WASP Levels in Platelets and Lymphocytes of Wiskott-Aldrich Syndrome Patients Correlate with Cell Dysfunction

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Wiskott-Aldrich syndrome (WAS) is an X-linked blood cell disorder classically characterized by thrombocytopenia, immunodeficiency, and eczema (1, 2). Platelets and lymphocytes are the principally affected cells. Abnormalities include small platelet size and decreased platelet number, typically 10% of normal, susceptibility to pyogenic and opportunistic infections, progressive lymphopenia after age 6, failure to produce Abs particularly to polysaccharide Ags, and increased incidence of autoimmune disorders and malignancies including lymphomas (reviewed in Ref. 3).

The mutated gene responsible for the disorder was identified in 1994 together with its protein product, WASP (Wiskott-Aldrich syndrome protein) (4), a 53-kDa intracellular hemopoietic cell protein (4–7). Subsequent biochemical studies characterized WASP as a multidomain molecule that appears capable of regulating the actin cytoskeleton, findings that resonate well with the defective cytoarchitecture of WAS patient blood lymphocytes (8–10) and their defective responsiveness to select stimuli (10, 11). Individual WASP domains bind phosphoinositol 4’5’-bisphosphate (PIP2) (12); WIP (WASP interacting protein), an actin-binding protein (13); cdc42, a GTPase that regulates filopodial and lamellipodial surface extensions (14–16); and Src homology (SH) 3-containing proteins including Grb2 and Fyn (Refs. 17 and 18, and references therein). WASP also contains a cofilin-like domain and a verprolin domain (12). Altogether, these findings hold promise that an integrated picture of normal WASP function will emerge in the near future, and with it, a better understanding of the pathological events in WAS patient cells.

On the other hand, certain findings are unexplained in current models. For example, WASP is expressed in all non-erythroid hemopoietic cells examined, including T cells, B cells, NK cells, monocytes (4–7), and neutrophils (this study), and indeed normal WASP levels are similar for many of these cells (this study), yet the pathology of the disease is primarily ascribed to platelets and T lymphocytes (reviewed in Refs. 3 and 18). Another unexplained feature is the variability of clinical symptoms. Whereas platelet defects are present from birth and are severe in all patients, the immune defects are extremely variable, both in age of onset and severity, ranging from negligible, in which case the disease has been also called X-linked thrombocytopenia, to life threatening.

If newly diagnosed WAS infants are to benefit from current and future therapeutic modalities, it is important that the basis of disease severity be understood. The present manuscript explores the premise that WASP expression levels in patients’ cells are an important determining factor for clinical outcome. The study takes advantage of WASP expression data established for EBV-transformed cell lines of a diverse patient panel (19). Fresh blood samples were obtained from these and additional patients, and WASP levels were separately quantified for lymphoid cells, platelets, and neutrophils and compared with established levels in EBV cell lines.

Materials and Methods

Patients

Patients were diagnosed with the WAS based on male sex, thrombocytopenia with small platelets, eczema, immunodeficiency of variable clinical
severities, and, in some cases, family history. WASP mutations for the patient donors of EBV cell lines P31-P35 and P37-P44 were previously described (19–21). Mutation identification for patients TS, OW, and WR was performed by Dr. Hans Ochs (University of Washington School of Medicine, Seattle WA) (unpublished data), for patients MR and LD by Dr. Sau-Ping Kwan (Rush Medical School, Chicago, IL) (unpublished data), for the kindred TM and SM by Dr. Alfons Meindl, Pediatric Clinic, University of Munich, Munich, Germany (unpublished data), and for patient AA by Drs. Silvia Gilliani and Luigi Notarangelo, Department of Pediatrics, University of Brescia, Brescia, Italy (unpublished data). Mutations for patients NK and SV were identified by sequencing amplified exons of blood cell DNA using a modified strategy (L. Jones and E. Remold-O'Donnell, unpublished observations).

**Blood cells**

Paired blood samples from WAS patients and normal healthy consenting individuals were collected in acid-citrate-dextrose (ACD; NIH formula A) and fractionated immediately or after overnight shipment at ambient temperature. The blood was centrifuged at 200 × g for 12 min to separate platelet-rich plasma (PRP) and pelleted cells. Additional ACC were added (1 part per 3 parts PRP), and platelets were pelleted at 800 × g for 15 min. The platelets were resuspended in 10 mM TES buffer (pH 7.2), 136 mM NaCl, 2.6 mM KCl, 0.5 mM NaHPO₄, 2 mM MgCl₂, 0.1% glucose, and 0.1% BSA; additional ACC (2%) was added, and prostacyclin (1 μg/ml) and the platelets were pelleted at 800 × g for 10 min.

Pelleted blood cells (after removal of PRP) were combined with equal volume 2% dextran in 150 mM saline for 30–40 min at −22°C to sediment erythrocytes. The supernatant was aspirated, and the leukocytes were fractionated by Histopaque 1077 centrifugation. PBMC were collected from the interface layer and pelleted with HBSS without Ca²⁺ and Mg²⁺ by pelleting at 200 × g for 15 min. Neutrophil pellet was washed with HBSS without Ca²⁺ and Mg²⁺ by pelleting at 200 × g for 15 min. and residual erythrocytes were removed by water lysis.

T and B lymphocytes were immunomagnetically isolated from fresh or frozen WAS patient and normal PBMC, and from frozen WAS patient or control spleen cells. The cells were preincubated at 4°C for 15 min in RPMI 1640 with 10% FCS. Following the manufacturer’s instructions, CD19 beads (Dynabeads; Dynal, Lake Success, NY) were added, and the cell suspension was incubated on a rotator at 4°C for 60 min. The CD19 beads were magnetically collected, washed with cold media, and incubated with Detach-a-Bead (Dynal) overnight at 4°C for 60 min. The CD4⁺ magnetically collected, washed with cold media, and incubated with Detach-a-Bead (Dynal) overnight at 4°C for 60 min. The CD4⁺ lymphocytes were immunomagnetically isolated from fresh or frozen WAS patient and normal PBMC, and from frozen WAS patient or control spleen cells. The cells were preincubated at 4°C for 15 min in RPMI 1640 with 10% FCS. Following the manufacturer’s instructions, CD19 magnetic beads (Dynal, Lake Success, NY) were added, and the cell suspension was incubated on a rotator at 4°C for 60 min. The CD19 beads were magnetically collected, washed with cold media, and incubated with Detach-a-Bead (Dynal) overnight at 4°C for 60 min. B cells. CD4⁺ T cells were isolated from the depleted cell suspension by adherence to CD4 immunomagnetic beads using the same protocol.

To harvest erythrocytes, pelleted blood cells remaining after removal of PRP and removal of the top half of the cell pellet were suspended in 10 vol of HBSS without Ca²⁺ and Mg²⁺ and washed by several cycles of centrifugation for 10 min at 1200 rpm.

**Cell lines**

EBV-transformed cell lines from WAS patients and normal individuals (19) were grown in RPMI 1640 with 10% FCS, penicillin, and streptomycin. HeLa epithelial carcinoma cells strain S3 were grown as adherent cells (20) were grown in RPMI 1640 with 10% FCS, penicillin, and streptomycin and were maintained in DMEM (high glucose), 10% FCS, penicillin, and streptomycin and were maintained in DMEM (high glucose), 10% FCS, and 1% nonessential amino acids. EBV-transformed cell lines from a normal donor, normal erythrocytes, and the HeLa epithelial cell line (2.7 × 10⁸ nucleated cells, 9 × 10⁶ platelets, and 12 × 10⁶ erythrocytes). The arrow (left) indicates WASP (m.w. ~53,000), which migrates variably at 54–59 kDa relative to protein standards (right). B. Mean WASP levels (units per mg of total cell protein) ± SEM of normal PBMC (n = 7), neutrophils (n = 5), platelets (n = 7), EBV cells (n = 4), erythrocytes, and HeLa cells (n = 3). The amount of WASP in PBMC is set as 1.0 unit per mg total protein.

**Protein quantification**

Protein in cell lysates was quantified with Nano-Orange (N-6666 kits; Molecular Probes, Eugene, OR) using bovine albumin as standard. Fluorescence was measured with excitation at 485 nm and emission at 590 nm.

**Results**

**WASP levels in normal blood cells**

Isolated peripheral blood cells and EBV-transformed B cell lines of normal individuals were lysed in the presence of protease inhibitors and examined for WASP content by quantitative Western blot (Fig. 1A). Because these cells differ in size, WASP levels are compared on a “per mg of total cell protein,” intended as an approximation of intracellular volume. With the content of normal PBMC set as 1.0 unit WASP/mg protein, 0.86 units per mg was found in platelets, 0.78 in neutrophils, and 0.93 units in platelets (Fig. 1B). WASP was nondetectable in erythrocytes and in HeLa cells, a nonhemopoietic line (Fig. 1A). When cells from seven normal donors were compared, WASP levels showed only minor variation, ±7% in PBMC, ±8% in platelets, and ±8% in neutrophils (5 donors). Mean WASP levels in normal peripheral T lymphocytes (CD4⁺) and B lymphocytes (CD19⁺) were 0.99 and 0.80 U/mg, respectively (presented below).

Others reported that normal neutrophils do not express WASP (Ref. 22); we cannot explain the discrepant findings.
First, expression vs non-expression of mutated WASP-negative EBV cell lines, two hypotheses were considered. To explain the group C pattern of WASP-positive PBMC and B cells, discordant expression of group C mutated WASP in T cells and levels, 10% of normal, in the group B cell line (19). P32, P41, and P44 cells, respectively, in contrast to the modest cells, 87%, 86%, 69%, and 85% of normal for EBV cell lines P31, also by the high levels of mature size WASP RNA in group A rantly increased degradation. This putative scenario is suggested result from (approximately) normal synthesis coupled with aber-
rent WASP levels in EBV cells and PBMC of group A patients summarized graphically in Fig. 3. We hypothesize that the differ-
ences would be expected to differ for transformed cells in culture with WASP-positive PBMC and the patient data were grouped to reflect these
forces would be expected to differ for transformed cells in culture.

**WASP-negative patient lymphoid cells**

When PBMC of 25 WAS patients were examined, seven patients were identified whose PBMC and EBV cell lines are WASP-neg-
ative (Table I). All of these patients have severe disease. For rea-
sons clarified below, the WASP-negative mutations are called group D.

**WASP-positive patient lymphoid cells**

Seventeen of 25 patients had WASP-positive PBMC. Two surpris-
ing features were noted. First, WASP expression levels in PBMC varied only within a narrow range, 7% to 20% of normal levels. Second, WASP levels in PBMC were in many cases substantially different (both higher and lower) than the levels in EBV cell lines from the same patients. On further inspection of the data, distinct patterns were discerned based on the ratio of WASP \(_{\text{EBV}}\) to WASP \(_{\text{PBMC}}\). The group data were grouped to reflect these expression patterns (Fig. 2).

In group A (9 patients), WASP levels in EBV cell lines are higher than corresponding PBMC (WASP \(_{\text{EBV}}\) > WASP \(_{\text{PBMC}}\)) (Fig. 2, upper panel). Although it is not the basis for the grouping, all identified group A patients have exon 1 and 2 missense mutations, and most have mild disease (8 of 9 patients).

In group B (2 patients), WASP levels are comparable in PBMC and EBV cell lines (Fig. 2, middle panel). The group B patients (one kindred) have an exon 10 frameshift mutation and moderate disease.

In group C (7 patients), WASP \(_{\text{PBMC}}\) levels are 7–16% of nor-
mal; however, EBV cell lines from these patients do not express WASP (Fig. 2, lowest panel). Mutations in this group are diverse, as is disease severity (see Discussion).

The four expression patterns for patient lymphoid cells, three with WASP-positive PBMC and the WASP-negative group, are summarized graphically in Fig. 3. We hypothesize that the differ-
ent WASP levels in EBV cells and PBMC of group A patients result from (approximately) normal synthesis coupled with aber-
antly increased degradation. This putative scenario is suggested also by the high levels of mature size WASP RNA in group A cells, 87%, 86%, 69%, and 85% of normal for EBV cell lines P31, P32, P41, and P44 cells, respectively, in contrast to the modest levels, 10% of normal, in the group B cell line (19).

**Discordant expression of group C mutated WASP in T cells and B cells**

To explain the group C pattern of WASP-positive PBMC and WASP-negative EBV cell lines, two hypotheses were considered. First, expression vs non-expression of mutated WASP might be determined by environmental forces acting on cells, and such forces would be expected to differ for transformed cells in culture compared with primary cells in circulating blood. Alternatively, the negativity of the EBV cell lines might reflect an intrinsic in-
ability of B lymphoid cells to express WASP with group C muta-
tions. In the latter case, peripheral blood B cells of group C pa-
tients should also be WASP negative.

To test the latter hypothesis, we separately evaluated CD4+ cells (T cells) and CD19+ cells (B cells) isolated by immunomagnet-
ic purification from patient blood. For normal individuals, the levels of WASP are 0.99 ± 0.07 U/mg protein in T cells (CD4+ cells) and 0.80 ± 0.07 U/mg protein in B cells (\(n = 5\)). When WASP levels in isolated patient T and B cells were each expressed

### Table I. WASP absence in PBMC of a subgroup of WAS patients

<table>
<thead>
<tr>
<th>Code</th>
<th>Patient</th>
<th>Mutation</th>
<th>Expected Effect</th>
<th>WASP in EBV Cells (%)</th>
<th>WASP in PBMC (%)</th>
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<tbody>
<tr>
<td>P37</td>
<td>MT</td>
<td>Intron 1, g→a (+1)</td>
<td>Donor site</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P35</td>
<td>OG</td>
<td>Exon 2, C298A</td>
<td>Tyr168 to stop</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P40</td>
<td>KS</td>
<td>Exon 3, G326 delete</td>
<td>Frameshift/stop</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P38</td>
<td>MWh</td>
<td>Intron 3, g→a (+1)</td>
<td>Donor site</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P43</td>
<td>SB</td>
<td>Intron 3, t insert (+2)</td>
<td>Donor site</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P51</td>
<td>SV</td>
<td>Exon 7A763 delete</td>
<td>Frameshift</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>P52</td>
<td>AA</td>
<td>Exon 12, T1519 delete</td>
<td>No stop</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

* WASP levels in EBV cell lines are from Ref. 19.
* WASP levels in PBMC are from ≥2 assays of ≥2 cell lysates except for the bracketed values from a single cell lysate
* MWh was the source of the EBV cell line; the PBMC are from his affected brother.
* NA, not available.

### FIGURE 2

Different WASP expression patterns, designated A, B and C, in patients with positive PBMC. WASP levels in EBV cell lines are from Ref. 19, except for G291A (Ref. 7). The first patient listed for each mutation was the source of the EBV cell line. Shown are mean levels ± SEM of ≥2 assays of ≥2 cell isolations; bracketed values are from a single cell lysate. The following patients are from the same kindreds: (WR and SW), (SM and NM), (MW and CW), and (OW and PB).
as a percentage of the corresponding normal population, no significant difference was found between the T cell level and the B cell level for group A patient P32, or group A patient P50, or group B patient P34 (Fig. 4). On the other hand, five group C patients with four different mutations had readily detectable WASP levels in their isolated CD4$^+$ T cells (6–13% of normal levels), but no detectable WASP in their isolated B cells (Fig. 4). These findings demonstrate that peripheral blood B lymphoid cells, like EBV-transformed B cell lines, are unable to express WASP with group C mutations.

**WASP levels in patient neutrophils**

Patients who have WASP-negative PBMC were found to have WASP-negative neutrophils (5 patients), and most patients with WASP-positive PBMC were found to have WASP at slightly lower levels in neutrophils. The latter pattern was found for eight group A patients (mean of 12.4% in PBMC, 8.6% in neutrophils) and six group C patients (mean of 11.0% in PBMC, 4.9% in neutrophils) (P33 and P39 mutations). However, the group B kindred with mean WASP$_{PBMC}$ of 12% have WASP-negative neutrophils, and the P54 group C kindred with mean WASP$_{T}$ cells of 15% also have WASP-negative neutrophils. Thus, the pattern of expression of mutated WASP genes in neutrophils is dissimilar to both T cells and B cells, i.e., does not correlate with the mutation grouping established here for lymphoid cells.

**WASP levels in patient platelets**

Isolated platelets in sufficient numbers for WASP assay were obtained from 18 patients. Included were splenectomized (n = 14) and eusplenic (n = 4) patients as well as patients with WASP-positive PBMC from group A (n = 6), B (n = 2), and C (n = 7), and also group D (WASP-negative PBMC) (n = 3). Platelets from all of these patients are WASP negative (Table II). This finding strongly suggests that WASP absence in patient platelets contributes to, or accounts for, the uniform severity of the platelet defect in this disease.

**Discussion**

Quantitation of WASP in isolated blood cells of 27 WAS patients revealed a surprising complexity. Lymphoid cells from individual patients represented one of four WASP expression patterns. In contrast, platelets from 18 of 18 patients were WASP negative. The finding of WASP absence in platelets of 18 patients, ages 3 years to 43 years, 14 splenectomized and 4 eusplenic, with platelet counts from 15,000 to 250,000 (platelets/μl), and representing 12 mutations and 4 lymphoid cell expression patterns, strongly suggests that WASP negativity of platelets is a universal feature of the disease. Absence of WASP in patient platelets appears to provide a molecular explanation for the severity and consistency of the platelet defect in the WASP.

Findings in the present study clearly demonstrate that different cell lineages can differ in their ability to handle the same mutated WASP gene. T cells and B cells are discordant for expression of P42, P39, P33, and P54 WASP (Figs. 4 and 5). Expression by...
neutrophils of WASP mutants correlates in most cases with expression by T cells; however, T cells and neutrophils are discordant in handling of the P34 and P54 mutations (Fig. 5). The phenomenon of cell lineage discordancy in expression of mutant genes, an epigenetic phenomenon, is unlikely to be limited to WASP. The mechanisms are largely unknown.

Given the existence of this discordancy, it is possible that patient platelets are WASP negative because the megakaryocytic lineage is particularly stringent and unable to express any mutant WASP gene. This scenario is unlikely, however, because mutated WASP protein was recently detected by immunofluorescence in megakaryocytes generated in vitro from CD34+ cells of two WAS patients (missense mutations) (23).

Alternatively, mutant WASP might be present in megakaryocytes and depleted in platelets due to enhanced proteolysis. Proteolytic depletion of platelet WASP could occur without depletion of megakaryocyte and lymphoid cell WASP because platelets have different proteases and/or because the minimal levels of protein synthesis (protein replacement) in platelets would be permissive for proteolytic depletion. In a previously described case of cell lineage discordancy in expression of a mutant gene, a subset of adenosine deaminase deficiency patients express the enzyme at undetectable or very low levels in erythrocytes, but at much higher levels in lymphocytes; this discordancy was attributed to the degradation of unstable mutated adenosine deaminase, which depletes the enzyme in cells, such as erythrocytes, that cannot synthesize new protein (24). For the putative case of mutated platelet WASP, calpain (Ca2+-dependent neutral protease) should be considered because its levels are high in platelets (25). Also, normal WASP is cleaved when platelet calpain is activated (26), and evidence indicates that WAS platelets have enhanced calpain activation (27). On the other hand, other sensitive calpain substrates, talin and actin binding protein, are not depleted in WAS platelets (27). Another putative mechanism for the absence of WASP in patient platelets is the possible failure of mutated WASP molecules to partition into platelets when the latter bud from megakaryocytes. This hypothesis is consistent with the postulate that megakaryocytes are more sensitive than other hemopoietic cells to loss of

**FIGURE 5.** Variable expression of mutated WASP in different cell lineages. Shown is WASP presence or absence in B cells, T cells, neutrophils, and platelets of patients with the indicated mutations; results are expressed as nondetectable WASP (−0) or positive (+), indicating 5–20% of normal blood cell levels and up to 60% for EBV lines. The 17 values for B cells are from 9 lysates of peripheral blood B cells and 14 values for EBV cell lines; where both were studied (six mutations), the same result was obtained.

<table>
<thead>
<tr>
<th>Lymphoid Cell Pattern</th>
<th>Patient</th>
<th>Spleenectomy</th>
<th>Mutation</th>
<th>WASP in PBMC (%)</th>
<th>WASP in Platelets (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CN</td>
<td>−</td>
<td>Exon 1, C163G</td>
<td>7.6 ± 3.0</td>
<td>−0</td>
</tr>
<tr>
<td>A</td>
<td>CD</td>
<td>+</td>
<td>Exon 2, C168T</td>
<td>11.9 ± 4.4</td>
<td>−0</td>
</tr>
<tr>
<td>A</td>
<td>SM</td>
<td>+</td>
<td>Exon 2, G257A</td>
<td>12.0 ± 0.9</td>
<td>−0</td>
</tr>
<tr>
<td>A</td>
<td>NM</td>
<td>+</td>
<td>Exon 2, G257A</td>
<td>(11.1 ± 0.5)</td>
<td>(−0)</td>
</tr>
<tr>
<td>A</td>
<td>MR</td>
<td>+</td>
<td>Exon 2, G291A</td>
<td>8.6 ± 4.0</td>
<td>−0</td>
</tr>
<tr>
<td>A</td>
<td>NK</td>
<td>+</td>
<td>Exon 2, G291A</td>
<td>9.3 ± 4.0</td>
<td>−0</td>
</tr>
<tr>
<td>B</td>
<td>MW</td>
<td>+</td>
<td>Exon 10, G1305 delete</td>
<td>12.6 ± 1.9</td>
<td>−0</td>
</tr>
<tr>
<td>B</td>
<td>CW</td>
<td>+</td>
<td>Exon 10, G1305 delete</td>
<td>11.3 ± 3.0</td>
<td>−0</td>
</tr>
<tr>
<td>C</td>
<td>LA</td>
<td>+</td>
<td>Exon 1, C71T</td>
<td>7.0 ± 1.3</td>
<td>−0</td>
</tr>
<tr>
<td>C</td>
<td>LA</td>
<td>−</td>
<td>Intron 6, g→a (+5)</td>
<td>16.2 ± 2.3</td>
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<tr>
<td>C</td>
<td>OW</td>
<td>−</td>
<td>Intron 6, g→a (+5)</td>
<td>8.8 ± 3.3</td>
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<tr>
<td>C</td>
<td>TS</td>
<td>+</td>
<td>Intron 6, g→a (+5)</td>
<td>15.4 ± 2.7</td>
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<tr>
<td>C</td>
<td>LP</td>
<td>+</td>
<td>Intron 6, g→a (+1)</td>
<td>9.8 ± 1.5</td>
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</tr>
<tr>
<td>C</td>
<td>TM</td>
<td>+</td>
<td>Intron 7, g→a (+5)</td>
<td>(15.3)</td>
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<tr>
<td>C</td>
<td>SM</td>
<td>+</td>
<td>Intron 7, g→a (+5)</td>
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<td>(−0)</td>
</tr>
<tr>
<td>D</td>
<td>OG</td>
<td>−</td>
<td>Exon 2, C298A</td>
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<td>−0</td>
</tr>
<tr>
<td>D</td>
<td>KS</td>
<td>+</td>
<td>Exon 3, G326 delete</td>
<td>−0</td>
<td>−0</td>
</tr>
<tr>
<td>D</td>
<td>SB</td>
<td>+</td>
<td>Intron 3, 1 insert &gt; (+2)</td>
<td>−0</td>
<td>−0</td>
</tr>
</tbody>
</table>

* Lyymphoblastoid cell expression patterns and WASP levels in PBMC are from Fig. 2 and Table I.
* WASP data are from ≥2 assays of ≥2 lysates except for the bracketed values from single lysates.
* WASP lymphoblastoid cell values for TM and SM are the average for single lysates of isolated peripheral T lymphocytes.

Table II. WASP absence in platelets of diverse WAS patients
WASP (28) and with studies of a megakaryoblastic cell line, which strongly suggest a functional role for WASP in megakaryocyte differentiation (29).

The finding of WASP negativity in the severely dysfunctional patient platelets supports the hypothesis that WASP levels in lymphocytes are also important in determining disease severity. Overall, the present findings support an inverse correlation of WASP lymphoid cell levels and severity of immune dysfunction in most cases. For example, all of the seven WASP-negative patients have severe disease, and the patients with WASP-positive lymphocytes (groups A and B) have mild or moderate disease.

The patients categorized as group A based on their lymphoid cell WASP expression patterns all have exon 1 and 2 missense mutations that map to the PH1 (pleckstrin homology 1) domain (12), which has been previously associated with milder disease (Refs. 7 and 28, and reviewed in Ref. 30). We hypothesize that enhanced posttranslational modification accounts for the decreased WASP levels in group A PBMC. The different WASP_{EBVL} and WASP_{EBV} levels for individual group A mutations may reflect different ratios of synthesis to proteolysis in PBMC and proliferating cell lines.

Evidence indicates that transcriptional or translational events are responsible for the decreased or absent WASP levels in most other mutations. Of the group D WASP-negative mutations, P35, an exon 2 nonsense mutation, and P40, an exon 3 frameshift, have no detectable mature RNA, indicating that their mutated WASP genes are not transcribed.

The group B P34 cell line has substantially decreased levels (10% of normal) of mature size RNA, strongly suggesting that the similarly decreased WASP protein levels are due to limiting events at the transcriptional level.

Most surprising are the group C mutations. These diverse mutations, donor splice site mutations in introns 6 and 7, an intron 6 acceptor site mutation, and an exon 1 nonsense mutation, were grouped initially based on discordant WASP expression in PBMC (positive) and EBV cell lines (negative). Additional experiments showed that the four mutations share discordant WASP expression at the level of peripheral blood cells, i.e., positive peripheral T cells and negative peripheral B cells (Fig. 4). Thus, WASP presence in group C PBMC is due to its presence in cells other than B lymphocytes.

RT-PCR of PBMC of a group C patient with the P39 mutation (intron 6, +5, g→a) revealed low levels of multiple RNA species, and sequencing showed that ~30% of the cloned products were without defect (7). This finding indicates that T cells correctly splice some P39 pre-RNA despite the intron 6 donor site mutation. The finding, together with absence of protein and mature size RNA in P39 EBV cells (19), suggests that T cells and B cells differ in the stringency of RNA splicing requirements. For a group C patient with the P33 mutation (intron 6, −1, g→a), it is likely that the significant discordant event in T cells and B cells is also at the transcriptional level because the patient’s B cell line lacks WASP RNA (19). RT-PCR of this patient’s PBMC showed multiple RNA species at low levels (L. Jones and E. Remold-O’Donnell, unpublished observations), suggesting that P33 T cells produce (some) mature WASP RNA despite the acceptor site mutation, either by low level use of the mutated splice site or by the use of an alternative splice site(s). The third group C splice site mutation (P54), intron 7, +5, g→a, has not been further studied.

In cells of the group C P42 patients (exon 1, Arg^{13}→stop), the discordant event responsible for WASP expression in T cells and non-expression in B cells, is apparently at the translational level because the P42 B cell line, which lacks WASP protein, has near normal levels of mature size WASP RNA (19). Inspection of the mutated gene sequence suggests that synthesis of WASP by P42 T cells occurs by initiation (or reinitiation) at an internal AUG codon downstream of the mutant stop codon, possibly at the Met^{38} codon. Thus, the discordant mechanisms that allow T cells, but not B cells, to generate WASP protein from group C mutated genes appear to be variable, which, if correct, hints at an overall greater stringency of gene expression mechanisms in B cells compared with T cells.

In terms of disease severity, group C patients are diverse. When seen as young children, the two P42 patients were categorized as severe, the P33 patient as moderate, and most of the P39 patients and P54 patients as mild phenotype (formerly called X-linked thrombocytopenia). This diversity is not surprising because, although all group C patients express some levels of WASP in their T cells, P42-WASP and probably P33-WASP are defective proteins, whereas P39-WASP protein may be normal.

In an apparently unfortunate feature of group C mutations, like group D, the clinical picture seems to be that, with time, a number of the patients develop B cell lymphomas, including one of the two P42 patients (the second underwent early bone marrow transplant), the P33 patient, and members of two of the three P39 kindreds described here. It is thus possible that the increased incidence of B cell malignancies in the WAS (31–33) includes disproportionate numbers of patients with pattern C mutations or patients with WASP-negative B cells (groups C and D). Additional data are needed to test this postulate and, if confirmed, to unravel the underlying mechanism.

Although there are four lymphoid cell expression patterns, the absolute WASP levels in patient PBMC varied only over the range 0 to 20%. Assuming that the patients studied here are representative and that WASP is not secondarily degraded in another disorder, assay of WASP levels in PBMC could be used as a criterion for diagnosis because patient levels would be readily distinguishable from normal. This suggestion has been made previously (e.g., Refs. 34 and 35) and, indeed, a flow cytometric method appropriate for clinical assay of WASP was recently developed (35). The present data on 27 patients provides substantial justification for clinical assay of WASP as part of diagnosis.

WASP levels have been assayed for EBV cell lines of >60 patients (5, 7, 19, 34), but levels in PBMC have been previously determined for only 15 patients (7, 34). We note, and cannot completely explain, the discrepancy between the present finding of 20 of 27 WAS patients with WASP-positive PBMC, but only 1 of 13 patients with WASP-positive PBMC in an earlier study (34). Some of the difference appears due to patient selection, which was almost random in the present study, whereas the earlier study involved primarily severe phenotype patients. On the other hand, the WASP-negative patients in the earlier study included two with the Val_{75}→Met mutation, characterized here for patients SM and NM as WASP positive.

The correlation of genotype/phenotype in the WAS, proposed by several investigators (3, 7, 28, 36, 37), remains controversial because of several cases of patients with the same mutation and different clinical outcomes (e.g., Refs. 38, and 39). Some disparities are caused by difficulty in assigning clinical scores and other confounding factors, including variable quality of medical care and deterioration of T cell number and function over time, so that clinical score can vary with patient age. Despite these problems, there is fairly general agreement that most patients with missense mutations, at least exons 1 and 2 missense mutations, have mild or mild/moderate disease, and most patients with null mutations and some frameshift and splice site mutations have severe disease (reviewed in Ref. 30).
The present study greatly enlarges the primary cell WASP database and demonstrates the absence of WASP in all patient platelets examined, providing a putative molecular explanation for the uniformly severe platelet defect in this disease. The study also identifies unexpected cell lineage discordancy in the processing of some mutated WASP genes and a need to separately analyze T cells and B cells to assess the mutational burden acting on individual patients’ immune cells.

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