Identification and Counting of Granulocytes by Peroxidase Reaction

By Tafio Rytömaa

PHLOXINE in propylene glycol provides a good and simple method of staining for the counting of eosinophilic granulocytes in the counting chamber. No comparable method has been available for the counting of neutrophils.

The identification of cell types in certain excreta of the organism, especially in the intestinal contents, is a cumbersome procedure.1,2 It has, however, proved necessary for quantitative studies of granulocyte kinetics.2,3 The present experimental investigation introduces a staining method based on the utilization of myeloperoxidase, an enzyme specific for the myeloid cell series.4-6 This method makes it possible to differentiate between granulocytes and other cells both in the counting chamber and in smears.

METHOD AND RESULTS

Human blood, rat blood and cell suspensions prepared from rat bone marrow, bowel contents and touch samples2 were used for the investigation. The bone marrow samples were suspended in 3.5 per cent polyvinyl pyrrolidine7 or 0.1M phosphate buffer (pH 7.0), the intestinal contents and touch samples in 0.1M phosphate buffer and the blood in 0.01M phosphate buffer (pH 7.0), to all of which one drop of heparin was added. The granulocyte counts of the cell suspensions were 100–5000 per cu.mm.

Peroxidase reagent: Fifty mg. of benzidine (Merck) was dissolved in 6 ml. of 96 per cent ethanol and diluted with 4 ml. of distilled water. From the 2,4-dichlorphenol used as catalase inhibitor,8 a 0.5 per cent solution was prepared in 5 per cent ethanol, and from the hydrogen peroxide a 0.01M solution in distilled water. The peroxidase reagent was prepared in the following way:

<table>
<thead>
<tr>
<th>Reagent Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzidine solution</td>
<td>0.15 parts</td>
</tr>
<tr>
<td>2,4-dichlorphenol</td>
<td>0.30 parts</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>0.50 parts</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td>10.00 parts</td>
</tr>
</tbody>
</table>

The reagent was prepared separately on each occasion.

Reaction for counting chamber: The peroxidase reagent was diluted 1:(4-9) with 0.01M phosphate buffer (pH 7.0). The cell suspension under examination was sucked up by an eosinophil pipette to the 0.5 mark and the diluted reagent to the 11 mark. The pipette was emptied into a small test tube and the contents mixed carefully. If the cell suspension under examination displayed numerous granulocytes, it was diluted with a suitable volume of 0.01M phosphate buffer before adding the reagent. The number of granulocytes in the reaction mixture was generally kept to a total of about 10–20 per cu.mm. The counting chamber of Fuchs-Rosenthal was used for the cell count.

Peroxidase-positive granulocytes were brown. The other cells remained unstained.
Reaction for smears: After air-drying, the smear preparations were fixed for 1 min. in a mixture (1:3) of 10 per cent neutral formalin and 96 per cent ethanol after which they were washed in 0.01M phosphate buffer (pH 7.0). After air-drying and 30-60 second treatment with ether, the preparations were rinsed with 0.01M phosphate buffer (pH 7.0). The preparations were then stained for 1-60 seconds, depending on their nature, with peroxidase reagent diluted 1:4 with 0.01M phosphate buffer. A few seconds generally sufficed for optimal staining. When necessary, the routine hematoxylin staining method of Mayer was used for staining the nuclei.

The peroxidase-positive granulocytes stained brown—neutrophils a lighter color than eosinophils.

When nitroprusside, which changes oxidized benzidine into a blue dye, was added to the peroxidase reagent (ad 0.3 per cent), the granulocytes stained an intense blue. However, the nonspecific background staining of the preparation as a whole increased at the same time, for it was necessary to prolong the reaction time considerably. The use of red nuclear stain (Kernechtrot, safranine) facilitated somewhat the evaluation of nonspecific staining.

Specificity of the methods: The following circumstances speak in favor of a reaction based on true peroxidase activity:
- If no hydrogen peroxide was added to the peroxidase reagent, no demonstrable staining occurred in a test tube within an observation period of 1 hour.
- Catalase functions in a living cell in the same way as peroxidase. However, 2,4-dichlorophenol inhibits catalase activity specifically without affecting peroxidase. In the present investigation, e.g., the red cells, which contain abundant catalase, failed to stain.
- The significance of hemoproteins (e.g., Hb) exerting a pseudoperoxidase effect was immaterial in the reactions employed owing to the low hydrogen peroxide concentration and short period of staining (see also below).
- The lipoid solubility of oxidized benzidine could not affect the result in smears as lipoid solvents were used prior to staining. Nor does the so-called stable sudanophilia involve lipoid solubility, but a chemical reaction between the dye and the cell component. Furthermore, secondary staining is never observed with benzidine brown.
- Only the granulocytes stained positively in test tubes. This was ensured by making a smear preparation of the suspension after the reaction and by staining it with routine methods (May-Grunwald-Giemsa, hematoxylin-eosin).

Other observations: It was possible to follow the formation of the dye under the microscope by pipetting the cell suspension in question into the counting chamber and adding a drop of peroxidase reagent diluted (1:9) with phosphate buffer onto the cover glass. The majority of the granulocytes (all neutrophils?) stained blue almost instantaneously and the brown dye formed only gradually. On the other hand, no intermediate blue phase was observed in cells identified as eosinophils but the cells stained brown directly. The final identification of eosinophils was performed after the peroxidase reaction by adding a drop of phloxine into the chamber. When staining with peroxidase reagent in the above-mentioned manner, a fairly sharply-defined area formed in the chamber, with all the cells at the periphery showing no stain. This observation indicates that the hydrogen peroxide contained in the reagent had been consumed, thus stopping the reaction before the reagent had had time to be diffused through the chamber (cf. specificity above).

If the cell suspension in the counting chamber was stained with peroxidase reagent diluted 1:99, only the eosinophils closest to the chamber edge stained positively. This observation, similar to those reported in the foregoing, also
support the view that the peroxidase reaction of eosinophils differs slightly from that of neutrophils. It is possible that the causative agent in these reactions is the alkaline pH of eosinophilic granules in which the primary oxidation result of benzidine, blue semiquinone, is dimerized immediately to a quinhydrone-like compound, benzidine brown.

Examination of the bone marrow samples of adult male rats (Sprague-Dawley strain) established that the number of peroxidase-positive cells was almost exactly two-thirds of the total number of cells in all the cases studied (18 samples from 10 rats). The total cell count was about \(2 \times 10^9\) per mg. of bone marrow. The result compares well with a ratio previously established for smear preparations.

**Summary**

The present investigation concerns a staining method based on peroxidase activity which can be used for the specific staining of granulocytes both in the counting chamber and in smears. The reagent employed at pH 7.0 contained low concentrations of hydrogen peroxide as substrate, benzidine as chromogenic hydrogen donator and 2,4-dichlorphenol as catalase inhibitor. The only positively reacting cells, granulocytes, stained brown. The other cells failed to stain.

The observations made support the view that the peroxidase reaction of eosinophils differs slightly from that of neutrophils.

Nearly exactly two-thirds of the bone marrow cells of adult male rats of the Sprague-Dawley strain belonged to the myeloid cell series.

**SUMMARIO IN INTERLINGUA**

Le presente investigation concerne un methodo de tincturation que es basate super le activitate de peroxydase e que pote esser usate pro le tincturation specific de granulocytos tanto in le camera de numeration como etiam in frottis. Le reagente usate a un pH de 7,0 contineva basse concentrations de peroxydo de hydrogeno como substrato, benzidina como donator de hydrogeno chromogene, e 2,4-dichlorphenol como inhibitor de catalase. Solmente granulocytos reageva possitivemente. Illos se tincturava brun. Le altere cellulas non se tincturava.

Le investigation supporta le these que le reaction peroxydasic de eosinophilos differe levemente ab illo de neutrophilos.

Quasi precisemente duo tertios del cellulas de medulla ossee ab adulte rattos mascule del racia Sprague-Dawley pertineva al serie de cellulas myeloide.

**REFERENCES**


Dr. Tapio Rytömaa, Department of Pathology, University of Helsinki, Helsinki, Finland.
Identification and Counting of Granulocytes by Peroxidase Reaction

TAPIO RYTÖMAA