Reactions of Rat Megakaryocytes in Mixed Cell Suspension*

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ABSTRACT

Rat megakaryocytes in mixed bone marrow cell suspension underwent morphologic change when exposed to adenosine diphosphate (ADP) and epinephrine. Viewed by phase microscopy, marginal membranes developed ragged or vacuolated appearance within one minute after exposure to ADP or epinephrine. Recovery from the change induced by ADP occurred within five minutes. These effects were inhibited by prior incubation of the cell suspension with dibutyryl adenosine cyclic monophosphate (DBcAMP) and were not produced by adenosine monophosphate (AMP) or adenosine triphosphate (ATP). The results suggest that megakaryocyte membranes react with agents known to effect platelets and lend support to the indications that demarcating membranes of megakaryocytes derive from the megakaryocyte plasma membrane. No observations were made which relate these observed changes to the release of platelets from megakaryocytes.

Introduction

On the basis of biochemical1 and morphologic2 evidence, it has been suggested that the demarcating membranes of megakaryocytes, and therefore platelet membranes, derive from the plasma membranes of megakaryocytes, and not from smooth endoplasmic reticulum3 or Golgi vesicles. If this were so, it would be reasonable to expect that the plasma membrane of megakaryocytes would be functionally similar to the platelet membrane and would be reactive to platelet-active substances. This report, therefore, describes the effect of adenosine diphosphate and epinephrine upon rat megakaryocytes in mixed bone marrow cell suspension.

Materials and Methods

Bone Marrow Cell Suspensions

The femoral bone marrow of 250 to 350 g Fisher CD strain rats was prepared for study by the method of Paulus and Mel.4 One entire freshly removed femoral marrow was diced into one mm cubes, and was digested for two hours at 37° in one ml of 200 U per ml crude collagenase† in Hanks solution. This procedure yielded a mixed cell suspension which contained 10,000 to 15,000 megakaryocytes per ml. Approximately 70 percent of the megakaryocytes

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† Worthington Biochemical Corporation.
appeared to have smooth, intact peripheral membranes when observed in a siliconized well slide, by phase microscopy.

**REAGENTS**

Epinephrine,† adenosine 5' diphosphate,§ adenosine 5' monophosphate§ and adenosine 5' triphosphate§ were dissolved in pH 7.4 Tris buffer to give final concentrations of $10^{-5} \text{ M}$ and $10^{-6} \text{ M}$ when mixed with cell suspensions. N6,02'-Dibutyryl adenosine 3':5' cyclic monophosphate§ was also prepared in pH 7.4 Tris buffer to produce a final concentration of $10^{-3} \text{ M}$ when added to cell suspension.

**EXPERIMENTAL PROCEDURE**

The cell suspension from digestion of a single femur was separated into two aliquots of 0.4 ml each. Each aliquot was sampled and studied as described. This is referred to as the "pre-addition" sample. At zero time, 0.04 ml of reagent or control solution was added to each aliquot. Each aliquot was sampled and evaluated 1, 5, 15 and 30 minutes after addition of reagent or control solution. In experiments in which DBcAMP was used, 0.04 ml DBcAMP or control solution was incubated with each aliquot for 10 minutes prior to the addition of a second reagent at zero time.

At the indicated time, a sample of approximately 0.05 ml of cell suspension was removed from the reaction tube and transferred to a siliconized well slide. The entire population of megakaryocytes in the sample was rapidly classified, according to whether or not the peripheral membrane appeared intact, appeared ragged or disrupted, or appeared altered by marginal vacuoles. These observations required about three minutes per sample and were performed at room temperature. Once the evaluation was completed, the sample in the well slide was discarded. In some additional experiments with ADP, individual cells were observed intermittently during the five minutes after addition of the reagent.

**Results**

Addition of ADP in final concentration of either $10^{-5} \text{ M}$ or $10^{-6} \text{ M}$ to the mixed cell suspension resulted in a rapid alteration of megakaryocyte margins. As shown in figure 1, there was a decrease in the percent of intact megakaryocytes within one minute of reagent addition. The statistical significance of these observations is presented in table I. These morphologic alterations consisted of both vacuolization of the marginal area and irregular disruption of the megakaryocyte membrane (figure 2). These changes were transient and were not apparent when compared to control suspensions five minutes after addition of reagent. Transient damage, followed by recovery of individual cells, was suggested by (1) intermittent observation of single cells over a period of five minutes and (2) the observation that the number of megakaryocytes in individual samples did not decrease with time.

Addition of $10^{-5} \text{ M}$ or $10^{-6} \text{ M}$ ATP or AMP did not result in similar changes (figure 3). Incubation of the mixed cell suspension with $10^{-5} \text{ M}$ DBcAMP for ten minutes prior to the addition of ADP apparently completely inhibited the reaction of megakaryocytes (figure 4).

Epinephrine, at concentrations of $10^{-5} \text{ M}$ or $10^{-6} \text{ M}$, also produced changes in megakaryocytes within one minute of addition (figure 5). Disrupted membranes were more commonly observed than were peripheral vacuoles. In contrast to ADP, the changes produced persisted for at least 15 minutes with $10^{-6} \text{ M}$ epinephrine and for 30 minutes, at $10^{-5} \text{ M}$ concentration. The statistical evaluation is presented in table I. In common with experiments using ADP, the changes induced by epinephrine were

† Parke, Davis and Company.
§ Sigma Chemical Company.
Figure 1. Reaction of megakaryocytes with (a) $10^{-6}$ M, (b) $10^{-6}$ M adenosine diphosphate.

Table I

<table>
<thead>
<tr>
<th>Additive</th>
<th>Mean (n)</th>
<th>95% Confidence Limit of Standard Error of Mean</th>
<th>Level of Significance*</th>
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</thead>
<tbody>
<tr>
<td>ADP $10^{-6}$</td>
<td>55.2 (6)</td>
<td>±4.2</td>
<td>p &lt; 0.05</td>
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<td>74.7 (6)</td>
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<td>ADP $10^{-6}$ M</td>
<td>58.5 (6)</td>
<td>±10.0</td>
<td>p &lt; 0.05</td>
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<td>37.3 (6)</td>
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<tr>
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<td>±12.3</td>
<td>p &lt; 0.05</td>
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<tr>
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<td>74.8 (6)</td>
<td>±4.8</td>
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</tbody>
</table>

* The Wilcoxon signed rank test was used to determine the significance of differences between paired experimental and control replicates.6
not clear. However, it is likely that they are "real" and not the result of mechanical trauma, in vitro cell degeneration or other artifact. The use of appropriate control solutions on paired cell suspensions from a single femur and the non-reactivity of megakaryocytes with AMP and ATP support this conclusion. Marginal change secondary to the addition of ADP appears to be transient, compared to the more prolonged alterations with epinephrine. None of the changes observed appeared to be related to release of platelets from megakaryocytes.

These studies suggest that megakaryocytes react with substances known to effect platelets. Because of the fragility of megakaryocytes, it was considered desirable to avoid additional steps necessary to obtain a purer megakaryocyte preparation. Therefore, in the mixed cell preparations used, an intermediate reaction involving other marrow cells cannot be ruled out. The pattern of reactivity, however, was similar to that of platelets and suggests a direct reaction between reagent and megakaryocyte: reactivity to ADP and epinephrine, inhibition of reactivity by DBcAMP, and lack of reactivity to AMP and ATP. Rat platelets aggregate upon addition of ADP, and this aggregation can be inhibited by prior incubation with DBcAMP (unpublished observation). Epinephrine is known to enhance aggregation of rat platelets by ADP, indicating the presence of receptors for the drug on rat platelets.5

Figure 2. Phase microscopy of megakaryocytes with (a) intact, (b) disrupted (arrow) and (c) vacuolated margins. (×225)
These observations are compatible with the reports by Barber and Jamieson that the biochemical constituents of platelet membranes resemble those of membranes of other cells and do not have characteristics of intracellular membranes. They are also compatible with Behnke's ultrastructural demonstration that demarcation membranes of megakaryocytes, and therefore platelet membranes are derived from the plasma membrane of megakaryocytes.

The observation that megakaryocyte membranes are responsive to substances which react with platelets provides functional evidence, in support of this chemical and ultrastructural evidence, that demarcating membranes are derived from the megakaryocyte plasma membrane.

An additional comment with implications for the clinical laboratory, should be made. The ease with which megakaryocytes were damaged by mechanical trauma, e.g., transfer between test tubes, was also
REATIONS OF MEGAKARYOCYTES

MINUTES AFTER ADDITION OF REAGENT

PERCENT INTACT MEGAKARYOCYTES

PREADDITION 1' 5' 15' 30'

Figure 5. Reaction of megakaryocytes with (a) $10^{-4}$ M, (b) $10^{-6}$ M epinephrine.

apparent in these experiments. Morphological observations of fragmentation or disruption of megakaryocytes in bone marrow aspirates, and particularly in smears of such aspirates, should be interpreted with caution.

Acknowledgment

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References