Expression of Specific Granule Markers on the Cell Surface of Neutrophil Cytoplasts

By Pamela R. Petrequin, Robert F. Todd III, James E. Smolen, and Laurence A. Boxer

The widespread assumption that cytoplasts generated from human polymorphonuclear leukocytes (PMNs) are vesicles consisting solely of cytoplasm surrounded by plasma membrane and devoid of granule activity remains to be tested. PMN cytoplasts were prepared by centrifugation of intact cells on a Ficoll step gradient in the presence of cytochalasin B. Two granule membrane markers, Mol, a fluorometrically detectable antigen, and cytochrome b, both of which have been shown to translocate to the plasma membrane during granule release, were compared for their activity in cytoplasts and intact PMNs. We found that the amount of Mol detected on the plasma membrane of intact PMNs, as compared with other membrane markers (such as antigens LFA-1 and \( \beta_2 \))m, increased 1.6-fold upon exposure of PMNs to Ficoll plus cytochalasin B prior to centrifugation. Another twofold increase in Mol expression occurred upon cytoplast preparation. Release of the granule enzymes, vitamin B\(_12\)-binding protein, and lysozyme were also followed and correlated well (\( r = .78 \) and .92) with the amount of Mol antigen present on the cell surface. Cytochrome b was also found to be higher (1.4-fold) on plasma membranes isolated from cytoplasts than on plasma membranes isolated from intact control cells. These results indicate that some fusion of granule membranes and plasma membranes occurred during treatment of PMNs with Ficoll plus cytochalasin b and during cytoplast preparation.

MATERIALS AND METHODS

Chemicals. Ionophore A23187 was purchased from Calbiochem-Behring Corp, La Jolla, Calif. Ficoll 70 and Percoll were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. (\(^3\)Co)-vitamin B\(_12\) and (\(^32\)P)-ATP were purchased from Amer sham Corp, Arlington Heights, Ill. All other reagents were purchased from Sigma Chemical Co, St Louis, unless otherwise stated. A23187 and cytochalasin B were dissolved in dimethyl sulfoxide and then added to aqueous solutions with vigorous mixing.

Monoclonal antibodies. Mouse ascites containing monoclonal antibodies specific for LFA-1\(^{1,8}\) (IgGl subclass, a gift from Dr Hergen Spits, Amsterdam, Holland), Mol\(^7\) (IgG2a subclass), \( \beta_2m\), Mol\(^2\) (IgG2b subclass, a gift of Dr Lee Nader, Boston), and la\(^3\) (IgG2a subclass) were used in indirect immunofluorescence analysis.

Preparation of cells. Suspensions of PMNs were prepared from heparinized (10 U/mL) venous blood from healthy adult donors. Standard techniques of Hypaque-Ficoll gradients\(^4\) followed by dextran sedimentation and hypotonic lysis of erythrocytes provided a PMN suspension that was 98% pure. Neutrophils were washed in phosphate buffered saline (PBS) (138 mmol/L NaCl, 2.7 mmol/L...
KCl, 8.1 mmol/L Na₂HPO₄, and 1.5 mmol/L KH₂PO₄, pH 7.4) before use.

**Plasma membrane preparation.** Approximately 2.5 x 10⁶ washed PMN were resuspended in 12 mL of relaxation buffer (100 mmol/L KCl, 3 mmol/L NaCl, 3.5 mmol/L MgCl₂, and 10 mmol/L piperoxan-N, N’-bis(2-ethanesulfonic acid), pH 7.3) and pressurized under N₂ for 20 minutes with stirring in a nitrogen bomb. The cavitating sample was collected dropwise into ethylene glycol tetra-aetic acid for a final concentration of 1 mmol/L. 

**Enzyme marker analysis.** Alkaline phosphatase was assayed according to DeChatelet and Cooper, with 10 mmol/L p-nitrophenol phosphate (Sigma) in a 50 mmol/L glycine buffer, pH 10, with 10 mmol/L MgCl₂. The enzyme activity was calculated using the extinction coefficient for p-nitrophenol (ε₈.₈/L cm⁻¹). Vitamin B₁₂-binding protein was measured by a modification of the method of Gottlieb et al. Sixteen picograms of (¹⁷⁶Co)-vitamin B₁₂ in 0.6 mL of saline with 200 pg of carrier vitamin B₁₂ was added to the protein sample in 1.0 mL of saline for a final specific activity of 90 cpd/pg. The reaction was run for 30 minutes at room temperature after which 1.0 mL of 2.5% charcoal, 0.5% vitamin B₁₂-deficient BSA (Sigma) in 0.9% NaCl was added to stop the reaction. Samples were centrifuged for ten minutes at 800 g at room temperature, and 1.0 mL aliquots of the supernatants were counted and collected in a Tractor Analytical Model 1190 (TM Analytic, Elk Grove, Ill) gamma counter to determine the amount of bound (¹⁷⁶Co)-B₁₂.

**Cytochrome b determination.** Cytochrome b was assayed by performing difference spectra from 600 to 400 nm on samples sonicated in PBS. Scans of samples reduced in sodium dithionite minus background scans of oxidized sample produced characteristic cytochrome b peaks at 559 nm and 428 nm. Although the 559-nm peak was more characteristic of cytochrome b activity, the 428-nm peak was larger and a more sensitive measure. Cytochrome b was quantitated using an extinction coefficient of 106 L·m⁻¹·cm⁻¹ for the 428-nm peak.

**RESULTS**

**Preparation of cytoplasts.** To examine the claim that cytoplasts are truly free of granule activity, a comparison of cytoplasmic membranes with those from intact cells for the appearance of two granule membrane markers was performed. It may be that during cytoplasmic preparation degeneration occurs and results in the fusion of granule membranes with the plasma membrane. Cytoplasts were prepared according to the method of Roos et al using a Ficoll step gradient containing cytochalasin B. The recoveries of the various soluble enzymes in the isolated fractions are shown in Table 1. The results are similar to those of Roos et al., with the largest amounts of LDH (39%) and protein (42%) being recovered in the cytoplasmic fractions. Examination of the material at this position using light microscopy showed only vesicles without nuclei. Since cytoplasts are believed to consist of only cytoplasm surrounded by plasma membrane, the activities of soluble granule enzymes were compared to the cytoplasmic marker LDH as an indication of the relative purity of the cytoplasm fraction (Table 2). Comparing the cytoplasm fraction to intact PMNs, decreases in all enzyme activities were observed. The largest decreases were in granule constituents, vitamin B₁₂ binding protein, β-glucuronidase, myeloperoxidase, and lysozyme, as was expected since cytoplasts have been shown to be deficient in these enzymes.

### Table 1. Marker Analysis of Ficoll Gradient Fractions From Cytoplast Preparations

<table>
<thead>
<tr>
<th>Marker</th>
<th>Band 1. Cytoplasts</th>
<th>Band 2. Whole Cells</th>
<th>Band 3. Nuclei and Granules</th>
<th>Total Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>42 ± 5</td>
<td>30 ± 13</td>
<td>34 ± 7</td>
<td>106 ± 11</td>
</tr>
<tr>
<td>LDH</td>
<td>39 ± 6</td>
<td>20 ± 3</td>
<td>22 ± 12</td>
<td>81 ± 3</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>5 ± 1</td>
<td>16 ± 5</td>
<td>68 ± 15</td>
<td>90 ± 10</td>
</tr>
<tr>
<td>Vitamin B₁₂-binding protein</td>
<td>7 ± 4</td>
<td>34 ± 12</td>
<td>51 ± 6</td>
<td>92 ± 22</td>
</tr>
<tr>
<td>β-glucuronidase</td>
<td>8 ± 1</td>
<td>23 ± 12</td>
<td>67 ± 18</td>
<td>98 ± 4</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>2 ± 1</td>
<td>33 ± 13</td>
<td>63 ± 23</td>
<td>98 ± 26</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>18 ± 1</td>
<td>33 ± 7</td>
<td>36 ± 8</td>
<td>87 ± 3</td>
</tr>
</tbody>
</table>

*Mean percentage (±SD) of activity recovered from fractions obtained during cytoplasm preparation. The results were obtained from three experiments.*
Alkaline phosphatase activity with respect to LDH did not change.

**Degranulation and Mol expression during cytoplast preparation.** Several steps during cytoplast preparation such as warming to 37°C and treatment with cytochalasin B could cause degranulation and translocation of granule membrane markers. To determine whether degranulation occurs during the early stages of cytoplast preparation, release of granule contents and surface Mol antigen expression were measured. Figure 1 shows a comparison of the release of two granule constituents, lysozyme and vitamin B12-binding protein, and Mol expression of intact cells at each step of cell treatment, then followed by stimulation with A23187. It can be seen that there was an increase in both enzyme release and Mol expression at every condition but the fifth (incubation for an additional 30 minutes in Ficoll with cytochalasin B). During the early preincubation steps of warming the PMNs for five minutes at 37°C in Ficoll plus cytochalasin B, which would be comparable to the conditions intact cells would be under when they were on the gradient except without centrifugation, did not result in an additional increase in Mol expression. However, treatment with A23187 caused a significant increase in Mol expression and enzyme release, indicating that further degranulation was possible.

Since both Mol and specific granule release increased during cytoplast preparation, the correlation between these two responses was calculated. For Mol expression and vitamin B12 binding protein release and for Mol and lysozyme release, the correlation coefficients were .78 and .92 respectively. These have a P value of less than .001 for n = 19 and, thus, are highly significant. As anticipated, the release of the two granule constituents vitamin B12-binding protein and lysozyme correlated well with each other (r = .89, P < .001).

**Expression of Mol and plasma membrane markers.** Although the mean Mol expression increased during the initial stages of cytoplast preparation, it was important to see how Mol behaved in relation to known plasma membrane markers during these manipulations and after cytoplasts had been generated. The two plasma membrane markers that were chosen, LFA-1 and β2m, have been shown to remain constant even after stimulation with f-Met-Leu-Phe. Table 3 shows the ratio of Mol to two plasma membrane markers, β2m and LFA-1, for cells at 4°C, after Ficoll plus cytochalasin B treatment and after cytoplast preparation. A comparison of the ratios of Mol to LFA-1 for PMNs incubated in Ficoll with cytochalasin B and for cytoplasts showed that there was an increase (1.9-fold) in the appearance of Mol after cytoplast preparation. Cells left at 4°C had a much lower Mol/β2m ratio than those exposed to Ficoll plus cytochalasin B at 37°C (Table 3). This observation confirmed the results shown in Fig 1, namely, that treatment with Ficoll and cytochalasin B caused a significant increase in Mol expression. In close agreement with the Mol/LFA-1 result, the ratio of Mol to β2m was significantly higher (1.6-fold) for cytoplasts than for PMNs treated with Ficoll plus cytochalasin B. The mean fluorescence intensities for LFA-1 and β2m on a per-particle basis did not change under any condition (data not shown). Stimulation by A23187 (10^-6 mol/L) of PMNs treated with Ficoll plus cytochalasin B increased the amount of Mol, indicating that further stores of Mol were available. The
Table 3. Ratio of Mol to LFA-1 and to β2 m During the Stages of Cytoplast Preparation

<table>
<thead>
<tr>
<th>Ratio</th>
<th>PMN</th>
<th>PMN Ficoll + CB†</th>
<th>Cytoplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mol/LFA-1</td>
<td>ND</td>
<td>6.24 ± 0.92</td>
<td>12.0 ± 3.28‡</td>
</tr>
<tr>
<td>Mol/β2 m</td>
<td>0.81 ± 0.05</td>
<td>2.65 ± 0.19‡</td>
<td>4.34 ± 1.94‡</td>
</tr>
<tr>
<td>Mol/β2 m + A23187 (10⁻⁸ mol/L)</td>
<td>ND</td>
<td>4.3 ± 0.56§</td>
<td>4.36 ± 2.8</td>
</tr>
</tbody>
</table>

Abbreviations: CB, cytochalasin B; ND, not determined.
*Ratio of mean fluorescence (±SD) of Mol to either of two plasma membrane markers from three experiments.
†PMN were incubated at 37°C in Ficoll with 20 μmol/L cytochalasin B for 30 minutes during cytoplast preparation.
‡P < .01 (using paired statistics) when compared to values for preceding cell treatment.
§P < .01 (using paired statistics) when compared to Ficoll + cytochalasin B–treated cells without A23187.

This comparison was crucial since the relative protein content of plasma membranes isolated from PMNs appeared to decrease after treatment with Ficoll plus cytochalasin B and, again, after cytoplast preparation. The activity of alkaline phosphatase also appeared to decrease, making the usefulness of this marker questionable. Another plasma membrane marker, Mg-ATPase, did not change during these treatments. Table 5 shows the ratios of cytochrome b content relative to the two plasma membrane marker activities. As predicted from the earlier results with Mol expression, the ratio of cytochrome b to alkaline phosphatase was significantly higher in plasma membranes from cells treated with Ficoll plus cytochalasin B than those from untreated PMN. A similar significant increase was found for the cytochrome b/Mg-ATPase ratio. The preparation of cytoplasts further increased the ratio of cytochrome b to alkaline phosphatase; the ratio of cytochrome b to Mg-ATPase was not altered by this final step.

Table 4. Enzyme Activity in Plasma Membrane Preparations Obtained at Different Stages of Cytoplast Preparation

<table>
<thead>
<tr>
<th>Marker</th>
<th>Specific Activity (±SE)</th>
<th>PMN</th>
<th>Ficoll + CB†</th>
<th>Cytoplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome b (× 10⁵)</td>
<td>1.4 ± 0.06</td>
<td>2.82 ± 0.27</td>
<td>3.87 ± 0.6‡</td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase (× 10⁵)</td>
<td>11.6 ± 4.0</td>
<td>6.77 ± 1.14</td>
<td>2.4 ± 0.15‡</td>
<td></td>
</tr>
<tr>
<td>Mg-ATPase (× 10⁴)</td>
<td>1.76 ± 0.7</td>
<td>2.08 ± 0.41</td>
<td>2.64 ± 0.21</td>
<td></td>
</tr>
</tbody>
</table>

*Mean specific activity was measured in terms of (μmol/mg protein) for cytochrome b and (μmol/min/mg protein) for plasma membrane enzymes (±SE) for at least three experiments. Marker descriptions include the exponents for the specific activities.
†PMNs were incubated at 34°C in Ficoll with 20 μmol/L cytochalasin B for 30 minutes during cytoplast preparation.
‡P < .01 as an indicator of significance when compared to the preceding stage.

This question of whether cytoplasts are devoid of all granule activities has been considered. Our preparation of cytoplasts was similar to that of Roos et al. in recovery of protein and marker enzyme activities. Our yield of cytoplast material was slightly lower than that reported by the latter investigators; recovery of LDH was 38% instead of 48% and that of alkaline phosphatase was 18% instead of 33%. In spite of all granule marker methods, the preparation of cytoplasts was similar to that of Roos et al. in recovery of protein and marker enzyme activities. This recovery of cytoplast material was slightly lower than that reported by the latter investigators; recovery of LDH was 38% instead of 48% and that of alkaline phosphatase was 18% instead of 33%.

Table 5. Ratio of Cytochrome b Activity to Two Plasma Membrane Enzymes Obtained at Different Stages of Cytoplast Preparation

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Ratio of Cytochrome b to Membrane Enzyme (±SE)</th>
<th>PMN</th>
<th>Ficoll + CB†</th>
<th>Cytoplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase (× 10⁴)</td>
<td>1.23 ± 0.4</td>
<td>4.2 ± 0.07‡</td>
<td>16.1 ± 1.3‡</td>
<td></td>
</tr>
<tr>
<td>Mg-ATPase (× 10⁴)</td>
<td>5.85 ± 0.09</td>
<td>16.0 ± 2.8‡</td>
<td>14.6 ± 1.0</td>
<td></td>
</tr>
</tbody>
</table>

*Ratio of the mean specific activity of markers from at least three experiments (±SD).
†PMNs were incubated at 34°C in Ficoll with 20 μmol/L cytochalasin B for 30 minutes during cytoplast preparation.
‡P < .01 as an indicator of significance when compared to the preceding stage.
of this, our cytoplast preparation was similar in having greatly reduced amounts of soluble granule enzymes.

Our studies indicate that degranulation and translocation of two granule membrane markers occurred during cytoplast preparation. In particular, we showed that the series of manipulations involved in cytoplast preparation increased Mol expression and the release of lysozyme and vitamin B$_{12}$-binding protein in a stepwise fashion. The good correlation between the release of the two specific granule constitu-
ents and Mol expression provided further evidence that Mol was a marker of degranulation. Significant increases in the ratio of Mol/β$_{12}$m (3.3-fold, Table 3) as well as that of cytochrome b/Mg-ATPase in the cell membrane (2.7-fold, Table 5) occurred upon incubation of PMNs with Ficoll plus 20 μmol/L cytochalasin B. The preparation of cytoplasts further increased the appearance of Mol on the cell surface (1.6-fold, Table 3) and the specific activity of cytochrome b in the plasma membrane (1.4-fold, Table 4). Therefore, our data suggest that cytoplast membranes contain significantly higher amounts of granule membrane activity than those of untreated cells.

Degranulation was shown to occur during the Ficoll and cytochalasin B pretreatment of cells for cytoplast prepara-
tion. The demonstration that Mol expression increased in a fashion similar to the release of lysozyme and vitamin B$_{12}$-binding protein is an important finding. Although degranulation had previously been shown to occur under conditions in which Mol expression is increased, this is the first report of a good correlation between Mol expression and enzyme release. This is not to be expected since Mol is a surface antigen that, unlike granule enzymes, has a low but significant level of expression on unstimulated cells (Fig 1). It should be noted that this good correlation does not necessarily support the hypothesis that Mol is located in specific granules.

The largest increase in Mol expression as compared to PMNs left at 4°C occurred during the five-minute incubation of cells with Ficoll and cytochalasin B at 37°C. The ability of cytochalasin B to enhance PMN responses is well docu-
mented.10,12-13 Cytochalasin B is thought to enhance stimulation by increasing influxes of Ca$^{2+}$ and Na$^+$ ions.14 There is one report showing that cytochalasin B alone causes the stimulation of oxidase activity.32 Our data also indicate that cytochalasin B can cause a significant amount of degranula-
tion in the presence of Ficoll.

Stimulation with the calcium ionophore A23187 in the presence of cytochalasin B increased the amount of Mol expressed and the amount of granule enzymes released from intact cells, but not from cytoplasts. The inability of A23187 to increase Mol in cytoplasts indicates that internal stores of Mol were not present in cytoplasts. This finding is in agreement with that of Lutter et al who showed that stimulation with PMA increased the content of cytochrome b in the membranes of intact cells but not those of cytoplasts.6

It is noteworthy that stimulation with A23187 did not increase Mol by the same proportion as it did the release of lysozyme and vitamin B$_{12}$-binding protein. This could to some extent be attributed to higher baseline levels of Mol, which, as already discussed, could be increased by low-level degranulation during the cell's history. The higher stimulated levels of lysozyme as compared to Mol might be explained by the contributions of enzyme from azurophil granules. That lysozyme might also be released from this other source was suggested by the release of β-glucuronidase, an azurophil granule enzyme, after stimulation by A23187 (data not shown).

The comparison of Mol to two membrane markers, LFA-1 and β$_{12}$m, demonstrated that the preparation of cytoplasts caused further translocation of Mol to the cell surface. The amounts of the two plasma membrane markers have been shown not to change after exposure to degranulating stim-
uli,26,29 making them suitable steady-state determinants for comparison to Mol. The higher ratio of Mol/β$_{12}$m for cells treated with Ficoll plus cytochalasin B as compared to untreated PMNs (Table 3) is consistent with the data in Fig 1 correlating the mean fluorescence of Mol to enzyme release. The relationship between Mol and the plasma mem-
brane markers LFA-1 and β$_{12}$m was analogous to the positive correlation between the specific activity of cytochrome b with respect to Mg-ATPase (which was also found to increase under similar conditions).

To determine whether another specific granule marker, cytochrome b, also translocated to the cell membrane during cytoplast preparation, plasma membrane fractions were pre-
pared. These fractions were enriched 12-fold in alkaline phosphatase and Mg-ATPase activities (data not shown). The plasma membrane fractions from cytoplast gradients had a smaller increase (sixfold) in specific activity, presumably because with most of the granules eliminated the plasma membrane protein could make a larger contribution to the total. As with Mol expression, the largest increase (twofold) in the specific activity of cytochrome b occurred when the cells were incubated in the presence of Ficoll with cytochalasin B; cytoplast preparation further increased its specific activity (1.4-fold). When the cytochrome b content was compared to Mg-ATPase activity, a significant increase in the ratio occurred upon incubation of cells in Ficoll with cytochalasin B. The comparison of cytochrome b to alkaline phosphatase activity magnified these differences, presumably because of the loss of enzyme activity with each treatment.

Our results differ somewhat from some other reports measuring cytochrome b content in cytoplasts as compared to intact cells.5,10 Some investigators used untreated, intact PMNs as controls, whereas we examined both these as well as cells treated with Ficoll plus cytochalasin B. Analyzing the specific activity of cytochrome b with respect to protein, Ohno et al10 found no difference between the plasma mem-
bres of intact cells and those of cytoplasts. In contrast, we demonstrated significant differences in cytochrome b activity among plasma membrane preparations from untreated cells, from cells treated with Ficoll plus cytochalasin B, and from cytoplasts. Since granule membranes have a density similar to plasma membranes,7 there may be a problem of contamination of plasma membrane fractions. In another report, Lutter et al compared cytochrome b to alkaline phosphatase activity and found that plasma membranes of intact cells had a ratio twice as high as that of cytoplast
membranes. Our results showed the opposite, with plasma membranes of cytoplasts having a higher cytochrome b/alkaline phosphatase ratio than those of either PMNs treated with Ficoll plus cytochalasin B or untreated cells. Contributing to these results are the large changes in alkaline phosphatase specific activity between the different fractions (Table 4). This could be due to differences in protein composition of the plasma membrane induced by the various treatments. However, this decrease in specific activity during cytoplast preparations was not seen with Mg-ATPase, indicating that the two plasma membrane markers were not distributing identically. Nonetheless, no matter how cytochrome b specific activity was calculated (eg, with respect to protein, alkaline phosphatase, or Mg-ATPase), a significant increase in activity was found after treatment with Ficoll plus cytochalasin B. Furthermore, this is in agreement with the results of Mol expression (Table 3). Thus, these data strongly suggest enrichment of granule membrane components in the plasma membrane during the steps of cytoplast preparation.

The intracellular sources of Mol and cytochrome b have not been determined. Mol has been shown to have an intracellular pool that migrates to the plasma membrane after activation of human PMNs.34 One source for Mol that has been implicated by some recent experiments is the tertiary granule, a gelatinase-containing granule originally described by Dewald et al.35 We have shown that by using stimuli under conditions in which specific and azurophilic granules are not released, both Mol expression and gelatinase release occur.36 We infer from these results that one intracellular source of Mol is the tertiary granule. One source of evidence locating cytochrome b to the specific granule comes from studies with a specific granule–deficient patient.37 The PMNs from this patient, which lack specific granules, do not contain cytochrome b in any granule fraction, implying that the specific granule is the source of cytochrome b. On the other hand, PMNs from this patient are capable of up-regulating Mol (unpublished data), indicating that other sources for Mol exist besides the specific granule. Another possible intracellular membrane source for these markers, which has not been excluded by these experiments, may be the receptor recycling system, receptosomes.28

In conclusion, degranulation occurred during the various stages of PMN cytoplast preparation as indicated by enzyme release and the translocation of granule membrane markers to the PMN plasma membrane. The increases in Mol expression and cytochrome b activity on the cell membrane upon incubation in Ficoll plus cytochalasin B and during cytoplast preparation indicate that cytoplasts are not devoid of granule membrane content.

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