Spontaneous In Vivo Reversion of an Inherited Mutation in the Wiskott-Aldrich Syndrome


*J Immunol* 2001; 166:5245-5249; doi: 10.4049/jimmunol.166.8.5245

http://www.jimmunol.org/content/166/8/5245

References

This article cites 33 articles, 14 of which you can access for free at:

http://www.jimmunol.org/content/166/8/5245.full#ref-list-1

Why The JI? Submit online.

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
The Wiskott-Aldrich syndrome (WAS), 1 (MIM 301000) is an X-linked primary immunodeficiency disease characterized by thrombocytopenia, immunodeficiency, eczema, and susceptibility of lymphoreticular malignancy (1–3). Varied immunological abnormalities in cellular and humoral immune systems were observed (4). In addition to the functional abnormalities, lymphocytes from WAS patients showed the morphological abnormalities of reduced number of microvilli on the surface examined by scanning electron microscopy (SEM) (5). The gene defective in WAS has been identified by the linkage studies (6) and subsequent positional cloning (7). The gene product, termed as WAS protein (WASP), was expressed mainly in all nonerythroid hemopoietic cells (8) and was characterized to play roles in signal transduction pathway for cell growth (9) and in cytoskeletal organization (10). The WASP mutation of the patient was A to G conversion at the 32nd codon of the gene had taken place in vivo. The patient was a 32-year-old male, whose clinical feature will be described in detail elsewhere (T. Kondoh and H. Moriuchi, manuscript in preparation). WAS diagnosis was made from the clinical evaluation when he was 1 year old and was confirmed by molecular studies at 29 years of age. The WASP mutation of the patient was A to G conversion at the nucleotide 354, which would change tyrosine 107 to cysteine. The same mutation was reported in another WAS patient (13). Our patient’s clinical grading score for WAS (14) got worse with aging, scoring 2 at the diagnosis at 2 years old, 3 at 7 years old, 4 at 11 years old, and 5 at 25 years old. During this study, he died from progression of lymphoma and severe infectious episodes. His mother was diagnosed as a WAS carrier by genetic studies as having the same mutant WASP allele as the patient’s.

Materials and Methods

Patient

The patient was a 32-year-old male, whose clinical feature will be described in detail elsewhere (T. Kondoh and H. Moriuchi, manuscript in preparation). WAS diagnosis was made from the clinical evaluation when he was 1 year old and was confirmed by molecular studies at 29 years of age. The WASP mutation of the patient was A to G conversion at the nucleotide 354, which would change tyrosine 107 to cysteine. The same mutation was reported in another WAS patient (13). Our patient’s clinical grading score for WAS (14) got worse with aging, scoring 2 at the diagnosis at 2 years old, 3 at 7 years old, 4 at 11 years old, and 5 at 25 years old. During this study, he died from progression of lymphoma and severe infectious episodes. His mother was diagnosed as a WAS carrier by genetic studies as having the same mutant WASP allele as the patient’s.

Cell lines

T cell lines were established using herpes virus saimiri, as described (15). Established lines were CD8+ predominately (95% were CD8+ referred as CD8+ line). We separated CD4+ cells from the line using MACS cell separation system (Miltenyi Biotec, Bergisch Gladbach, Germany), and subsequently established CD4+ line (98% were CD4+). Purity of each line was repeatedly confirmed. EBV-transformed B cell line was also established by a standard procedure.
FCM-WASP

FCM-WASP was performed as previously described (11, 12) with minor modification. In brief, cells washed in PBS containing 1% FBS were treated with Cytofix/Cytoperm solution from CytoStain kit (PharMingen, San Diego, CA) at 4°C for 20 min. After washing twice with Perm/Wash solution, they were reacted with 1/200 diluted mouse anti-WASP mAb (3F3-A54) (16) or 1/5 diluted mouse IgG1 Ab (Becton Dickinson, San Jose, CA) at 4°C for 30 min. They were then reacted with FITC-labeled goat anti-mouse IgG1 Ab (Southern Biotechnology Associates, Birmingham, AL). For the surface/intracellular dual staining, we first stained the cell surface, followed by washing twice before intracellular staining. Abs used for surface staining were as follows: PE conjugate anti-human CD3, PE conjugate anti-human CD4, PE conjugate anti-human CD8 (Southern Biotechnology Associates), PE conjugate anti-human CD20 (Beckman Coulter, Fullerton, CA). Because 3F3-A54 Ab belongs to murine IgG1 subclass, all murine Abs used for cell surface staining were IgG2a to prevent a crossing reaction. To examine the clonality of established T cell lines, murine Abs to human αβ TCR V regions (Endogen, Cambridge, MA) were used.

Detection of the reverse mutation

The fragment including the WASP exons 3 and 4, in which the patient’s mutation was located, was PCR amplified with the primers (TGAAAA CTCTCCTACCTTCCACCGAC, ACTCTACCTCTGCCCAACTTCCTC), as described previously (17). The purified fragment was directly sequenced using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and an automated ABI 373A DNA sequencer. The mutation eliminates the AccI recognition site in the fragment. Then the different PCR products were digested with restriction enzyme AccI (Takara Shuzo, Kyoto, Japan) and were electrophoresed on agarose gel.

HLA typing studies

DNA typing for identification of HLA class I and DRB1 alleles was performed by PCR sequence-specific primer methods and PCR microsatellite plate hybridization methods (18), respectively. Type of HLA class I was determined using Micro SSP class I Generic Typing Kit (Veritas, Tokyo, Japan), and type of HLA-DRB1 was determined using a kit for HLA-DR (Tutzing, Germany). The detection sensitivity of this method was reported (19) as 1:10³.

Microsatellite polymorphic analysis

Microsatellite polymorphic markers in six individual loci (20–25), HGH (17q22–24), INT2 (12q13), D6S89 (6p), CCG (2q36–37), AluVpA1 (1q32), and ACTBP2 (6), were used. After PCR amplification with specific primers reported, the products were electrophoresed on 10% polyacrylamide gel, and visualized by silver staining.

SEM studies

T cell lines from the patient, another WAS patient, and a normal control were subjected for SEM studies, as previously described (26). Briefly, the cells were washed in Ca⁴⁺/Mg²⁺-free PBS and allowed to bind to 3-aminopropyltriethoxysilane (Sigma, St. Louis, MO)-coated slides before fixation in 1.25% of glutaraldehyde in PBS for 30 min. The cells postfixed in 1% osmium tetroxide for 30 min, were dehydrated with graded ethanol, subjected to critical point drying from carbon dioxide, and finally coated 1% osmium tetroxide for 30 min, were dehydrated with graded ethanols, and propyl-triethoxysilane (Sigma, St. Louis, MO)-coated slides before fixation in 1.25% of glutaraldehyde in PBS for 30 min. The cells postfixed in 1% osmium tetroxide for 30 min, were dehydrated with graded ethanol, subjected to critical point drying from carbon dioxide, and finally coated with gold. Specimens were visualized in a scanning electron microscope (Hitachi, Tokyo, Japan), and photographed at a magnification of 7,000 to 10,000.

Results

The WAS⁺bright cells belong to CD3⁺/CD8⁺ and CD3⁺/CD4⁺ population

We found a WAS patient who possessed the small population of intracellular WASP⁺bright lymphocytes by FCM-WASP (Fig. 1A), which was a very unique finding we have never observed (11). The population was ~10% of lymphocytes. When monocytes were gated and analyzed, we could not detect a definite number of the WASP⁺bright cells. To characterize which class of lymphocytes was involved, the surface/intracellular dual staining was performed. The WASP⁺bright cells belong to CD3⁺/CD8⁺, but not to the CD20⁺ population cells (Fig. 1B). Although a few numbers of CD3⁺/CD4⁺ cells seemed to be WASP⁺bright, this could not be unequivocally established. Then we established CD3⁺/CD8⁺ and CD3⁺/CD4⁺ lines and examined whether the WASP⁺bright cells were detectable or not in the lines. Just after establishment of each cell line, the WASP⁺bright population was detected in ~60% of the CD3⁺/CD8⁺ line, but 0.5% in the CD3⁺/CD4⁺ line. However, the WASP⁺bright cells in the CD3⁺/CD4⁺ line rapidly expanded to 80% after 2 wk of cultivation (Fig. 2). No WASP⁺bright cell was detected in CD8⁺ cells. To characterize which class of lymphocytes was involved, the surface/intracellular dual staining was performed. The WASP⁺bright cells belong to CD3⁺/CD8⁺, but not to the CD20⁺ population.
Detection of the reverse mutation in the WASP<sup>bright</sup> cells

Sequencing results of the mutation site, using DNAs from the varied WASP<sup>bright</sup> populations from the patient, are shown (Fig. 3A). When DNA from the patient’s PBMC (mixture of lymphocytes and monocytes) was examined, the mutation of A354G was repeatedly observed. With a careful observation, however, a small peak of “A” was seen under a big peak of “G.” It turned out that the small “A” was not a noisy signal, because when DNA from the T cell lines of the 60% WASP<sup>bright</sup> population was examined, the “A” and “G” were almost equal level just like the results of the patient’s mother. With DNA from the 80% WASP<sup>bright</sup> population, the “G” was dominated by the “A.” The ratio of the WASP<sup>bright</sup>/WASP<sup>dim</sup> population was correlated with the nucleotide peaks of “A/G” ratio. The same results were obtained with the AccI digestion studies of the PCR fragment including the mutation site (Fig. 3B). Because the AccI cut the normal PCR fragment into two small pieces, the detection of the small pieces represents the normal “A” nucleotide at the mutation site. The very faint small pieces were seen with the digestion of the fragment derived from the patient’s PBMC. When the fragment from the T cell lines with 40% or 80% WASP<sup>bright</sup> population was digested, the digested small pieces were clearly observed. The amount of the small pieces increased in proportion to the WASP<sup>bright</sup> population.

Studies for the origin of the WASP<sup>bright</sup> cells

HLA DNA typing studies and DNA microsatellite studies were performed using both the WASP<sup>bright</sup> (T cell line) and the WASP<sup>dim</sup> (B cell line) populations. No HLA class I or HLA DRB1 alleles other than the patient’s own type were detected using the WASP<sup>bright</sup> samples (data not shown). Moreover, the identical pattern between the WASP<sup>bright</sup> and the WASP<sup>dim</sup> populations was observed in six microsatellite polymorphic loci (Fig. 4). These results clearly prove that the WASP<sup>bright</sup> cells were derived from the patient himself.

Structural abnormality was corrected in some cells from the patient’s WASP<sup>bright</sup> T cell lines

The results of the SEM studies of the patient T cell lines, the T cell lines from another WAS patient, and a normal individual were used as control. When DNA from the patient’s PBMC (mixture of lymphocytes and monocytes) was examined, the mutation of A354G was repeatedly observed. With a careful observation, however, a small peak of “A” was seen under a big peak of “G.” It turned out that the small “A” was not a noisy signal, because when DNA from the T cell lines of the 80% WASP<sup>bright</sup> population was examined, the “A” and “G” were almost equal level just like the results of the patient’s mother. With DNA from the 80% WASP<sup>bright</sup> population, the “G” was dominated by the “A.” The ratio of the WASP<sup>bright</sup>/WASP<sup>dim</sup> population was correlated with the nucleotide peaks of “A/G” ratio. The same results were obtained with the AccI digestion studies of the PCR fragment including the mutation site (Fig. 3B). Because the AccI cut the normal PCR fragment into two small pieces, the detection of the small pieces represents the normal “A” nucleotide at the mutation site. The very faint small pieces were seen with the digestion of the fragment derived from the patient’s PBMC. When the fragment from the T cell lines with 40% or 80% WASP<sup>bright</sup> population was digested, the digested small pieces were clearly observed. The amount of the small pieces increased in proportion to the WASP<sup>bright</sup> population.

Discussion

In this study, we report a WAS patient who had some T cells with spontaneous in vivo reversion of an inherited mutation. The unusual FCM-WASP finding (Fig. 1) forced us to study the small population of WASP<sup>bright</sup> mononuclear cells from the patients, and the molecular analysis of these cells revealed that the mutation was disappeared (Fig. 3, A and B). We attempted to define the mechanism of how the WASP<sup>bright</sup> cells occurred in the patient. His mother had been molecularly diagnosed as a WAS carrier in a previous study, and we again verified that she had the mutant allele of nucleotide A354G. Thus, the possibility that the WASP<sup>bright</sup> cells resulted from somatic mosaicism due to de novo mutation during the embryogenesis was ruled out. The WASP<sup>bright</sup> cells were identified to originate exclusively in the patient himself by a HLA typing study. No HLA class I or HLA DRB1 alleles other than the patient’s own type was detected (data not shown). We further confirmed it by analysis of six microsatellite polymorphic loci (Fig. 4). The identical pattern in the six loci between the WASP<sup>bright</sup> and WASP<sup>dim</sup> cells indicated that both the cells were the same origin. Therefore, the possibility of the engraftment of his mother’s cells in utero or other individuals’ cells by blood transfusions, was excluded. The possibility of mosaicism of XXY/XXY in the patient may be proposed. However, this is not the case.

Table I. Results of T cell αβ receptor panel analysis<sup>a</sup>

<table>
<thead>
<tr>
<th></th>
<th>Vα2</th>
<th>Vα12.1</th>
<th>Vβ3.1</th>
<th>Vβ3.1 (a)</th>
<th>Vβ5 (b)</th>
<th>Vβ5 (c)</th>
<th>Vβ6.7</th>
<th>Vβ8 (a)</th>
<th>Vβ12</th>
<th>Vβ13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Established CD8&lt;sup&gt;+&lt;/sup&gt; T line</td>
<td>1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.8</td>
<td>31.5</td>
<td>1.2</td>
<td>1.4</td>
<td>32.6</td>
<td>0.9</td>
<td>0.4</td>
<td>0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Control PBMC</td>
<td>2.5</td>
<td>2.1</td>
<td>5.1</td>
<td>8.0</td>
<td>1.0</td>
<td>3.5</td>
<td>1.6</td>
<td>3.5</td>
<td>2.6</td>
<td>3.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>The clonality of T lines (the CD8<sup>+</sup> WASP<sup>bright</sup> cells) from the patient was studied by TCR panel analysis described in Materials and Methods. PBMC from healthy individuals were used as control.

<sup>b</sup>All values are presented as percentages.
because when we studied the patient’s cells with almost 100% WASPbright, the mutation signal of the “G” was hardly detected. Mixture of the “A/G” should be equally shown if WASPbright cells consisted of XXY in the case. Finally, although in vitro reversion detected in cell lines from a WAS patient had been reported (27), this is not our case, because we detected the WASPbright cells before in vitro culture. The observation that the WASPbright cell population exhibited the polyclonal phenotype by TCR panel analysis also argues against an in vitro reversion (Table I). Taking these results together, we conclude that the WASPbright cells originated from his own hemopoietic cells with spontaneous in vivo reversion of an inherited mutation in the WASP gene.

Then, which hemopoietic cell was implicated for the event of the reverse mutation? We detected the WASPbright cells in T cells, CD3+CD4+ and CD3+CD8+ cells, but not in CD20+ B cells or in monocytes (Fig. 1B). The clonality study of the WASPbright cell line also indicated polyclonal character. Therefore, the reversion in a pre-T cell precursor could account for these unusual findings; however, we think a more primitive progenitor cell could be responsible. The reversion event probably took place at the level of a single cell. We were only able to identify this event when the reverted cells could gain a growth advantage over the mutant cells and expand in vivo. Recently, we suggested that the WASP had a more critical role in growth and development of lymphocytes (T cells) than those of monocytes (12). Although we do not know whether the WASP also has the critical role for B cells as it does for T cells, a study on WASPdeficient mice revealed a critical role for WASP in T cell but not B cell activation (28). Therefore, our inability to find WASPbright B cells and monocytes might come from a lack of a critical growth advantage in the WASPbright B cells and monocytes. The WASPbright T cells, moreover, showed a growth advantage during in vitro culture. Established T cell lines showed dominant for CD8+ cells, which already consisted of the 60% WASPbright cells. As for CD4+ line, it dramatically changed its WASPbright population from 0.5% to 80% during 2 wk of cultivation (Fig. 2). However, we do not know why CD4+ cells showed such a low number of the WASPbright cells in vivo. Another possibility is that the WASP may contribute to T cell transformation process in vitro.

We were curious whether there might be a structural change of the WASPbright cells from the patient. It has been shown previously that lymphocytes from WAS patients show the abnormalities on SEM studies (5). Lymphocytes from WAS patients were devoid of fine cell surface microvilli that were densely observed on cells from normal individuals. The results of the morphological studies on SEM of the patient’s T cell lines, the T cell lines from another WAS patient, and a normal individual are shown (Fig. 5A–D). The cells with dense microvilli surface projections were frequently observed in the patient’s T cell lines containing the 80% WASPbright population (Fig. 5A), just like normal T cell lines (Fig. 5D). However, some cells with ruffled or ridgelike surface projections, which were reported as the characteristic abnormalities of WAS lymphocytes (5) (Fig. 5C), were still detected in the patient’s T lines (Fig. 5B). Thus, it was shown that the spontaneous reverse mutation resulted in a restoration of the structural abnormalities of the patient’s cells.

Spontaneous in vivo reversion of mutation has been reported in a patient with adenosine deaminase deficiency (29) and a patient with X-linked SCID (30). Both the patients were characterized with their progressive mild clinical course probably due to the reverse mutation in some lineage cells. Recently, we also detected reverse mutation in T cell lines from two patients with adenosine deaminase deficiency (31). In this study, the patient could survive >30 years without receiving bone marrow transplantation. Because few WAS patients survived such a long period without bone marrow transplantation, the reversion event may have some beneficial effects on the patient’s clinical course. However, the effects were not enough because he died from progression of lymphoma and severe infectious episodes at 32 years old. We cannot estimate when the reversion event occurred in the patient; thus, it could be too late for him to get a sufficient benefit from it. Alternatively, the normalization of some of the T cells might not be enough. In fact, the WASPdeficient macrophages/monocytes may be responsible for the most critical immune defects observed in WAS patients (32).

The present studies may have significant implications regarding the prospects of the future gene therapy for WAS patients. First, it was shown that the gene-corrected T cells perhaps revealed growth advantage over other cells. Our findings also indicate that if only the WASP gene is introduced into a single progenitor cell and is expressed, it will make a significant population of T cells in vivo. We previously reported a WAS patient with dual mutations, an inherited and a de novo mutation (33). We found that the patient had major population of mononuclear cells with the dual mutations because additional de novo mutation could partially restore the WASP function and make a growth advantage in preference to the
cells with the single mutation. Second, the WASP may not contribute to growth and development of B cells or monocytes as much as of T cells. If it is the case, the expansion of gene-introduced B cells or monocytes will be delayed in a gene therapy with targeting hematopoietic stem cells. The conditioning to provide advantage for the gene-introduced cells of both lineages may be required. Finally, because of the complexity of the WASP function in vivo, it may be difficult to assess the function of gene-introduced cells. In this regard, SEM studies could be useful for evaluating the efficacy of the gene therapy (34).

Acknowledgments

We thank Dr. Hideaki Kikuta (Department of Pediatrics, Hokkaido University School of Medicine) for providing us with herpes virus saimiri.

References