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Wiskott-Aldrich syndrome (WAS) is an X-linked disorder characterized by thrombocytopenia, eczema, and progressive decline of the immune function. In addition, lymphocytes and platelets from WAS patients have morphologic abnormalities. Since chemokines may induce morphologic changes and migration of leukocytes, we investigated the monocyte response to chemoattractants in cells from WAS patients with an identified mutation in the WAS protein gene. Here, we report that monocytes derived from four patients with molecularly defined typical WAS have a severely impaired migration in response to FMLP and to the chemokines monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α compared with normal donors. Conversely, neither MCP-1 binding to monocytes nor induction of the respiratory burst by MCP-1 and FMLP is significantly different between WAS patients and normal donors. Within a few minutes of stimulation, monocytes respond to chemokines with increased expression of adhesion molecules and with morphologic changes such as cell polarization. Although up-regulation of CD11b/CD18 expression following stimulation with FMLP or MCP-1 is preserved in WAS patients, cell polarization is dramatically decreased. Staining of F-actin by FITC-phalloidin in monocytes stimulated with chemoattractants shows F-actin to have a rounded shape in WAS patients, as opposed to the polymorphic distribution of F-actin in the polarized monocytes from healthy donors. These results suggest that WAS protein is involved in the monocyte response to the chemokines MCP-1 and macrophage inflammatory protein-1α.


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2 Address correspondence and reprint requests to Dr. Raffaele Badolato, Clinica Pediatrica, Universita' di Brescia, Udine, Italy. E-mail address: badolato@master.cci.unibs.it

3 Abbreviations used in this paper: WAS, Wiskott-Aldrich syndrome; DTH, delayed-type hypersensitivity; WASp, Wiskott-Aldrich syndrome protein; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; SOD, superoxide dismutase.
Materials and Methods

Patients

Four patients with molecularly defined WAS were included in this study. Patients were evaluated and treated at the Department of Pediatrics, University of Brescia. Monocytes constituted 5 to 15% of all leukocytes in the patients studied. As a control, PBMC were obtained, after informed consent was granted, from age-matched subjects who were hospitalized for minor head trauma.

Cell culture

PBMC were purified by Ficoll separation medium (Lymphocyte-H, Cedarlane Laboratories, Hornby, Canada) gradient centrifugation as described previously (22). Monocytes constituted 30 to 40% of PBMC as determined by a direct immunofluorescence assay using the mAb CD14 (Dako, Glostrup, Denmark). When indicated, monocytes were purified by Percoll separation medium (Pharmacia Biotech, Uppsala, Sweden) as described previously (23). Cells were cultured in RPMI 1640 (HyQ, HyClone Europe, U.K.) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 20 mM HEPES (Imperial, Andover Hants, U.K.), and 10% heat-inactivated FCS (Boehringer Mannheim, Mannheim, Germany). Human recombinant MCP-1, MIP-1α, and RANTES were obtained from Pepro Tech (Rocky Hill, NJ). All reagents and media, tested by The Endotoxin Kit (Sigma, St. Louis, MO), contained endotoxin at levels <12 pg/ml.

Cytokine determination

The MCP-1 concentration was assessed by an ELISA kit purchased from R&D Systems Europe (Abingdon, U.K.), following the manufacturer’s instructions.

Migration assays

Migration of monocytes (1.5 × 10^6 cells/ml in RPMI 1640 and 1% BSA) was evaluated by a microchamber technique as described previously (24). For monocytes, 5-μm pore size polycarbonate filters (Neuro Probe, Cabin John, MD), were employed. Under the assay conditions employed, only monocyte in PBMC preparations migrated across the filter. At the end of the incubation (90 min), filters were removed, fixed, and stained with Diff-Quick (Harleco, Gibbstown, NJ), and three oil immersion fields were counted in a blind evaluation after coding samples. In each assay, FMLP (Sigma) at a concentration of 10 nM was used as a standard chemoattractant for monocytes.

MCP-1 binding

Biotin-conjugated MCP-1 (R&D Systems, Minneapolis, MN) was used, following manufacturer’s instructions. Briefly, cells (10^9/100 μl) were washed twice with PBS, resuspended in 50 μl of RPMI 1% BSA, and incubated at 16 to 20°C with 10 μl of biotin-conjugated-MCP-1 (R&D, Minneapolis, MN). After 15 min, 10 μl of avidin-FITC (R&D Systems) was added to each sample, and the incubation was continued at 4°C for 30 min. Cells were then washed with cold medium (RPMI/1% BSA) twice, resuspended in PBS plus 1% paraformaldehyde, and analyzed by a flow cytometer FACSscan (Becton Dickinson, San Jose, CA). Binding activity was expressed as the mean channel fluorescence of MCP-1-avidin binding cells (>90% of monocytes were positive for MCP-1 binding).

FACS analysis

Whole blood treated with FMLP was washed and incubated with saturating concentrations of CD11b (Dako) or control mouse-IgG (Dako) for 30 min at 4°C. Cells were washed twice with PBS, resuspended in 100 μl of PBS, and incubated at 4°C for 30 min with 4 μl of FITC-conjugated rabbit anti-mouse IgG (Dako). RBCs were then lysed by incubating the blood with 4 ml of FACS lysing buffer (Becton Dickinson) for 5 min at room temperature. After three washes with PBS, cells were resuspended in PBS plus 1% paraformaldehyde and analyzed with a FACSscan (Becton Dickinson). At least 5000 events were acquired, and on the basis of forward and side scatter, the window for monocyte-gated cell was set.

Monocyte polarization assay

The polarization assay was performed with purified monocytes in suspension stimulated with chemoattractants as described previously (25). Briefly, purified monocytes were stimulated, in duplicate, with chemoattractants or with medium alone for variable lengths of time (2–10 min) at room temperature in polypropylene tubes. Stimulation was stopped by adding an equal volume of 10% formaldehyde to the medium. After coding samples, at least 200 cells were counted and classified on the basis of their morphology (spherical or head-tail shape) by an independent investigator. Data were expressed as a percentage.

Immunofluorescence staining of F-actin

The monocyte polarization assay in response to FMLP, MCP-1, and MIP-1α was performed as described above. At the end of the incubation period, monocytes were centrifuged over a slide at 700 rpm for 10 min by cytospin. Monocytes adherent to the slide were washed twice with PBS, incubated with 0.165 μM FITC-phalloidin or with a control IgG conjugated to FITC for 30 min at 4°C, and washed. Slides were stained with FITC-phalloidin and then photographed by a fluorescence microscope. Slides stained with FITC-IgG did not display any detectable fluorescence.

Superoxide anion production

Superoxide production by monocytes was measured as superoxide dismutase (SOD; Sigma)-inhibitable cytochrome c reduction by a modified

Table I. Summary of clinical aspects and mutations detected in WAS patients included in the study

<table>
<thead>
<tr>
<th>Patientsa</th>
<th>Age (yr)</th>
<th>Scoreb</th>
<th>Mutation</th>
<th>Predicted Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>W1</td>
<td>6</td>
<td>2</td>
<td>del C907</td>
<td>Y291X</td>
</tr>
<tr>
<td>W2</td>
<td>3</td>
<td>5</td>
<td>C155T</td>
<td>R41X</td>
</tr>
<tr>
<td>W3</td>
<td>4</td>
<td>2</td>
<td>g→a at +1, intron 8</td>
<td>Splicing defect</td>
</tr>
<tr>
<td>W4</td>
<td>1</td>
<td>2</td>
<td>Ins C364 fs</td>
<td>H111 X126</td>
</tr>
</tbody>
</table>

a Patient W1 was 6 mo old, patient W2 was 3 yr old, W3 was 4 yr old, and W4 was 1 yr old when the study started. At diagnosis, all patients had severe thrombocytopenia with platelet counts ranging from 5 to 38 × 10^9/l and reduced mean platelet volume (4.5 to 5.9 fl; normal values 7 to 9.5). All patients had mild eczema; in addition, W2 developed severe autoimmune manifestations (ulcerative colitis). Mutations of WASP gene were identified in all patients by SSCP analysis and PCR sequencing, performed as described (39). Patient W1 has a deletion of nucleotide C 907 that introduces a stop codon at nucleotide 954 of the cDNA sequence. Patients W2 and W3 (who have a nonsense mutation in exon 1 and a splice mutation in exon 8, respectively) are described as patient 4 and patient 12 in a previous publication (39). Patient W4 has an insertion of a C nucleotide at position 361 in exon 2.

b Clinical score was determined according to Zhu et al. (40).
Pick and Mizel method (26). Briefly, monocytes, contained in PBMC preparations were resuspended in medium (Hanks’ containing 5 mM glucose, 0.5 mM calcium chloride, 4 mM sodium azide, and 80 μM cytochrome c; Sigma) and preincubated in 96-well microtiter plates for 5 min at 37°C. Stimulation of cells was performed in triplicate with or without addition of SOD (250 μg/ml) at 37°C for 60 min. At 5-min intervals, the OD at 550 nm of each reaction mixture was determined in an ELISA reader. Superoxide production was calculated from the difference in the ODs at 550 nm between the wells with and without SOD and was converted to nanomoles of superoxide anion per 2 x 10^5 monocytes.

**FIGURE 2.** Chemotaxis of monocytes in response to MCP-1 in WAS patients. MCP-1 (50 ng/ml) or medium alone was placed in the lower wells of a microchemotaxis chamber. Monocytes (1.5 x 10^6 cells/ml in RPMI 1640 containing 1% BSA) were added to the upper wells. The results are expressed as the mean (±SD) number of cells that migrated across the filter in three high power fields (HPF), counted in triplicate, after subtraction of the number of cells that migrated in response to medium alone. Statistical analysis of the chemotactic response to MCP-1 between WAS patients and normal donors was performed using nonparametrical analysis (Mann-Whitney test).

**FIGURE 3.** Chemotaxis of monocytes in response to MIP-1α in WAS patients. MIP-1α (50 ng/ml) or medium alone was placed in the lower wells of a microchemotaxis chamber. Monocytes (1.5 x 10^6 cells/ml in RPMI 1640 containing 1% BSA) were added to the upper wells. The results are expressed as the mean (±SD) number of cells that migrated across the filter in three high power fields (HPF), counted in triplicate, after subtraction of the number of cells that migrated in response to medium alone. Statistical analysis of the chemotactic response to MIP-1α between WAS patients and normal donors was performed using nonparametrical analysis (Mann-Whitney test).

**FIGURE 4.** Chemotaxis of monocytes in response to MCP-1, MIP-1α, and FMLP in WAS patients: dose response. Increasing concentrations of MCP-1 (10, 50, and 200 ng/ml), MIP-1α (10, 50, and 200 ng/ml), or FMLP (10^-7, 10^-8, and 10^-9 M) were placed in the lower wells of a microchemotaxis chamber. Monocytes (1.5 x 10^6 cells/ml in RPMI 1640 containing 1% BSA) were added to the upper wells. The results are expressed as the mean (±SD) number of cells that migrated across the filter in three high power fields (HPF), counted in triplicate. Results shown are representative of three independent experiments performed on three patients and three normal subjects. Statistical analysis of the chemotactic response to the chemoattractants was based on nonparametrical analysis (Kruskal Wallis test). Asterisks indicate a significant difference (p < 0.05).
Comparisons between normal donors and WAS patients were performed where indicated using nonparametric analysis for unpaired data.

Results
We studied the chemotactic response of monocytes obtained from WAS patients and from normal donors in response to the bacterial product FMLP. This agent, used at a concentration of 10 nM, induced a reduced chemotactic response in monocytes obtained from WAS patients compared with normal donors (Fig. 1). FMLP, like most of chemotactic agonists, including C5a, platelet-activating factor, and chemokines, induces migration of leukocytes by activating G protein-coupled receptors (27).

To determine whether WAS patients have a generalized defect of monocyte chemotaxis, we studied monocyte responses to the chemokines MCP-1 and MIP-1α. With both chemokines, the chemotactic responses of monocytes obtained from WAS patients were strongly reduced compared with those of monocytes from normal donors (Figs. 2 and 3). To investigate the extent of the defect, we used different concentrations of the three chemotactic agents (10, 50, and 100 ng/ml) or different time points for the assay (Figs. 4 and 5). We found that the chemotactic responses of monocytes from WAS patients to the chemokines or to FMLP were consistently defective at concentrations that induced an optimal response. In addition, monocyte chemotaxis was significantly reduced in WAS patients at all points analyzed.

A reduction of the chemotactic response might be caused by prolonged exposure of cells to chemoattractants, resulting in receptor desensitization. High concentrations of MCP-1 or other chemokines in the blood of WAS patients might reduce chemotactic response of leukocytes to a second stimulation (17, 28).

### Table II. Monocyte desensitization in WAS patients

<table>
<thead>
<tr>
<th>Cells Preincubated with</th>
<th>Medium</th>
<th>FMLP</th>
<th>MCP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>5.9 ± 1.9³</td>
<td>1.2 ± 0.9³</td>
<td>4.9 ± 1.3</td>
</tr>
<tr>
<td>WAS</td>
<td>3.1 ± 1.2³</td>
<td>2.4 ± 1.6</td>
<td>2.0 ± 0.6³</td>
</tr>
<tr>
<td>MCP-1</td>
<td>3.1 ± 1.0³</td>
<td>1.4 ± 1.9³</td>
<td>3.5 ± 0.9³</td>
</tr>
<tr>
<td>MCP-1</td>
<td>1.5 ± 2.5</td>
<td>1.5 ± 1.7</td>
<td>1.0 ± 0.7³</td>
</tr>
</tbody>
</table>

*Monocytes (2 × 10⁶ cells/ml) were in medium, with or without FMLP (100 nM), with MCP-1 (200 ng/ml) or with medium alone, at 37°C for 30 min. After two washes, their chemotactic response to the same chemoattractants FMLP (10 nM), and MCP-1 (50 ng/ml) was assessed. Results are the means (± SE) chemotaxis index derived from three experiments performed in three WAS patients and three normal donors.

³ Significant chemotactic response in comparison to medium (p < 0.05).

Statistical analysis
Comparisons between normal donors and WAS patients were performed where indicated using nonparametric analysis for unpaired data.
concentrations were consistently significant levels of MCP-1 in the plasma of WAS patients may be mediated at least in part through down-regulation of chemokine receptors on monocytes (29). Although we could not detect significant levels of MCP-1 in the plasma of WAS patients (concentrations were consistently <500 pg/ml; tested in four WAS patients), we investigated MCP-1 binding to monocytes and desensitization by FMLP or by MCP-1. Saturating concentrations of biotinylated MCP-1 were incubated with PBMC obtained from three WAS patients or from three normal donors. After a second staining with FITC-avidin, the comparison of fluorescence intensity of MCP-1-stained monocytes did not demonstrate any significant difference between WAS patients (199 ± 12 as mean channel units) and normal subjects (210 ± 16; data not shown). However, a study of binding activity to monocytes would not detect possible functional defects. Thus, we studied whether the mechanisms of receptor desensitization were functioning in WAS patients. Monocytes obtained from three normal donors or from three WAS patients were incubated for 30 min with FMLP, MCP-1, or medium alone, then washed with medium and tested for their chemotactic responses to the same chemotactants. Under these experimental conditions, the monocyte response to regulation with FMLP and MCP-1 decreased as expected in both normal donors and WAS patients (Table II). This result indicated that receptor desensitization was not involved in the mechanism of reduction of monocyte chemotaxis in WAS patients. Thus, we studied other functions elicited by chemotactants in monocytes. FMLP and MCP-1 are also potent activators of respiratory burst and cell surface integrin expression, two events that are crucial for the induction of antibacterial properties and for monocyte adhesion (21). We investigated whether the defective chemotactic response to chemokines in monocytes from WAS patients is associated with reduced activation of the respiratory burst. We found that the production of superoxide anion, as measured by cytochrome c reduction, is induced by FMLP and MCP-1 to a similar extent in patients and controls (Fig. 6). Since stimulation of monocytes with chemotactic factors such as FMLP and Serum amyloid A (rSSA) increases their surface expression of CD18/CD11b (22), we investigated the effect of FMLP on the expression of CD11b on monocytes from normal donors or WAS patients. Whole blood was used in these assays because in many cases isolation of monocytes already results in considerable surface expression of CD18/CD11b (22). Incubation of cells with FMLP for 15 min induced an enhancement of cell surface expression of CD18/CD11b in both monocytes from normal donors and those from WAS patients, indicating that the observed defect in the monocyte response to chemokines is not dependent on a defect in CD11b expression (Fig. 7).

Induction of monocyte chemotaxis by chemokines is preceded by cell polarization, an event that occurs within 10 min after the stimulation. This event is marked by several morphologic changes, including formation of cell protrusions and pseudopodia, possibly mediated by GTPases such as Rho, Cdc42, and Rac (14, 27). Moreover, some of these GTPases have been reported to interact with WASp in a transfected cell system (9–12). Indeed, monocytes from WAS patients displayed a severe defect in cell polarization after stimulation with either FMLP or MCP-1 compared with the response of monocytes from normal donors (Table III).

Table III. Monocyte polarization in response to FMLP and MCP-1

<table>
<thead>
<tr>
<th>Subject</th>
<th>Medium</th>
<th>FMLP</th>
<th>MCP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>27.0 ± 3.2</td>
<td>51.9 ± 4.1</td>
<td>48.6 ± 3.6</td>
</tr>
<tr>
<td>N2</td>
<td>20.5 ± 4.6</td>
<td>38.3 ± 4.3</td>
<td>43.4 ± 6.5</td>
</tr>
<tr>
<td>N3</td>
<td>10.6 ± 1.6</td>
<td>54.3 ± 4.5</td>
<td>43.7 ± 3.1</td>
</tr>
<tr>
<td>W1</td>
<td>17.1 ± 4.1</td>
<td>20.2 ± 3.7</td>
<td>19.7 ± 2.2</td>
</tr>
<tr>
<td>W2</td>
<td>9.5 ± 4.8</td>
<td>12.7 ± 6.1</td>
<td>6.9 ± 7.9</td>
</tr>
<tr>
<td>W3</td>
<td>20.4 ± 4.9</td>
<td>21.3 ± 3.2</td>
<td>19.7 ± 5.5</td>
</tr>
</tbody>
</table>

*Monocytes (2 × 10⁶ cells) were stimulated in 200 µl of medium (RPMI 1% FCS), with FMLP (10 nM), with MCP-1 (100 ng/ml), or with medium alone, at 20 to 22°C. After 10 min the stimulation was stopped with an equal volume of 10% formaldehyde, and the number of cells with polarized morphology were counted. Data are expressed as the percentage of polarized cells above the total number of cells counted. Experiment representative of three performed.

Significant difference of polarization response between normal controls and WAS patients after stimulation with the chemotactrant compared to medium (p < 0.05).

Significant polarization response in comparison to medium (p < 0.05).
Cell polarization and locomotion require a co-ordinated series of intracellular events including polymerization of filamentous actin (F-actin) (30). To better evaluate the differences in shape change in response to chemokines in WAS patients, we stained monocytes stimulated by MCP-1, MIP-1α, or FMLP with FITC-conjugated phalloidin, a fluorescent probe specific for F-actin (Fig. 8). Normal monocytes stimulated by FMLP, MCP-1, or MIP-1α showed a polarized distribution of F-actin with pseudopod formation. In contrast, monocytes obtained from WAS patients maintained a rounded shape upon stimulation.

**Discussion**

We report that monocytes from WAS patients have a reduced response to FMLP and to the chemokines MCP-1 and MIP-1α. Monocyte refractiveness to chemotactants is not related to receptor desensitization in vivo as hypothesized by Altman et al. (31), as we failed to detect increased levels of MCP-1 in the plasma of WAS patients, and their monocytes display a normal desensitization pattern in response to both FMLP and MCP-1. This observation was further confirmed by our finding that monocytes from WAS patients display a binding activity to MCP-1 comparable to that of monocytes derived from normal donors, as shown by flow cytometric analysis of biotinylated MCP-1 binding. Furthermore, monocytes from WAS patients, stimulated with MCP-1 or by FMLP, release superoxide anion and up-regulate cell surface expression of CD11b/CD18 to the same extent as monocytes obtained from normal donors, indicating a selective defect in cell motility rather than a generalized abnormality of chemokine-mediated responses.

In leukocytes, chemotacticants induce actin polymerization, integrin up-regulation, and superoxide anion production through G protein-coupled receptors; these receptors activate a cascade of intracellular events, including changes in cytosolic...
free calcium and release of phosphoinositides (30). In this signaling pathway, proteins with GTPase activity, such as Rho, Cdc42, and Rac, are involved (14, 27). These proteins belong to the Ras superfamily and determine membrane ruffling and pseudopodia formation in fibroblasts (14). The active form of Cdc42 was found to interact with WASp (9–11). Following interaction with Cdc42, WASp forms clusters of aggregation of F-actin (9). We found that monocytes from WAS patients, after stimulation with MCP-1 or with FMLP, lack the capability of cell polarization and show a diffuse distribution of F-actin in cells with a rounded shape, in contrast to the accumulation of F-actin in pseudopods as observed in normal donors after stimulation with FMLP, MCP-1, or MIP-1α. These results demonstrate that monocytes from WAS patients have a defective cell polarization in response to chemotaxotants and suggest that WASp is involved in the regulation of F-actin polymerization in vivo (9). In addition, our results suggest that the defect of cell polarization observed in WAS patients may determine the reduction of leukocyte chemotaxis in response to chemotaxotants. A possible explanation for the monocye defects observed in WAS patients is based on the hypothesis that in normal individuals, following stimulation with chemokines, Cdc42 would interact with WASp and thereby induce F-actin polymerization. A similar role has been postulated for the GTP binding protein Rac in the induction of respiratory burst in response to chemokines in polymorphonuclear cells, but has not been described for Cdc42 (32). The small GTP binding proteins, Rho and Rac, are essential intracellular components of the superoxide anion synthase that translocate to the membrane and activate the enzyme after polymorphonuclear cells stimulation with FMLP or IL-8 (27, 33). We speculate that chemokines such as MCP-1 and MIP-1α might activate Cdc42, and that, in turn, WASp, bound to activated Cdc42, might constitute the anchor for G-actin polymerization.

The decreased chemotactic response of monocytes in WAS patients suggests possible defects of leukocyte recruitment in inflamed tissues in these subjects. Leukocyte migration and homing are induced by local production of chemokines in inflammatory reactions, as observed in dermatitis and in DTH; in these cases, chemokine release in the derma was shown to be required for leukocyte recruitment (34, 35). Our observation that monocytes from WAS children have deficient chemotactic responses to MCP-1 and to other chemokines, but produce normal amounts of MCP-1 when activated by LPS (data not shown) provides a possible explanation for the impaired DTH that is often observed in WAS patients, even when the in vitro lymphocyte proliferative response to mitogens is preserved (1, 3). Furthermore, chemokines such as MIP-1α, IL-8, and Stromal derived factor-1 are involved in the regulation of chemotaxis and the proliferation of hemopoietic precursors (36, 37). Whether interactions with narrow stromal cells and a proliferative advantage of hemopoietic precursors with intact WASp function account for the nonrandom pattern of X-chromosome inactivation reported in CD34+ hemopoietic progenitor cells from WAS carrier females remains to be seen (38).

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References