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R F Todd 3rd, … , W A Peters, B M Babior

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Research Article

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Subcellular Localization of the Large Subunit of Mo1 ($Mo1_\alpha$; formerly gp 110), a Surface Glycoprotein Associated with Neutrophil Adhesion

Robert F. Todd III, M. Amin Arnaout, Richard E. Rosin, Carol A. Crowley, Wendy A. Peters, and Bernard M. Babior

Departments of Medicine and Pediatrics and the Blood Research Laboratory, Tufts-New England Medical Center, Boston, Massachusetts 02111; and Divisions of Nephrology and Cell Biology, Children's Hospital, and of Tumor Immunology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115

Abstract. $Mo1_\alpha$ (formerly gp 110) is a neutrophil glycoprotein whose deficiency is associated with abnormalities in several neutrophil functions, including defects in adherence, chemotaxis, and phagocytosis. Examination of whole cells and subcellular components by the use of both immunological and electrophoretic techniques demonstrated that $Mo1_\alpha$ was located primarily in the specific granules but that a small portion was present in the plasma membrane, where it is exposed to the extracellular environment and can bind to anti-Mo1 antibody. During degranulation, $Mo1_\alpha$ is translocated from the specific granules to the plasma membrane, resulting in a 5–10-fold increase in the surface expression of this glycoprotein. These findings plus previous work suggest that plasma membrane-associated $Mo1_\alpha$ is needed for a normal interaction between neutrophils and underlying surfaces, and raise the possibility that the increase in surface adhesiveness of neutrophils that have discharged their specific granules might be due in part to the increase in the amount of $Mo1_\alpha$ in the plasma membranes of these degranulated cells.

Introduction

We have recently described two patients with an inherited abnormality of neutrophil function leading to recurrent pyogenic infections accompanied by a leukemoid reaction (1–3). Gel electrophoresis showed that neutrophils from both of these patients lacked a specific particle-associated glycoprotein. In one patient, the missing protein was assigned a molecular weight of 110,000 (1), whereas the molecular weight of the protein missing in the other patient was estimated to be 150,000 (3); reanalysis has indicated that the two missing proteins are similar in size, migrating with apparent molecular weights of 155,000 in 7% polyacrylamide gels and 180,000 in 12% gels[1] (Arnaout and Babior, unpublished observations). In one of the patients, this glycoprotein has been found to represent the α-subunit of a heterodimeric glycoprotein designated Mo1 (gp 94, 155) which is expressed on the surface of normal neutrophils, monocytes, and null cells (5, 6).

The association between the deficiency of the glycoprotein and the various functional defects observed in neutrophils from deficient patients has led to the speculation that the glycoprotein may itself be required for the performance of these functions (1–3). This idea is supported by the observation that the exposure of normal neutrophils to anti-Mo1 monoclonal antibody produces functional defects that closely parallel those exhibited by the two patients (7).

In this paper we report that the α-subunit of Mo1 (hereafter designated $Mo1_\alpha$), now shown to be deficient in both patients, is a constituent not only of neutrophil plasma membranes but also of specific granules, which in fact contain most of the glycoprotein found in the cell. Moreover, degranulation of normal neutrophils leads to a 5–10-fold increase in the amount of $Mo1_\alpha$ in the plasma membrane, suggesting that specific granule-associated $Mo1_\alpha$ is transferred to the plasma membrane during specific granule discharge. The possible physiological

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1. This gel concentration-dependent variation in the apparent molecular weight as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis is typical of glycoproteins (4). To assign a true molecular weight to the protein will require more extensive structural studies.

significance of this shift of $Mo1_\alpha$ from granules to membrane is discussed.

Methods

Dextran 70, 6% (wt/vol) in 0.9% saline (Macrodex) and Ficoll-Hypaque were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Bovine erythrocyte superoxide dismutase, catalase (bovine liver, thymol free), dried *Micrococcus lysodeikticus*, A23187, o-dianisidine, diisopropylphosphofluoridate (DFP),[2] NADH, and pyruvate substrate were obtained from Sigma Chemical Co. (St. Louis, MO). CN-^{3}H-Cbl (5.1 Ci/mmol) and carrier-free Na^{125}I were purchased from New England Nuclear (Boston, MA). Gel electrophoresis equipment and reagents, including Coomassie Blue dye, were supplied by Bio-Rad Laboratories (Richmond, CA). Other reagents were the best quality commercially available, and were used without further purification.

Neutrophils. Neutrophils were prepared by dextran sedimentation followed by Ficoll-Hypaque centrifugation as previously described (8). Preparations contained >99% granulocytes.

Tube gel electrophoresis of whole particulate fractions. Particles were prepared and analyzed by tube gel electrophoresis (Laemmli method, 10% gels [9]) as previously described (1). Gels were stained with Coomassie Blue or periodic-acid Schiff (10) and destained in 7% acetic acid.

Separation and slab gel electrophoresis of subcellular fractions. Neutrophils (5×10^8 cells in 10 ml Dulbecco's phosphate-buffered saline [PBS] without calcium or magnesium but with 1 mM glycylglycine buffer pH 8.0) were mixed with 0.1 M DFP in dry ethylene glycol (1) at a ratio of 0.05 ml/ml suspension and incubated at 0°C for 10 min to inhibit proteases. They were then centrifuged, resuspended in 10 ml of fresh calcium- and magnesium-free PBS, and incubated for 1 hr at 0°C to destroy residual DFP. The cells were centrifuged again, suspended at 2×10^7 cells/ml in 0.25 M sucrose which had been brought to pH 7.4 with 0.1 M NaOH, and disrupted at 0°C in a sonicator (model W-220F; Heat System-Ultrasonics, Inc., Plainview, NY) fitted with a water-jacketed cup horn by use of one 15-s burst at full power. Subcellular components were separated by sucrose density gradient centrifugation according to Wright and Gallin's modification (11) of the method of West et al. (12), except that the concentrations of sucrose at the extremes of the gradient were 31.3 and 75.5% (wt/vol). The composition of each sucrose density gradient fraction was determined by the measurement of myeloperoxidase (azurophil granules) (13), lysozyme (azurophil and specific granules) (14) or cobalamin-binding protein (specific granules), alkaline phosphatase (plasma membrane) (13), and lactate dehydrogenase (cytosol) (15). These assays indicated that granule rupture was slight; the specific granules in particular remained almost entirely intact, as shown by the very limited amount of cobalamin-binding protein that appeared in the fractions migrating with the cytosol (2.0 and 6.3% of the total in two separate preparations). The fractions were pooled as indicated below, then centrifuged to yield pellets consisting primarily of azurophil granules, specific granules, or plasma membrane. Whole 105,000 *g* pellets were prepared as previously described (1). The pellets were dissolved by suspending in 0.3 ml Laemmli sample buffer (9) and boiling for 4 min. Samples were analyzed by electrophoresis on 7 or 12% polyacrylamide slab gels according to the method of Laemmli (9). Stained gels were scanned with a gel scanner (model 1310; ISCO, Lincoln, NE). Integration was accomplished by the cutting out and weighing of peaks of interest from copies (Xerox Corp., Stamford, CT) of the scans, as described elsewhere (1).

Analysis of degranulation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Neutrophils were treated with DFP as described above, except that after the residual DFP was destroyed, the cells were suspended in 1.5–5 ml Hank's balanced salt solution (HBSS) at a concentration of 5×10^7 cells/ml. Discharge of specific granules was induced by incubation of the cell suspension with 0.1 vol of 10 μM A23187 in HBSS for 25 min at 37°C in a shaking water bath. As a resting control, cells were incubated similarly with 0.1 vol of HBSS alone. The reaction mixtures were then centrifuged for 2.5 min at low speed in a tabletop centrifuge, the supernatants were frozen, and the cells were resuspended in 1 ml HBSS. A 50-μl portion of this cell suspension was frozen after a further dilution with 0.95 ml HBSS. The rest of the suspension was then centrifuged again, and the cells were suspended in 0.25 M sucrose at a concentration of 2×10^7 cells/ml and fractionated as described in the preceding section. The subcellular fractions were analyzed by electrophoresis on 7% polyacrylamide slab gels according to the method of Laemmli.

Proteins in the supernatants from the A23187-treated neutrophils were subjected to electrophoresis on 7 and 12% polyacrylamide gels according to the Laemmli procedure. For this purpose, the proteins were concentrated by acetone precipitation. The frozen supernatants were thawed, and 0.1-ml portions were reserved for enzyme and protein assays. The remaining portions were mixed with equal volumes of acetone, allowed to stand at 0°C for 30 min, then centrifuged at 25,000 *g* for 30 min at 4°C. The supernatants were carefully poured off, and the precipitates were dried in air at room temperature for 10 min to remove most of the remaining acetone. The precipitated proteins were then dissolved in sample buffer and analyzed by electrophoresis.

Samples of the supernatants and frozen cell suspensions were analyzed for protein, enzymes, and cobalamin binder as described elsewhere in this section. After they thawed, the cell suspensions were homogenized by sonication (one 20-s pulse at maximum power in a sonicator fitted with a cup horn) before the assays were carried out.

Cobalamin-binding protein. Cobalamin-binding protein in the pooled fractions and in the cells and supernatants from the degranulation experiments was measured by a modification of the albumin-coated charcoal technique of Gottlieb et al. (16). Albumin-coated charcoal was prepared by the mixing of a 5% (wt/vol) suspension of activated charcoal powder (unwashed; Mallinckrodt, Inc., St. Louis, MO) in 0.9% saline with an equal volume of 1% (wt/vol) bovine serum albumin in 0.9% saline. The assay was begun by mixing 50 μl pooled fraction with 0.585 ml 0.9% saline, then adding 15 μl 1% Triton X-100 in water followed by 2.5 ng CN-^{3}H-Cbl in 0.1 ml 0.9% saline. After 30 s at room temperature, 0.5 ml of albumin-coated charcoal was added to the assay mixture. After 30 s more the charcoal was removed by centrifugation (12,000 *g* for 15 min), and the amount of cobalamin in 0.625 ml of the filtrate was measured by liquid scintillation counting. This value was corrected for background by subtracting the value obtained when the pooled fraction was replaced with 0.9% saline. The amount of cobalamin-binding protein in the assay mixture (expressed as nanograms bound cobalamin) was then calculated by multiplying the corrected value by 2 (equal to the assay mixture volume divided by the volume of filtrate counted). From this value it was possible to calculate the concentration of cobalamin-binding protein (nanograms bound cobalamin per milligram protein) in the pooled fraction.

2. *Abbreviations used in this paper:* DFP, diisopropylphosphofluoridate; HBSS, Hank's balanced salt solution; LFA, lymphocyte function-associated antigen; PAGE, polyacrylamide gel electrophoresis.

Monoclonal reagents and immunofluorescence analysis. Monoclonal antibodies to Mo1 and Mo5 antigens were developed and characterized as previously reported (6, 7, 17). Indirect immunofluorescence as a measure of antigen expression in neutrophils was carried out by exposing the cells to saturating concentrations of anti-Mo1, anti-Mo5, or a negative control antibody, and then incubating them with a saturating concentration of fluorescein-conjugated goat anti-mouse IgG (Meloy Laboratories Inc., Springfield, VA) as previously described (6). Cells and antibodies were diluted in medium containing 2.5% pooled human AB serum to block nonspecific Fc receptor-mediated antibody binding (6). Immunofluorescence intensity as a quantitative measure of relative antigen expression was analyzed on an Ortho Cytofluorograf FC 200/4800A (Ortho Diagnostic Systems Inc., Westwood, MA) by use of a logarithmic amplifier. The channel number (log scale) representing the peak fluorescence intensity of 10,000 cells exposed to either experimental (anti-Mo1 or Mo5) or control antibodies was determined. Linear fluorescence intensity channels were calculated from a logarithmic-linear calibration curve.

Separation and immunological analysis of subcellular fractions. Subcellular fractions for immunological analysis were prepared exactly as described by Borregaard (18). These were solubilized in NP40 0.5% in PBS for 10 min at 4°C. 50 μg of protein from each fraction was then labeled with $Na^{125}I$ by the chloramine T method (19). ^{125}I incorporation was measured by trichloroacetic acid precipitation and was similar in the three fractions. After extensive dialysis against PBS, each fraction was subjected to immunoprecipitation by anti-Mo1 monoclonal antibody (7) and the specific precipitates were analyzed by autoradiographic examination of material run on SDS 7% polyacrylamide gels. A labeled band at 155,000 mol wt representing Mo1 was detected in the specific granule and plasma membrane fractions but not in the azurophil granule fraction. The relative amount of immunoprecipitated Mo1 in each of these fractions was determined by the cutting out of the regions of the original gel that contained the 155,000 bands (in the case of the azurophil granules, a corresponding region of the gel was excised) and the counting of the excised portions of gel in a gamma scintillation counter.

Protein. Protein was measured by the Bradford method (20) or by the microassay of Schaffner and Weissmann (21).

Results

Deficiency of $Mo1_\alpha$ in neutrophils from the patient described by Crowley et al. (1). The abnormal neutrophils used in the present studies were obtained from the patient of Crowley et al. (1). With respect to his clinical manifestations and the functional abnormalities and glycoprotein composition of his neutrophils, this patient closely resembled the patient whose neutrophils were found by immunological techniques to be deficient in $Mo1_\alpha$ (22).[3] The Crowley patient's neutrophils were therefore examined for $Mo1_\alpha$ by means of the fluorescence-activated cell sorter, using the monoclonal anti-Mo1 antibody previously described (6). The results (Fig. 1) showed that the amount of $Mo1_\alpha$ antigen on the surface of these neutrophils

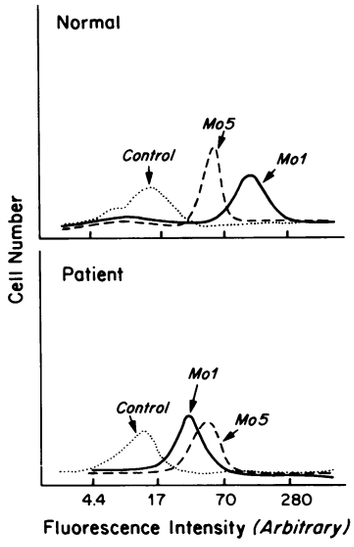

Figure 1. Comparison of Mo1 antigen expression by $Mo1_\alpha$-deficient and normal granulocytes as measured by indirect immunofluorescence. *Top,* immunofluorescent staining of normal granulocytes exposed to negative control (· · · · ·), anti-Mo1 (——), or anti-Mo5 (– – –) antibodies. *Bottom,* immunofluorescent staining of $Mo1_\alpha$-deficient granulocytes exposed to the same reagents.

was greatly decreased,[4] though the cells contained normal amounts of a surface antigen recognized by another antibody, anti-Mo5. This finding strongly suggests that $Mo1_\alpha$ and the glycoprotein missing from the neutrophils of both of these patients are one and the same, and indicate that at least a portion of this glycoprotein is localized to the plasma membrane where it is exposed to the extracellular environment.

$Mo1_\alpha$ in subcellular fractions. The location of $Mo1_\alpha$ was established by comparison of SDS gel electrophoretic patterns of subcellular fractions prepared from normal and $Mo1_\alpha$ deficient neutrophils. Cells were disrupted by gentle sonication and fractionated by sucrose density gradient centrifugation as described in Methods. The concentrations of marker enzymes in the gradient fractions are shown Fig. 2. The fact that only small amounts of lysozyme and myeloperoxidase were found in the lactic acid dehydrogenase-containing (cytosolic) fraction indicates that the granules remained mostly intact during cell disruption.

Gel electrophoretograms of these subcellular fractions are shown in Fig. 3. Each of the fractions from the normal preparation showed a band at apparent molecular weight 180,000 (12% polyacrylamide gel) which was lacking in the corresponding $Mo1_\alpha$-deficient fractions (arrowhead). This band appeared to be much more prominent in the specific granule fraction than in either of the other two fractions, suggesting that $Mo1_\alpha$ is mainly a component of the specific granules. This suggestion was confirmed by comparison of the concentration of $Mo1_\alpha$ in each of these subcellular fractions (measured by gel scanning) with the actual specific granule content of the

3. Minor differences in clinical features between the two patients could be explained on technical grounds, since the patients were studied in two different laboratories. Plans are underway to study the two patients in the same laboratory at the same time.

4. Recent observations show that $Mo1_\alpha$ expression by neutrophils and monocytes from both patients is greatly diminished but not totally absent (Arnaout, M. A., H. Spits, C. Terhorst, J. Pitt, and R. F. Todd III, submitted for publication).

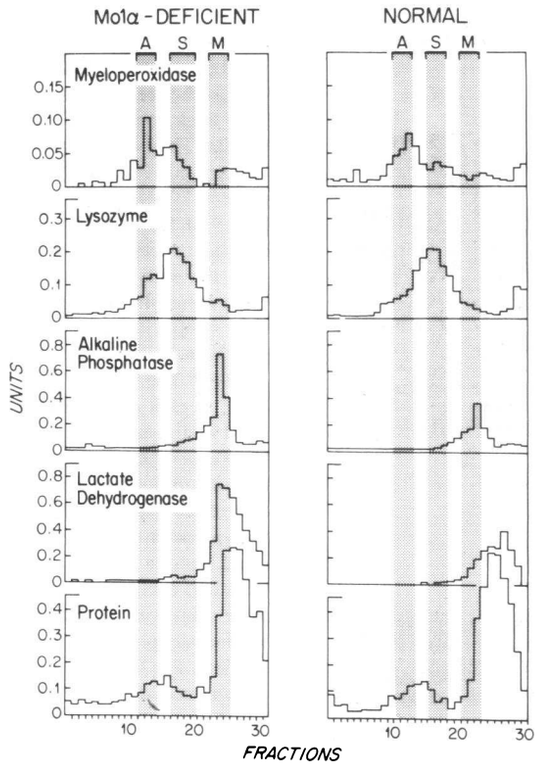

Figure 2. Protein and marker enzyme concentrations in the fractions obtained by sucrose density gradient centrifugation of neutrophil sonicates. Fractions from $Mo1_\alpha$-deficient neutrophils are on the *left*, normal neutrophils on the *right*. 50-μl samples of each fraction were used for each determination. Units are as follows: myeloperoxidase, $(\Delta A_{540}-\Delta A_{600})$/min; lysozyme, $\Delta A_{450}/2$ min; alkaline phosphatase, $\Delta A_{450}/10$ min; lactate dehydrogenase, $\Delta A_{340}/2$ min; and protein, milligrams per fraction. The stippled areas show how the fractions were pooled to prepare samples consisting primarily of azurophil granules (A), specific granules (S), and plasma membrane (M).

fractions (measured as the concentration of cobalamin-binding protein, a neutrophil constituent restricted to the specific granules [23]). The results (Table I) established that the preparation referred to as the specific granule fraction actually consisted primarily of specific granules, and showed that by far the highest concentration of $Mo1_\alpha$ was found in this fraction. They also suggested that $Mo1_\alpha$ was present in small amounts in the plasma membranes but was probably absent from the azurophil granules, since it could be shown from calculations based on the levels of cobalamin-binding protein that specific granule contamination accounted for <15% of the $Mo1_\alpha$ in the plasma membrane preparation but for the entire amount found in the azurophil granules.

$Mo1_\alpha$ was initially identified in the 105,000 *g* pellets obtained from neutrophil homogenates, where it appeared as a relatively isolated band that moved only a small distance into an SDS-polyacrylamide gel (1). To confirm the identity of the protein missing from the $Mo1_\alpha$-deficient specific granules, normal and deficient specific granule fractions were electrophoresed on slab gels side by side with the previously characterized 105,000 *g* pellets from normal and $Mo1_\alpha$-deficient neutrophils. The results (Fig. 4) show that the mobility of the protein missing from the specific granules is identical to that of authentic $Mo1_\alpha$ (arrowhead).

The anti-Mo1 antibody was used to carry out an immunological analysis of the subcellular localization of $Mo1_\alpha$. For this analysis, subcellular particulate fractions (azurophil granules, specific granules, and plasma membranes) from normal neutrophils were prepared and labeled with ^{125}I, then solubilized in NP-40 and treated with anti-Mo1 as described in Methods. The amount of antibody-associated label from each of the subcellular fractions is shown in Table II. These results again indicate that $Mo1_\alpha$ is divided between the specific granules and the plasma membrane, most being found in the specific granules; the azurophil granules appear to be devoid of the glycoprotein.

Transfer of $Mo1_\alpha$ to the plasma membrane during degranulation. When exposed to sufficiently high concentrations of an appropriate stimulus such as C5a or a chemotactic peptide, neutrophils display a complex activation response whose features include the secretion of the specific granule contents into the extracellular medium. Experiments were conducted to examine the fate of the specific granule-associated $Mo1_\alpha$ during this process of degranulation. Degranulation was elicited by the calcium ionophore A23187, an agent that has been shown to provoke the discharge of the specific granule contents but not of the azurophil granule contents into the extracellular environment (24), and the distribution of $Mo1_\alpha$ in resting and degranulated cells was studied by immunological and gel electrophoretic techniques.

Studies with the anti-Mo1 antibody strongly suggested that when neutrophils were treated with A23187, $Mo1_\alpha$ was transferred from the specific granules to the plasma membrane. Fig. 5 compares the amount of $Mo1_\alpha$ available for binding with anti-Mo1 before and after exposure of neutrophils to the ionophore. Treatment with 0.1 μM A23187 was found to increase the amount of immunoreactive $Mo1_\alpha$ on the surface of the neutrophils by a factor of almost 10. The source of this additional immunoreactive material was probably the discharged specific granules, whose membranes were incorporated into the plasma membrane as a result of the membrane fusion event which is integral to the degranulation process; however, an ionophore-induced conformational change in the portion of glycoprotein initially in the plasma membrane that renders that portion more accessible to the antibody cannot be ruled out from these data. The availability of $Mo1_\alpha$ as a function of ionophore concentration and incubation time is shown in Fig. 6; these results show that the augmentation in $Mo1_\alpha$ availability was maximal under the conditions employed for the experiment presented in Fig. 5.

Further studies on the translocation of $Mo1_\alpha$ during degranulation were carried out by SDS-PAGE of subcellular

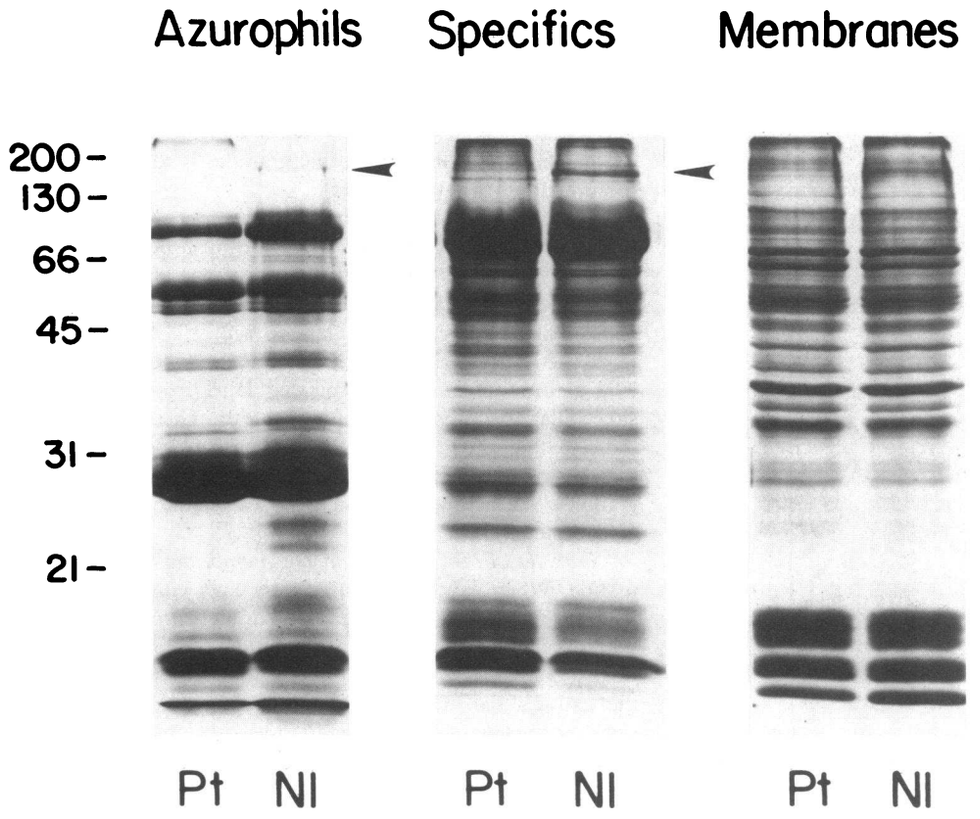

Figure 3. SDS-PAGE patterns of subcellular components from normal and Mol_α-deficient neutrophils. Each track contained 75 μg protein. The positions of molecular weight standards are given in kilodaltons at the left of the figure. The running gel concentration was 12%. For each subcellular component, the Mol_α-deficient sample is shown on the *left*, the normal sample on the *right*. The location of Mol_α in each pattern is indicated by the arrowhead. In these experiments, recoveries were determined by summation of the quantities of protein or enzyme activity found in each of the gradient fractions and comparison of these sums with the quantities initially placed on the gradient. For the experiment shown, recoveries were as follows (protein or enzyme, percentage recovered from the Mol_α-deficient sample, percentage recovered from the normal sample): protein, 91.1, 79.8; myeloperoxidase, 48.2, 55.8; lysozyme, 69.8, 76.3; alkaline phosphatase, 32.6, 41.8. Similar gel electrophoretic patterns were obtained in a second experiment in which the recoveries were: protein, 73.9, 48.0; myeloperoxidase, 17.8, 36.0; lysozyme, 30.6, 51.5; and alkaline phosphatase, 47.6, 66.5.

fractions obtained from resting and A23187-treated neutrophils. At the concentration used in these studies (1.0 μM), the ionophore induced the release of >50% of the specific granule contents, as determined from the amount of cobalamin-binding protein liberated into the medium (Table III). To establish that Mol_α was not one of the constituents released from the specific granules, the proteins in the medium from the degranulated neutrophils were subjected to SDS-PAGE. Measurements of protein concentration showed that during degranulation, the neutrophils released 2.1±0.4 μg protein/10^6 cells into the medium, whereas less than one-fourth that amount (0.46±0.08 μg protein/10^6 cells) was found in medium from resting neutrophils incubated under similar conditions. An SDS-PAGE analysis of the proteins released from resting and A23187-activated neutrophils is shown in Fig. 7. The degranulated supernatant showed a major component of molecular weight 94,000, along with smaller quantities of several lighter components. Proteins >100,000 mol wt were not detected in either supernatant. In particular, there was no evidence that degranulation had caused the release of Mol_α from the specific granules into the external medium.

Table I. Concentrations of Cobalamin-binding Protein and Mol_α in the Subcellular Fractions from Normal Neutrophils

Fraction	Cobalamin-binding protein	Mol_α
	ng bound cobalamin/mg protein	*ng/mg protein*
Azurophil granules	42±21	1.2±0.7
Specific granules	298±80	9.6±1.3
Plasma membrane	7±4	2.0±1.1

Measurements were made as described in Methods. The concentrations of cobalamin-binding protein and Mol_α are expressed as the mean±SE from four and three experiments, respectively, each using neutrophils from a different subject.

The transfer of Mol_α from the specific granules to the plasma membrane during the act of degranulation was further established by SDS-PAGE analysis of neutrophils that had been fractionated into the subcellular components before and

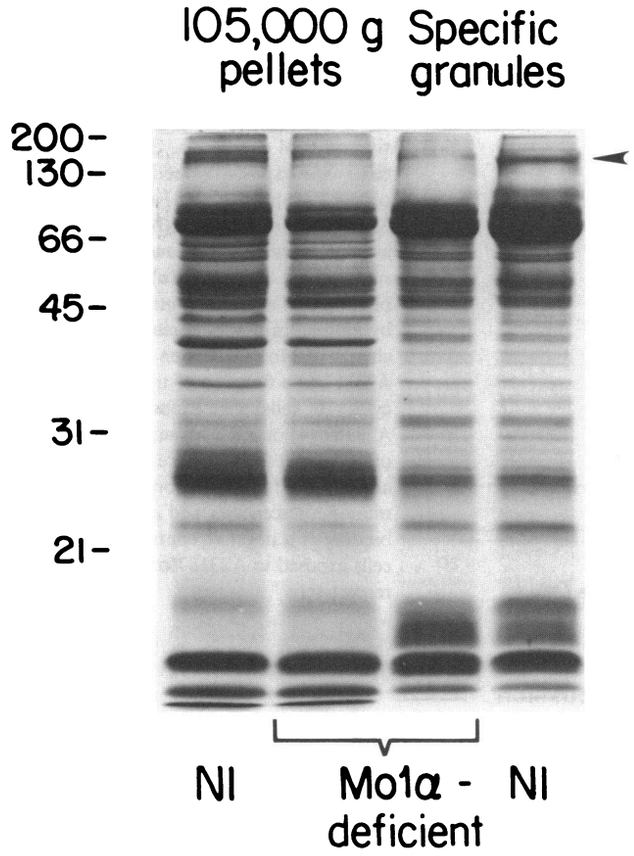

Figure 4. Comparison of the SDS-PAGE patterns of 105,000 *g* pellets (60 μg protein/track) and specific granules (46 μg protein/track) from normal and $Mo1_\alpha$-deficient neutrophils. The running gel concentration was 12%. The location of $Mo1_\alpha$ is indicated by the arrowhead.

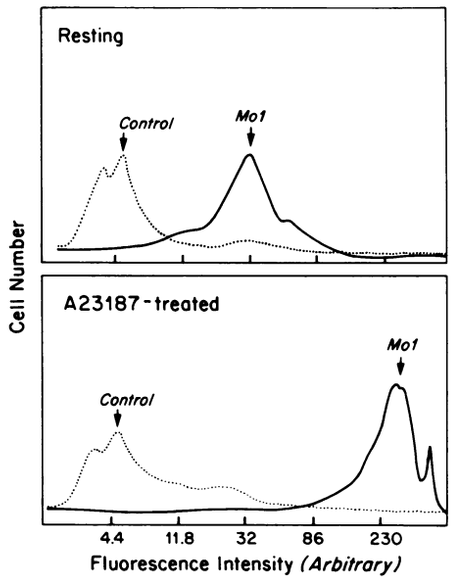

Figure 5. Increase in surface Mo1 antigen expression by normal granulocytes after exposure to A23187, as measured by indirect immunofluorescence. *Top,* immunofluorescent staining of untreated granulocytes exposed to negative control (· · · · ·) or to anti-Mo1 (——) antibodies. *Bottom,* immunofluorescent staining of granulocytes treated with 0.1 μM A23187 for 10 min at 37°C and then exposed to negative control (· · · · ·) or anti-Mo1 (——) antibodies.

after treatment with A23187. For better quantitation of $Mo1_\alpha$, the electrophoresis was carried out with 7% gels, into which the glycoprotein migrated far enough that it was reasonably well separated from other high molecular weight bands and could be easily identified and scanned. 7% SDS-PAGE patterns of the specific granule fractions from the $Mo1_\alpha$-deficient patient and a normal control are shown in Fig. 8. In the gel from the normal subject, $Mo1_\alpha$ appears as a rather broad, fuzzy band (arrow). This band is absent from the $Mo1_\alpha$-deficient pattern, which shows instead two bands of lower molecular weight that are not seen in the normal pattern (arrowheads). The relationship between these two bands and the missing glycoprotein remains to be determined.

Table II. Concentrations of $Mo1_\alpha$ in the Subcellular Fractions from Normal Neutrophils as Determined by Immunoprecipitation with Anti-Mo1 Antibody

	$Mo1_\alpha$ (*cpm/50 μg protein*)	
Fraction	Experiment 1	Experiment 2
Azurophil granules	49	45
Specific granules	850	8,959
Plasma membrane	830	4,353

For details, see text. The amount of $Mo1_\alpha$ (in counts per minute) immunoprecipitated from the specific granule and plasma membrane fractions was 0.086 and 0.056%, respectively, of the total proteins labeled in each fraction.

Fig. 9 shows 7% SDS-PAGE patterns of the specific granules and plasma membranes from resting and A23187-treated neutrophils. The plasma membranes from the A23187-treated cells clearly show more $Mo1_\alpha$ than do the plasma membranes from resting cells, consistent with a transfer of the glycoprotein to the plasma membrane during the course of degranulation. This increase was confirmed by scans of SDS-PAGE patterns from four such experiments, which indicated that the $Mo1_\alpha$ content of the plasma membrane increased from 1.6±0.2 SE to 4.4±0.8 SE μg/mg total protein during the course of degranulation. There appeared to be no difference in the $Mo1_\alpha$ content of specific granules from resting vs. A23187-treated neutrophils, not a surprising finding since the residual granules isolated from partially degranulated cells could not have participated in the degranulation process. The residual granules,

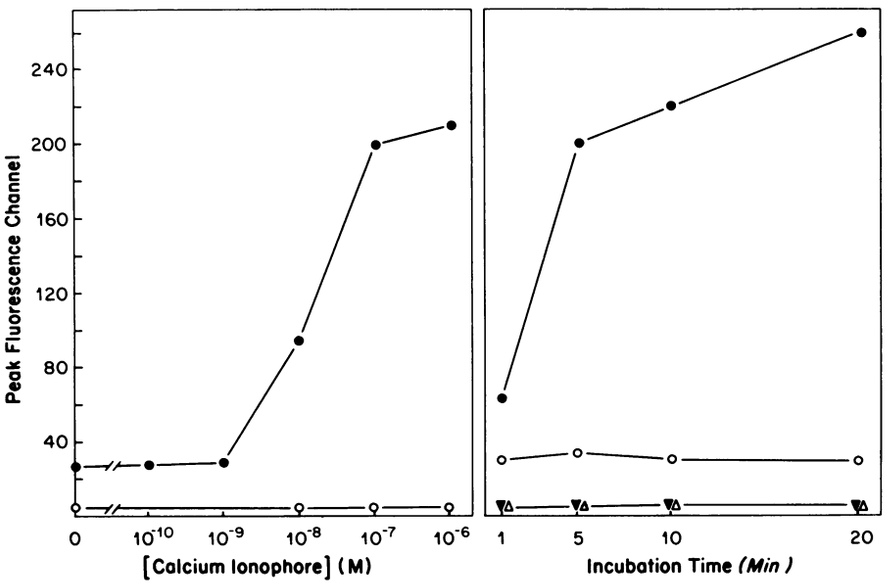

Figure 6. Increased surface expression of Mo1 antigen by normal granulocytes exposed to A23187 as a function of A23187 concentration and duration of A23187 exposure. *Left*, effect of A23187 concentration on Mo1 expression. Granulocytes (10^7 cells) were exposed to the indicated final concentrations of A23187 in 1 ml of Krebs-Ringer phosphate (KRP) for 10 min at 37°C before washing and analysis of surface Mo1 expression (●) relative to the binding of a negative control antibody (○) by indirect immunofluorescence. *Right*, the change in Mo1 expression in response to A23187 as a function of time. Granulocytes (10^7 cells) were exposed to 0.1 μM A23187 (●) or KRP alone (○) for 1, 5, 10, or 20 min at 37°C, before washing and analysis of Mo1 expression by indirect immunofluorescence. ▲ and △ indicate the binding of a negative control antibody to cells exposed to A23187 or KRP alone, respectively.

however, were not identical in composition to specific granules from resting neutrophils. As indicated by the arrowhead, the residual granules were found to lack a prominent 100,000 mol wt protein that was present in granules isolated from unstimulated cells.

Table III. Release of Enzymes and Cobalamin-binding Activity from Neutrophils in Response to the Calcium Ionophore A23187

	Amount in supernatant (% *of total*)	
Activity	Resting	Treated with A23187
Myeloperoxidase	0.9±0.5	4.9±1.1
Cobalamin-binding protein	6.0±2.5	54.2±8.7
Lactate dehydrogenase	8.3±3.2	7.5±2.6

Supernatants and cell pellets from resting and A23187-treated neutrophils were assayed for myeloperoxidase, cobalamin-binding protein, and lactate dehydrogenase (markers for azurophil granules, specific granules, and cytosol, respectively) as described in Methods. Release of activity was calculated from these figures after correction for the fraction of the total volume of supernatant or cell pellet suspension represented by the quantity of material assayed. Results are given as the mean±1 SE for five experiments. The total activity (supernatant plus cell pellets) for each of the three markers was as follows (mean±1 SE per 10^8 cells for five samples); myeloperoxidase, 53.3±9.4 ΔA_{540}/min; cobalamin-binding protein, 27.9±7.4 ng bound CNCbl; and lactate dehydrogenase, 41.0±11.4 ΔA_{540}/min.

Discussion

In these experiments, most of the $Mo1_\alpha$ in human neutrophils was found in the specific granules, but a substantial quantity appeared in the plasma membrane as well. Although it could be argued that the $Mo1_\alpha$ in the plasma membrane preparations was a reflection of contamination by specific granules, two considerations militate strongly against this possibility. First is the finding that in whole cells the protein was accessible to the anti-Mo1 antibody; this is difficult to explain without proposing that $Mo1_\alpha$ is an intrinsic component of the plasma membrane. Second are calculations based on the SDS-PAGE analysis of the subcellular fractions (Table I). These calculations show that whereas the plasma membrane preparations contained <3% of the specific granule marker (cobalamin-binding protein) that the specific granules did, they contained almost 20% as much $Mo1_\alpha$. This amount of $Mo1_\alpha$ seems too large to be accounted for by specific granule contamination of the plasma membrane preparation. We therefore conclude that $Mo1_\alpha$ is an intrinsic constituent of the plasma membrane as well as of the specific granules.

When the specific granules were induced to discharge,[5] they transferred their $Mo1_\alpha$ to the plasma membrane. This protein transfer probably occurred through fusion of the

5. The ionophore A23187 also causes the discharge of a newly described secretory component of human neutrophils which can be distinguished from the specific granules both by kinetics and by sedimentation analysis (25). Whether $Mo1_\alpha$ is present in this as yet morphologically unidentified secretory component remains to be established.

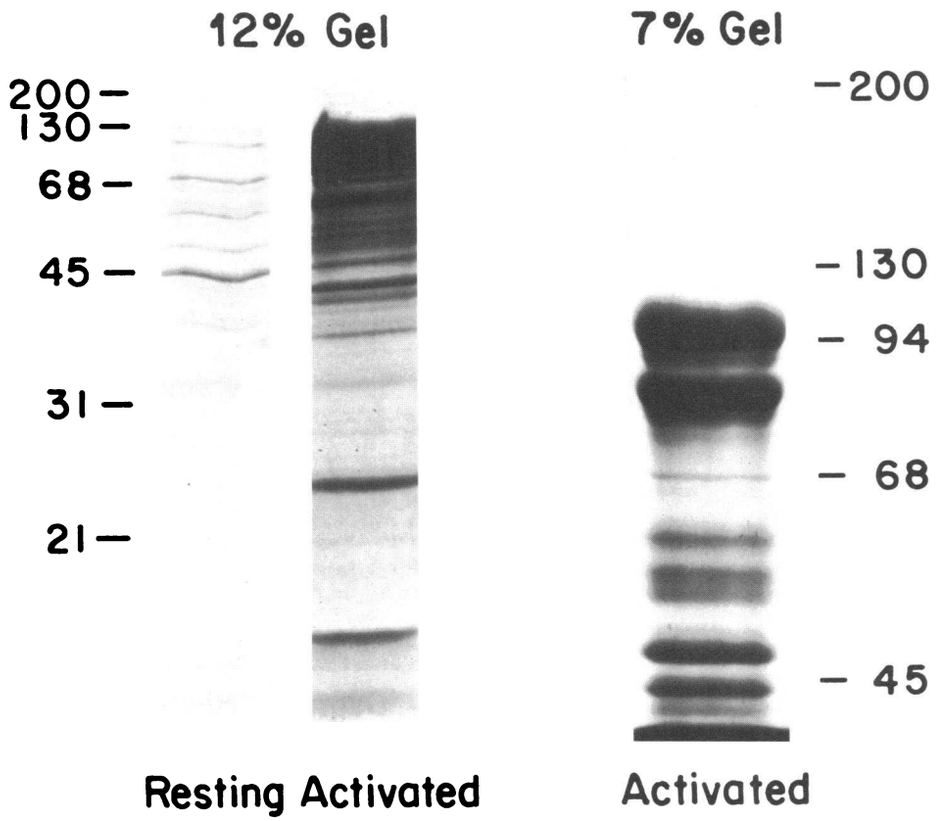

Figure 7. SDS-PAGE pattern of proteins secreted by neutrophils in response to the calcium ionophore A23187. Experiments were performed as described in the text. The two patterns on the left were obtained with a 12% running gel. The quantities of acetone-precipitated protein loaded onto the gel were 10 and 65 μg for the resting and activated supernatants, respectively. The pattern on the right was obtained with a 7% running gel and 100 μg acetone-precipitated protein. The positions of molecular weight standards are given in kilodaltons. These results are representative of four experiments with resting supernatants and four experiments with activated supernatants.

specific granule membranes with the plasma membrane during degranulation, a process that would cause proteins originally intrinsic to the specific granule membranes to become components of the plasma membrane. There is even a possibility that some of the $Mo1_{\alpha}$ found in the membranes of resting neutrophils might have originated in the specific granules, since the slow discharge of specific granules appears to take place even in resting cells, at least in vitro (26).

Of interest is the finding that a 100,000-mol wt protein that was found in specific granules from resting cells was missing from the residual specific granules isolated from partly degranulated neutrophils. One possible explanation for this finding is that this protein is present only in a special set of specific granules that are programmed to discharge in response to a stimulus, and that granules devoid of this protein represent a different set destined to remain behind. Against this explanation, however, are results from other studies showing that at least 70% of the specific granules can discharge in response to a maximal stimulus (27, 28), whereas in the experiment shown in Fig. 9, <30% of the granules had secreted their contents into the extracellular medium. An alternative explanation that does not require subpopulations of specific granules postulates that agents able to provoke degranulation cause the 100,000-mol wt protein to be stripped away from the specific granules, perhaps preparing them for outward migration and fusion with the membranes. Further work will be necessary to test the validity of this explanation.

Previous studies have shown that substances that provoke specific granule discharge also elicit an increase in the adhesiveness of neutrophils to surfaces (26–36). It is possible that the transfer of $Mo1_{\alpha}$ to the plasma membrane is at least partially responsible for the increased adhesiveness characteristic of degranulated neutrophils. In addition, the immobilization of neutrophils exposed to high concentrations of chemotactic factor might be explained in part by the increase in surface adhesion resulting from the transfer of $Mo1_{\alpha}$ to the plasma membrane during the specific granule discharge induced by these concentrations of chemotactic factor.

The patients with neutrophil $Mo1_{\alpha}$ deficiency demonstrate in vitro functional defects not only in adhesion but also in chemotaxis and in the phagocytosis of opsonized particles (1–3). Normal neutrophils pretreated with anti-Mo1 monoclonal antibody exhibit decreased phagocytosis and reduced binding to C3bi-coated particles (7). These findings suggest that $Mo1_{\alpha}$ may be involved in a variety of neutrophil functional activities that depend upon cell-ligand or cell-substrate adhesion. Whereas these studies have focused on the $Mo1_{\alpha}$ molecule (readily apparent in Coomassie Blue-stained plasma membrane and

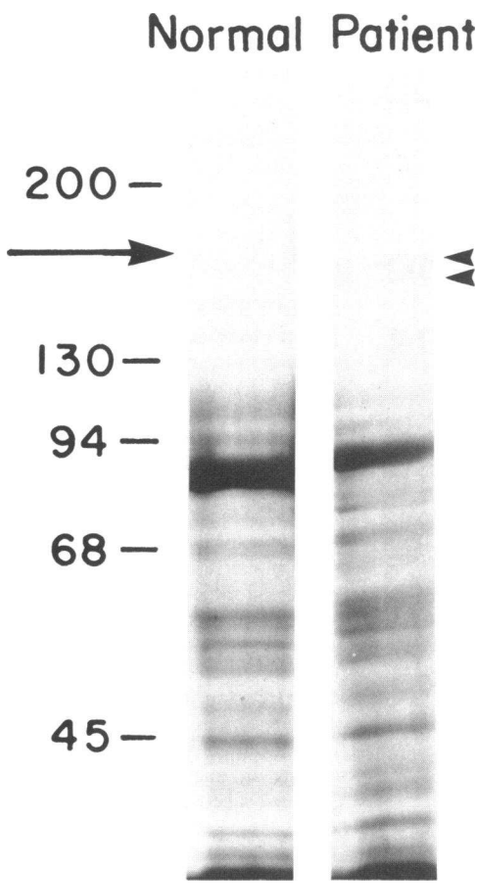

Figure 8. SDS-PAGE of specific granules from normal and $Mo1_\alpha$-deficient neutrophils with a 7% running gel. The normal and $Mo1_\alpha$-deficient tracks contained 63 and 45 μg protein, respectively. The positions of molecular weight standards are given in kilodaltons at the left of the figure. The location of $Mo1_\alpha$ is indicated by the arrow. Arrowheads indicate the lower molecular weight bands that are seen in the $Mo1_\alpha$-deficient, but not in the normal, track.

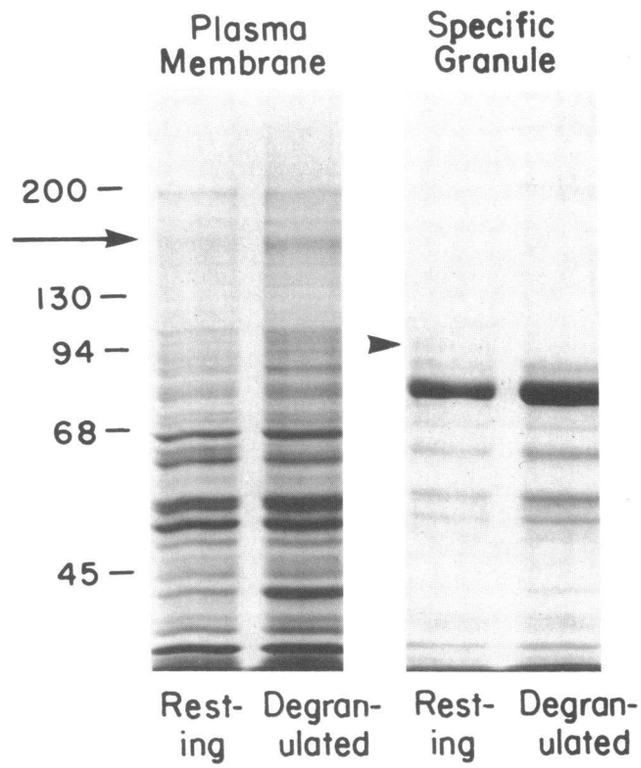

Figure 9. SDS-PAGE of specific granules and plasma membranes from resting and A23187-treated neutrophils. The running gel concentration was 7%. The specific granule and plasma membrane tracks contained 100 and 70 μg protein, respectively. For each subcellular component, the resting sample is shown on the *left*, the degranulated sample on the *right*. The location of $Mo1_\alpha$ in each pattern is indicated by the arrow; the 100 kD-protein that is present in resting granules but not in activated granules is indicated by the arrowhead. Similar gel electrophoretic patterns were obtained in three other experiments with three different neutrophil preparations.

specific granule fractions), immunoprecipitation by monoclonal antibodies specific for this glycoprotein brings down a heterodimer containing nonconvalently associated subunits of molecular weight 155,000 ($Mo1_\alpha$) and 94,000 ($Mo1_\beta$) (7% gels) from both the plasma membrane and specific granular fractions of neutrophils (5, 36a). Since immunological analysis has demonstrated that both subunits are deficient in functionally impaired neutrophils from $Mo1_\alpha$-deficient patients (22; Arnaout, M. A., and R. F. Todd, unpublished observations), the relative functional significance of the two subunits molecules has yet to be resolved.

Mo1 may represent one member of a family of structurally related molecules that also includes lymphocyte function-associated antigen (LFA-1) (gp 177, 94), which itself is an adhesion-promoting molecule involved in lymphoid cellular interactions (37). This relationship between Mo1 and LFA-1 is further suggested by our recent observations that patients who are deficient in Mo1 expression also lack LFA-1 (36a). These findings are similar to those reported by Beatty et al., who have identified a patient lacking a series of leukocyte surface polypeptides as defined by monoclonal antibody 60.3 in association with phagocyte in vitro defects similar to those exhibited by the patients we have examined (38). They also parallel the observations of Thorne et al., who previously described an increase in the membrane expression of a 150,000-mol wt glycoprotein in rabbit neutrophils stimulated with endotoxin, and suggested that this glycoprotein may have been mobilized from a granule compartment (39).

The finding that only a fraction of $Mo1_\alpha$ resides on the

plasma membrane of unstimulated neutrophils parallels the observations of others who have found that the number of leukocyte receptors for C3b and f-Met-Leu-Phe rapidly increases after exposure of cells to various stimuli, including those that result in degranulation (40, 41). The increased plasma membrane expression of $Mo1_{\alpha}$ and other receptor molecules that results from degranulation may augment the functional activity of neutrophils. This phenomenon may therefore represent a process that has considerable physiological significance in vivo.

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