X-linked agammaglobulinemia (XLA) have only the normal X active, while skewed X inactivation is specific to T and B cells of carriers of X-linked severe combined immunodeficiency (SCID). Similarly, nonrandom X inactivation is found in several bone marrow derived cell lineages, including T cells, of obligate carriers of WAS.

This report describes two unrelated boys who presented with isolated sporadic thrombocytopenia. Testing maternal T cells for nonrandom X inactivation provided a means to establish the hereditary nature of the disorder in each case. Moreover, the evidence that their mothers carried a primary T-cell defect supported the concept that these boys had a form of WAS, and one of the boys was subsequently shown to have defective lymphocyte activation via CD43. Together, these findings suggest that at least some cases of X-linked thrombocytopenia represent an attenuated form of WAS.

PATIENTS AND METHODS

Patient 1. This term infant of Middle Eastern extraction was the first son of a woman with two daughters, eight sisters, and one brother, all in good health. At 2 weeks of age, he developed rectal bleeding and was found to have platelet counts as low as 58,000/μL. At 3 months, although developing well, he had persistent rectal bleeding and petechiae; bone marrow examination showed normal megakaryocytes. Coombs' test, antinuclear antibodies, and rheumatoid factor were negative, but platelet-associated antibodies were increased.
present. He experienced six bouts of otitis in his first year and had neutropenia. At times profound (0 to 220 neutrophils/µL). Immunologic evaluation at 1 year showed intact blastogenic responses to mitogens. Cell surface markers were as follows: CD4+ (T helper) 21%, CD8+ (T cytotoxic/suppressor) 23%, CD11a+ (pan-T cell) 70%, and CD19+ (pan-B cell) 7%. Quantitative immunoglobulins (in milligrams per deciliter, followed by age-adjusted normal ranges) were: IgG 1845 (383-1070), IgA 124 (27-169), and IgM 136 (43-113). IgE was elevated at 260 IU/mL (normally less than 15). Specific antibody responses positive included titers to cytomegalovirus and Epstein-Barr virus, and a low positive isohemagglutinin titer of 1:4 to blood type A. HIV titer was negative. Up through age 2½, when lost to follow-up, he had no eczema. His last platelet count was 20,000, with mean platelet volume by Coulter counter measurement of 7.1 µm (normal range, 7.4 to 10.4).

Patient 2. This term white male developed persistent bleeding for 7 days after circumcision. Clotting studies were normal, but the platelet count was 41,000/µL. Family history was negative for bleeding or problems related to the immune system. By four months of age he had petechiae and a palpable spleen tip, but was otherwise normal. Bone marrow contained increased megakaryocytes; Coombs' test, anti-nuclear antibodies, and anti-platelet antibodies were negative in the patient and his mother. Titers to cytomegalovirus, HIV, and toxoplasmosis were negative. By 1 year of age, episodes of severe rectal bleeding requiring transfusions began. An intrinsic platelet defect rather than increased consumption was suspected initially when transfused platelets were observed to have a normal survival time. However, in spite of chronic platelet transfusions, the patient had several life-threatening hemorrhages and was treated with plasma exchange, prednisone, vincristine, and intravenous gamma globulin. He developed transient eczema after age 1 year, which responded promptly to topical steroids. He suffered from oral thrush and herpes stomatitis while on immunosuppressive medications and had one episode of impetigo followed by cutaneous abscesses. Although immune dysfunction, either primary or secondary, was considered, lymphocyte surface markers and mitogen and alloantigen blastogenic responses were normal, and immunoglobulins were as follows: IgG 1320, IgA 124, and IgM 118. After splenectomy at 17 months of age, the platelet volume increased and the thrombocytopenia tended to resolve. Starting at age 2, he has had intermittent Coombs' positive hemolytic anemia and thrombocytopenia with anti-platelet antibodies; nonender subcutaneous nodules; swollen, painful joints; and soft tissue swelling of the feet. At age 3, he developed transient cerebellar ataxia with a fourth cranial nerve palsy, which totally resolved. At 5 years, his disease became quiescent; off all therapy, immunologic evaluation showed positive skin test reactivity to candida and intact lymphocyte blastogenic responses. Lymphocyte surface antigens included 90% CD3+ (pan-T cell), 86% CD4+, 7% CD8+, and 2% CD19+. Quantitative immunoglobulins showed an age-adjusted normal IgG of 1020 (539-1506), and IgA of 277 (27-169), and IgM of 1020 (539-1506) and IgA of 277 (27-169) and IgM of 1020 (539-1506). Isohemagglutinins were absent.

**RESULTS**

We previously developed a strategy for using human T cell/hamster hybrids to assay X chromosome inactivation patterns in carriers of SCID and XLA. To apply this technique to study T cell X inactivation in the mothers of our patients with thrombocytopenia, and to clarify the relationship between their disease and WAS, we first studied X inactivation patterns in three known carriers of typical WAS from two unrelated families as compared with 37 controls. Figure 1 shows representative Southern blot autoradiograms of genomic DNA (A, B, and C: lane G) and DNA from hybrids containing the active X chromosome from T cells. The three T cell donors shown in each of the panels (A, B, and C) were heterozygous for the RFLP DXS10, which has alleles detected as fragments of 7 and 5 kilobases (kb) in Tag I restriction digests. Of the 13 hybrids from the normal control woman shown in panel A, six had an active human X chromosome bearing the 7 kb DXS10 allele; five had an active X bearing the 5 kb allele; and two retained both Xs and thus could not be scored. This normal pattern of clearly random X inactivation was in contrast to the skewed pattern apparent in panels B and C, in which T-cell hybrids were

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**Note:** The text contains scientific and medical terminology and may require a background in immunology and genetics for full understanding. The content is presented as accurately as possible based on the given data.
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made from an obligate carrier of typical WAS and the mother of Patient 1, respectively.

Of note, an active WAS-bearing X chromosome was detected in 4.3% of the 46 hybrids with a single human X from the three known carriers of WAS (data not shown); this frequency would be expected if about 10% randomly inactivated cells were admixed with a completely skewed population. T cells of the 37 control women assayed in our laboratory by the same hybrid technique have shown wide variation in X inactivation ratio. Maximum likelihood analysis was done using the control hybrid data to determine the value for the parameter N of the binomial distribution to best describe normal female X inactivation patterns. (The binomial distribution of female X inactivation is a function of the probability of finding the paternally derived X active in a given cell [which is 0.5], and N, the number of T cell precursors with random X chromosome inactivation. The maximum likelihood value of N was found to be 10.) For a woman whose carrier status is unknown, an odds ratio can be formed comparing the probabilities that the hybrids represent WAS versus normal X inactivation patterns.

Table 1 shows results of the hybrid analysis carried out with T cells from the mothers of our two thromocytopenic patients. Mother 1 showed markedly skewed X inactivation. Her hybrids are also shown in the two autoradiograms in panel C of Fig 1. Hybrid 2, with both X chromosome DXS10 alleles, was not scored; hybrid 3 had the minority 5 kb allele on its active human X; and all of the remaining 20 hybrids had an active X with the 7 kb DXS10 allele. It is very unlikely that this degree of skewed X inactivation would be seen by chance in a female who does not carry an X-linked mutation deleterious to T cells. In fact, as shown in Table 1, the odds ratio was 20:1 in favor of the WAS carrier state for Mother 1.

Mother 2's T cells were also used to make hybrids; all of the 21 hybrids obtained had the same RFLP allele at DXS1 and DXS14, loci at which her genomic DNA had shown her to be heterozygous. The odds ratio was calculated to be 33:1 in favor of her being a carrier. DNA samples prepared from Patient 2 and Mother 2 were analyzed by Southern blotting for RFLP markers near the WAS locus. Although Mother 2 was homozygous at DXS7, distal to WAS on the short arm of the X chromosome, she was heterozygous at DXS14, on the centromeric side of WAS, and DXS255, which has been shown not to recombine with WAS with a lod score of greater than 10.52 At both these loci, Patient 2 inherited the RFLP allele from the X chromosome that was never active in Mother 2's T cells (data not shown), indicating that Patient 2 inherited the WAS locus from his mother's affected X.

Because neither patient had sufficient clinical or laboratory evidence of immune dysfunction to secure a diagnosis of WAS, the finding of nonrandom maternal T-cell X inactivation prompted further T-cell investigations of Patient 2. Blood mononuclear cells from Patient 2, and also from a boy with typical familial WAS and two normal controls were cultured with either PHA or B1B6, a monoclonal anti-CD43 antibody, which has been observed to stimulate proliferation by normal53 but not WAS lymphocytes.56 These subjects' cells were also exposed to neuraminidase and galactose oxidase (NA-GO), which stimulates T-cell proliferation by interacting with membrane galactosyl residues, as well as to periodate (NaIO4), which interacts with terminal α-linked
sialic acid residues, notably on the CD43 molecule. As shown in Table 2, T cells of Patient 2 proliferated in response to PHA and NA-GO. However, no significant increase in thymidine uptake over unstimulated cells was seen after exposure to anti-CD43 or treatment with periodate. This was the same response pattern seen in lymphocytes from the boy with typical, familial WAS. Control lymphocytes treated in the identical manner showed positive proliferative responses to all stimuli. Interestingly, the PHA response of Patient 2, but not the typical WAS patient, approximated that of normal controls, a finding consistent with both the less severe disease expression in Patient 2 and the considerable variability in lectin responses in WAS.

**DISCUSSION**

Both our patients presented with clinically significant bleeding and thrombocytopenia, but without clear immunodeficiency as required to make the diagnosis of WAS.

Patient 1 had some indication of immune dysfunction with recurrent otitis, neutropenia, low CD4/CD8, and high serum IgG. However, the absence of eczema, the high, rather than typically low, IgM, and positive, albeit low isohemagglutinins, precluded a definite diagnosis of WAS. Patient 2 had mild eczema and infections, but the latter may have been related to his immunosuppressive treatment. The hemolytic anemia and arthritis experienced by Patient 2 suggested an autoimmune disease, such as has been reported occasionally in patients with typical WAS. His low IgM and negative isohemagglutinins at age 5 are also characteristic of WAS.

Moreover, absent lymphocyte proliferation upon exposure to periodate or anti-CD43 appears to be specific for WAS and is thought to reflect an impairment in o-linked glycosylation.

While neither of our thrombocytopenic boys (particularly, Patient 1) had striking abnormalities of cellular immunity by routine laboratory evaluation, both their mothers had highly skewed X chromosome inactivation in their T cells. Although normal lyonization of female embryos is expected to result in occasional individuals with skewed X inactivation patterns, the mothers of our patients had profoundly skewed X inactivation, similar to that observed in known carriers of WAS. Our assay using hybrids to determine X inactivation pattern indicated that Mother 1 was 20-fold and Mother 2 was 33-fold more likely to be a carrier of an X-linked mutation hindering T-cell proliferation or survival than to be a normal female with random X inactivation. A phase-known odds calculation for Mother 2, incorporating the information that her affected son inherited the WAS locus from her inactive X chromosome, increases her odds of being a carrier to 66:1.

These results show the utility of the hybrid technique in assessing X chromosome inactivation patterns. Not only do the hybrids lend themselves to quantitative estimation of carrier risk, but also in contrast to assays based on differential methylation, the hybrid assay described here can be used for all women, not just those who are heterozygous for one of the RFLPs near differentially methylated cytosine residues. In fact, of the three WAS carriers and two mothers of patients presented in this report, none was informative for the RFLP-methylation assay.

Our studies of maternal T cell X inactivation indicate that there is a relative, but not absolute, selective disadvantage for T cells expressing the WAS mutation, both in carriers of typical WAS and in the mothers of the thrombocytopenic patients in this report. The absence of a normal gene product at this locus may produce immunodeficiency by hindering T-cell proliferation or survival during thymic maturation, through accelerated splenic sequestration of underglycosylated CD43-bearing lymphocytes, as postulated by Kenney et al., or by as yet unknown mechanism(s). In some cases, such as with our Patient 1, the defect may have been too mild to produce clinical or routine laboratory evidence of T-cell dysfunction. Nonetheless, we were able to show an abnormality in Patient 2 with the failure of his lymphocytes to respond to activation via CD43.

Patients such as ours emphasize the clinical variability of WAS and support the concept that WAS and other X-linked thrombocytopenic syndromes may represent a spectrum of allelic mutations of differing severity at a single locus on the proximal short arm of the X chromosome. Such variability may complicate the already difficult problem of selection of patients for early bone marrow transplantation for WAS. It would be of interest to evaluate members of pedigrees with simple thrombocytopenia showing clear X linkage with regard to T-cell X inactivation patterns of carriers, CD43-induced lymphocyte activation in affected males, and genetic linkage studies. Mapping of X-linked thrombocytopenia in a single reported kindred was consistent with a mutation in proximal Xp, similar to WAS, but a locus anywhere on the entire long arm of the X was not ruled out.

The detection of skewed T-cell X inactivation in the mothers of our patients increased our clinical suspicion that the patients did have significant lymphocyte abnormalities in addition to thrombocytopenia and strongly suggested that they had inherited an X-linked disorder. In this context, it is most likely that they both had a form of WAS.

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<th>Table 2. Lymphocyte Proliferative Responses</th>
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Values shown are mean cpm of triplicate cultures with SD less than 15%. Assay conditions were as described in Methods.
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interpretation not only gave us a better understanding of the clinical illnesses in these patients, it also allowed us to provide genetic counselling for both families. Both mothers were given a 50% recurrence risk in subsequent male pregnancies and were offered the possibility of carrier determination in other females at risk and of prenatal diagnosis of WAS by linkage analysis in subsequent pregnancies. Until these genetic defects are completely understood at the molecular level, a determination of maternal T-cell X inactivation pattern will be the only way to diagnose the carrier state for WAS in mothers of sporadic cases.

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Atypical presentation of Wiskott-Aldrich syndrome: diagnosis in two unrelated males based on studies of maternal T cell X chromosome inactivation [see comments]

JM Puck, KA Siminovitch, M Poncz, CR Greenberg, M Rottem and ME Conley

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