Morphological Abnormalities in the Lymphocytes of Patients With the Wiskott-Aldrich Syndrome

By Dianne Kenney, Lloyd Cairns, Eileen Remold-O'Donnell, Jeffrey Peterson, Fred S. Rosen, and Robertson Parkman

Lymphocytes from 18 patients with the Wiskott-Aldrich Syndrome (WAS) were examined by scanning electron microscopy (SEM). Most peripheral blood lymphocytes from normal individuals are covered with slender microvillus projections, but a large proportion of lymphocytes from WAS patients were found to be devoid of microvilli. A lymphocyte morphology scoring system was developed to quantify the density of microvilli: Grade 4 classified those lymphocytes with <75% of the surface covered with microvilli with progressive decrements to grade 1, which were those without microvilli. The mean lymphocyte morphology score of eight normal individuals was 3.62 ± 0.22. The mean lymphocyte score of WAS patients was substantially lower (2.89 ± 0.27, P < 0.001). In addition, WAS lymphocytes often were qualitatively abnormal, with short, blunt microvilli. These morphologic criteria were used to diagnose WAS from the cord blood lymphocytes of one "at-risk" patient. Thus, WAS is the first primary immunodeficiency in which morphological abnormalities have been identified that can aid in diagnosis.

THE WISKOTT-ALDRICH syndrome (WAS) is an X-linked recessive disorder characterized by thrombocytopenia with platelets of reduced size and function, progressive deterioration of T lymphocyte function, an inability to produce antibodies to carbohydrate antigens, and eczema. It is difficult to make a definitive clinical diagnosis in some patients because of variability in the immunologic manifestations of the disease.

We previously reported that WAS is characterized by a molecular defect in a lymphocyte membrane surface component, a sialoglycoprotein, gp115. To determine if lymphocyte abnormalities could be detected at the morphological level, lymphocytes from 18 WAS patients were examined by scanning electron microscopy (SEM) and their morphology was compared with that of lymphocytes from eight normal individuals.

METHODS

Patients. The diagnosis of WAS was based on male sex, severe thrombocytopenia with platelets of reduced size and function and, in some patients, a family history of the disorder. Many of the patients had eczema and clinical immunodeficiency. Six patients had been splenectomized; 16 had platelet counts of <100,000/µL. Their median age was 4 years (range one day to 25 years; five patients, ≤1 year).

Cell isolation. Peripheral blood was collected with parental consent in preservative-free heparin (100 U/mL) or acid-citrate-dextrose (ACD; NIH, formula A) (0.17 mL/mL). Blood specimens were maintained at room temperature and either processed immediately or the day after shipment. All patient specimens were obtained by teasing thymic tissue removed as a routine part of open heart surgery. Thymocytes were resuspended in RPMI with 2% FCS for one hour at 37 °C and resuspended in RPMI without serum. Lymphocytes from 18 patients with the Wiskott-Aldrich Syndrome (WAS) were examined by scanning electron microscopy (SEM). Most peripheral blood lymphocytes from normal individuals are covered with slender microvillus projections, but a large proportion of lymphocytes from WAS patients were found to be devoid of microvilli.

Morphology. Preliminary examination of lymphocytes from two WAS patients revealed an apparent decrease in the frequency of cells densely covered with microvillus surface projections. To evaluate the morphological alterations of the lymphocytes, a scoring system (summarized in Table I and in the Results section) was established. When morphology was intermediate, the higher score was given. One of the observers scored lymphocytes from 12 of 19 patients and 6 of 8 normal subjects in a blind fashion. (ie, without knowledge of their origin). The mean difference in the scores of the two observers for all patient and normal lymphocytes was minimal (0.17 ± 0.11). Scores reported are the mean of the two observers' scores. Scores were not influenced by choice of anticoagulant or whether lymphocytes were isolated immediately or after overnight shipment. Statistical significance was determined by the nonparametric Wilcoxon rank-sum test comparison of two groups in independent samples, and results are expressed as the mean ± SD.

RESULTS

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WAS lymphocytes and to compare them with normal lymphocyte morphology, a grading system was established based on the quality and density of surface projections on normal lymphocytes (Table 1). Lymphocytes with microvillus projections on >75% of the surface were scored as grade 4, whereas lymphocytes with microvillus projections on <75% of the cell surface were scored as grade 3 (Fig 1A). Lymphocytes with only ruffled or ridge-like projections (thymocyte-like) were scored as grade 2, whereas cells with no projections were scored as grade 1 (Figs 1B, C, and D). Normal peripheral blood lymphocytes are primarily grade 4 (65.8% ± 18.9%) and grade 3 (29.4% ± 21.0%) cells with occasional grade 2 (2.8% ± 4.1%) or grade 1 (1.9% ± 2.5%) cells (Table 1). The mean score of lymphocytes from normal individuals was 3.62 ± 0.22 (n = 8). Normal thymocytes were composed of >95% grade 2 cells with occasional grade 3 or grade 1 cells and had a mean score of 1.99 ± 0.01 (n = 2) (Figs 1B and 2). Peripheral blood lymphocytes from 18 WAS patients were analyzed; the mean score was 2.89 ± 0.27 (n = 18, P < .001 as compared with normal lymphocytes). The lower score was due to a lower percentage of grade 4 cells and a higher percentage of grade 3 and grade 2 cells (Table 1). The change in the percentage of grade 1 cells was not significant. There were no differences between the absolute lymphocyte counts of the patients and the normal individuals. The lymphocyte score of 17 of 18 patients did not overlap with the normal range (Fig 2). No change in the morphology of WAS (or normal) lymphocytes resulted from holding whole blood overnight prior to isolating lymphocytes; for example, the mean morphology scores of a single patient's lymphocytes isolated immediately and, on a separate occasion, after 24-hour shipment of whole blood, were 3.03 and 3.11, respectively. The extent of morphological alterations

<table>
<thead>
<tr>
<th>Grade</th>
<th>Surface Morphology</th>
<th>Normal Lymphocytes (%)</th>
<th>WAS Lymphocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Villus projections on &gt;75% of cell surface</td>
<td>65.8 ± 18.9</td>
<td>22.5 ± 13.0, P = .001</td>
</tr>
<tr>
<td>3</td>
<td>Villus projections on &lt;75% of cell surface</td>
<td>29.4 ± 21.0</td>
<td>54.1 ± 15.3, P = .005</td>
</tr>
<tr>
<td>2</td>
<td>Ridgelike projections</td>
<td>2.8 ± 4.1</td>
<td>15.7 ± 7.9, P = .001</td>
</tr>
<tr>
<td>1</td>
<td>No projections</td>
<td>1.9 ± 2.5</td>
<td>7.7 ± 7.9, NS</td>
</tr>
</tbody>
</table>

Scoring system to evaluate lymphocyte morphology was based on the quality and density of surface projections found in scanning electron micrographs of peripheral blood lymphocytes of eight normal individuals and 18 WAS patients.

Fig 1. (A) Normal lymphocytes, grades 4 and 3; (B) normal thymocytes, grade 2; (C) WAS lymphocytes, grades 3 and 2; (D) WAS lymphocytes, grade 1. Bars = 1 μm.
MORPHOLOGICAL ABNORMALITIES IN WAS

Fig 2. Mean lymphocyte morphology scores of peripheral blood lymphocytes from eight normal individuals (PBL), and 18 WAS patients (WAS). Eusplenic (x) and splenectomized (o) WAS patients are indicated. Arrows indicate mean of the scores for the normal and patient groups: PBL = 3.62 ± .22; WAS = 2.89 ± .27.

and lower numerical scores in WAS lymphocytes did not correlate with the severity of the patient's thrombocytopenia or clinical immunodeficiency (not shown). The mean morphology score of lymphocytes from the six splenectomized patients was 3.16 ± 0.13, whereas the mean score of the 12 eusplenic patients was lower—2.81 ± 0.3. Although the numerical difference between the mean scores of the two patient subgroups was not statistically significant, scores of all splenectomized patients are clustered above 3, whereas scores of eusplenic patients ranged broadly from 2.46 to 3.37 (Fig 2).

In addition to the quantitative difference reflected by the lymphocyte morphology score, qualitative differences were also noted between normal and WAS lymphocytes. The microvillus projections of many WAS lymphocytes were shorter and blunter relative to the more filamentous projections characteristic of normal peripheral blood lymphocytes (Figs 1C and D). WAS lymphocyte surface morphology was clearly different from the ruffled or ridgelike projections evident on almost all normal thymocytes, however, (compare Figs 1B and C).

Neonatal diagnosis. The neonatal diagnosis of WAS can be difficult to make even if thrombocytopenia is present; T lymphocyte function is usually normal and eczema is never present. Adequate cell numbers for surface radiolabeling and/or electrophoretic studies are unobtainable. The cord blood lymphocytes of the male sibling of a WAS patient were collected and analyzed by SEM. The mean score of the cord blood lymphocytes was 2.65 ± 0.14. Further, the neonate's grade 4 and 3 lymphocytes were qualitatively abnormal, with blunted microvilli (Fig 3). The surface morphology of cord blood lymphocytes from two normal neonates could not be distinguished from that of normal peripheral blood lymphocytes (not shown). The patient's cord blood platelet count was 75,000/μL. During the first week of life, his platelet count decreased to 20,000/μL. The patient died of WAS at 4 months of age.

DISCUSSION

The Wiskott-Aldrich Syndrome is a multi-faceted clinical syndrome in which affected males have thrombocytopenia with platelets of reduced size and function, eczema, and immunodeficiency with decreased T lymphocyte function and inability to make antibodies to carbohydrate antigens. The demonstration of glycoprotein abnormalities in both the platelets and lymphocytes of WAS patients suggested a common basis for the platelet and T lymphocyte functional deficiencies. The present observations demonstrate that the peripheral blood lymphocytes of WAS patients, like their platelets, are morphologically abnormal.

In the present study, SEM demonstrated that lymphocytes from WAS patients had a marked deficiency of surface microvilli. In agreement with a previous study, >95% of normal lymphocytes were found to be densely covered with microvillus projections. Only 77.8% of WAS lymphocytes displayed the same density of microvillus projections. A scoring system for evaluating surface morphology was established. The mean score for the lymphocytes from 17 of 18 (neonate excluded, see below) WAS patients was below the range of scores of normal lymphocytes. The one patient whose score overlapped with the low normal range (Fig 2) did not differ in any clinical characteristics (degree of immunodeficiency, eczema, or thrombocytopenia) from the other WAS patients.

The immunologic deficiency in WAS is progressive, and the problems of recurrent infections and eczema are the most
variable clinical manifestations and often the last to appear. Of the 18 WAS patients studied (excluding the one neonate), 11 had fully developed eczema and 6 were clinically immunodeficient. Immune abnormalities were detected in most patients, however, by anergy in delayed hypersensitivity skin testing, abnormal circulating T cell phenotypes, poor T cell proliferative responses to specific antigens and mitogens (spontaneous or after immunization), and failure to make antibodies after immunization with specific antigens. In this study, patients were diagnosed as WAS patients based on the dependable criteria of male sex, family history of WAS, and thrombocytopenia with platelets of reduced size. The finding of defective lymphocyte morphology in all these patients suggests that lymphocyte structural alterations represent a previously undescribed and invariable manifestation of the syndrome. This morphological defect is evident early, before clinical immunodeficiency and eczema are manifest, and is independent of splenectomy status and the severity of thrombocytopenia and clinical immunodeficiency. Thus, defective lymphocyte morphology as assessed by SEM represents an additional diagnostic criterion to distinguish WAS in very young or mildly affected patients.

Only 1 to $2 \times 10^6$ purified lymphocytes are required for SEM. The morphological defect in WAS lymphocytes is independent of the manipulations used to isolate lymphocytes, since SEM analysis of the unfractionated buffy coat layer (at the plasma/red cell interface after gravity sedimentation of whole blood) can detect the presence of morphologically defective WAS lymphocytes (not shown). Therefore, it is possible to evaluate patients from whom only small blood samples are available. SEM was also used to diagnose WAS in one at-risk neonate who could not have been diagnosed on clinical grounds until later. Eventually, it may be possible to apply this diagnostic method to blood samples drawn prenatally.

The relationship between abnormal lymphocyte morphology and immune dysfunction in WAS is not clear. As the simplest interpretation, the lower lymphocyte morphology scores suggest that WAS patients have increased numbers of circulating thymocytes. The surface morphology of WAS lymphocytes, however, with fewer and more blunt surface projections than normal lymphocytes, is qualitatively different from that of thymocytes, which have ridgelike or ruffled surface projections. Furthermore, lymphocyte morphological abnormalities and lower morphology scores were found in all patients whether or not immature T cells could be detected by phenotypic analysis of peripheral blood lymphocytes (not shown). SEM analysis of the lymphocytes of patients with some other primary immunodeficiencies [ataxia telangiectasia (2 patients), common variable agammaglobulinemia (1 patient), hyper-IgM syndrome (2 patients), severe combined immune deficiency (2 patients)] has not revealed any morphological lymphoid abnormalities, indicating that altered lymphocyte morphology is not a generalized characteristic of the immunodeficiency state (not shown). Successful bone marrow transplantation corrected the lymphocyte morphological abnormality in three WAS patients studied posttransplant (not shown).

A small percentage of lymphocytes devoid of microvilli are consistently found in normal individuals (ref 6 and Table 1). We hypothesize that the higher percentage of lymphocytes devoid of microvilli in WAS patients results from removal of lymphocyte membrane and represents an accentuation in the patients of the normal mechanisms of lymphocyte turnover. A recent study by Corash and co-workers demonstrated a role for the spleen in an immunologically mediated process that results in the structurally abnormal, small platelets of the WAS. Thus, one interpretation of our findings is that the abnormalities in lymphocyte membrane glycoprotein, gpL115, lead to the splenic removal of membrane fragments and result in the loss of microvillus projections from the lymphocyte surface. The differences in the mean morphology scores of nonsplenectomized and splenectomized WAS patients examined in this study, however, were not statistically significant. We have not yet serially examined lymphocytes of WAS patients after splenectomy, when even a transient improvement in the morphology score would indicate a role for splenic removal of microvilli.

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REFERENCES
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