Expression of 5’Nucleotidase Activity and Wheat-Germ Agglutinin Binding Sites in Mononuclear Phagocytes From Bone Marrow Cultures

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The question as to whether the various types of mononuclear phagocyte found in bone marrow cultures and recognized by specific peroxidatic (PO) activity patterns differ in the expression of binding sites for the lectin wheat-germ agglutinin (WGA) and the activity of the ectoenzyme 5’nucleotidase (5’N) was investigated. Monoblasts, promonocytes, monocytes, and/or exudate macrophages, and exudate-resident macrophages generally showed a high level of WGA binding, and a considerably lower level was found in the PO-negative cells and in resident macrophages. 5’N activity was absent in monoblasts, promonocytes, and in the great majority of the monocytes and/or exudate macrophages, but was demonstrable in exudate-resident macrophages and resident macrophages, as well as in PO-negative macrophages after 4 days of culture. On the basis of the successive occurrence of the above-mentioned phenotypes in cultures, the possibility that this diversity in WGA binding and 5’N activity is related to modulation during cell differentiation is discussed. The present findings led to the conclusion that the PO-negative macrophages, whose origin was previously not entirely certain, are precursors of resident macrophages.

Key words: phagocyte, bone marrow culture, 5’ nucleotidase, wheat-germ agglutinin binding

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INTRODUCTION

The use of a cytochemical method for the demonstration of peroxidatic (PO) activity makes it possible to recognize various types of monocytes and macrophages at the electron microscopical level, i.e., monocytes and/or exudate macrophages (which have PO activity in granules), resident macrophages (PO activity in the RER and nuclear envelope), exudate-resident macrophages (PO activity in the RER, nuclear envelope, and granules), and macrophages devoid of PO activity [1,8,10,17]. All these types of cells have precursor cells in the bone marrow, because they appear in bone marrow cultures in the presence of the appropriate growth factors [17–19]. This assumption is also supported by kinetic and cytochemical data [5–7,9,10,24].

In previous studies [2,11–13] on monocytes and macrophages from the peritoneal cavity, use was made of two cytochemical techniques, each combined with the demonstration of PO activity. One of these, a gold labeling procedure, offers a quantitative method for the assessment of binding sites for the lectin wheat-germ agglutinin (WGA); the other concerns the ectoenzyme 5' nucleotidase (5'N). These experiments yielded the following observations: The resident macrophages showed a low level of WGA binding and were positive for 5'N, whereas monocytes and exudate macrophages showed high WGA binding and no 5'N activity. PO-negative macrophages showed moderate WGA binding and both 5'N-negative and 5'N-positive cells. Exudate-resident macrophages were scarce, but the great majority of those observed showed a pattern similar to that of monocytes and exudate macrophages.

These studies raised the question whether the diversity of the expression of WGA binding sites and 5'N activity in monocytes and macrophages is related to the differentiation of a monocyte into a resident macrophage [1,17,23] or to the presence of separate lineages, i.e., monocytes and exudate macrophages on the one hand and resident macrophages on the other [5–10]. The above-mentioned cytochemical techniques were applied to bone marrow cultures in which differentiation and maturation of mononuclear phagocytes takes place [15,19] to find out whether under these experimental conditions the WGA binding and 5'N activity patterns of these cells would be similar to those of the various types of macrophages of the peritoneal cavity [12,13]. An attempt was also made to obtain more information about the differentiation pathway of these cells in culture, with special attention to the status of the PO-negative macrophage.

MATERIAL AND METHODS

For this collaborative study, bone marrow cultures were performed in the Department of Infectious Diseases (J.W.M.V.D.M., J.S.V.D.G.) and the electron microscopy was performed in the Laboratory for Electron Microscopy (R.D.W., J.M.V.N., J.J.M.O., L.A.G.).

Animals

Male Swiss mice (specific pathogen free; 5–6 weeks old) obtained from the Central Institute for the Breeding of Laboratory Animals TNO (Zeist, The Netherlands) were used in all experiments.

Bone Marrow Cultures

Cells were collected from the femur by gentle flushing with medium and washed and the concentration was adjusted to $5 \times 10^4$ nucleated cells per milliliter; 2-ml
Aliquots were then plated in 35-mm culture dishes (Falcon Plastics, Cockeysville, MD). The culture medium was composed of Dulbecco modified Eagle’s medium (Grand Island Biologicals, Grand Island, New York), 20% horse serum (Flow Laboratories, Irvine, Scotland), and 30% conditioned medium originating from a WEHI-3 cell line and from embryonic mouse fibroblasts in a ratio of 1:2 [14,15,19]. The cultures were incubated in a water-saturated atmosphere with a constant flow of 10% CO₂ in air for 2, 4, 7, 11, 14, and 17 days. Duplicate cultures were used in each experiment. At the indicated timepoints the cells adhering to the culture dish were washed three times with physiological saline and incubated according to one of the following cytochemical procedures:

**PO cytochemistry combined with WGA labeling.** The cells were fixed with 1.5% glutaraldehyde in 0.14 M cacodylate buffer (pH 7.4) for 10 min, rinsed three times with 0.14 M cacodylate buffer (the first two at pH 7.4, the third at pH 6.9), and incubated for the demonstration of PO activity as described elsewhere [11]. The presence of WGA binding sites was determined by a two-step method in which ovomucoid-labeled gold particles were used as electron-dense markers [11]. Briefly, the cells were incubated in a solution of WGA (0.1 mg/ml) for 30 min at 37°C, washed, and then reincubated (45 min at room temperature) with an ovomucoid-gold conjugate adjusted to an absorbance of 1.0 (at 518 nm). Postfixation was performed for 30 min at 4°C in 1% OsO₄ in Millonig’s buffer [16]. In control experiments the cells were treated similarly except that in the first incubation step the lectin was omitted or N-acetylglucosamine added to a final concentration of 0.2 M.

**Combined peroxidase and 5’ nucleotidase cytochemistry.** After fixation for 60 min at 4°C in 1% glutaraldehyde in buffer composed of 0.05 M Tris-maleate and 5% sucrose (TMB, pH 6.0) and three rinses (15 min each) in TMB (pH 7.2, 0°C–4°C), the cells were incubated for 15 min at 37°C in TMB (pH 7.2) containing 1.4 mM 5’AMP (Sigma Chemical Co.), 10 mM Mg(NO₃)₂, and 1 mM CeCl₃ for the demonstration of 5’N activity [2]. The cells were then rinsed with cacodylate buffer, reincubated for the demonstration of PO activity as described above, and postfixed in 1% OsO₄ in 0.14 M cacodylate buffer (pH 7.2) for 30 min at 4°C.

**Electron Microscopy**

The cells adhering to the plastic Petri dish were dehydrated in a graded ethanol series and embedded in Epon 812. Ultrathin sections were cut with a Reichert microtome (Vienna, Austria), briefly stained with lead hydroxide, and then examined in a Philips EM 300 operated at 80 kV.

**Quantification Methods**

Monoblasts, promonocytes, and the various types of monocytes and macrophages were identified on the basis of their morphology and PO activity pattern as described elsewhere [8,11,17]. At a number of timepoints during culture (see Fig. 2) the cellular composition of the population of adherent cells was determined in 500 cells obtained from four to five specimen blocks.

The degree of WGA binding was determined in electron micrographs made at random and enlarged to a final magnification of ×35,000, as calibrated by a carbon replica grating (30,000 lines per inch). The number of cells photographed for each cell category is indicated between parentheses in Table 1. For each cell, the number of gold particles present on a length of 8 μm of perpendicularly cut cell membrane (2 μm per cell quadrant) was calculated (n/μm); for this purpose, use was made of a
TABLE 1. WGA Binding Sites and 5'N Activity in Bone Marrow Mononuclear Phagocytes at 7 Days Culture

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Exp. I (0.1 mg WGA/ml)</th>
<th>Exp. II (0.1 mg WGA/ml)</th>
<th>Exp III (0.01 mg WGA/ml)</th>
<th>5'N activity b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Monoblast</td>
<td>115 ± 36 (8)</td>
<td>-c</td>
<td>-c</td>
<td>0.3</td>
</tr>
<tr>
<td>Promonocyte</td>
<td>123 ± 21 (43)</td>
<td>91 ± 22 (62)</td>
<td>88 ± 15 (56)</td>
<td>0.3</td>
</tr>
<tr>
<td>Monocyte</td>
<td>111 ± 36 (20)</td>
<td>69 ± 20 (32)</td>
<td>60 ± 20 (26)</td>
<td>2.9</td>
</tr>
<tr>
<td>Ex.-resident macrophage</td>
<td>110 ± 11 (8)</td>
<td>59 ± 17 (10)</td>
<td>-c</td>
<td>0</td>
</tr>
<tr>
<td>PO-negative macrophage</td>
<td>45 ± 24 (50)</td>
<td>33 ± 17 (44)</td>
<td>23 ± 10 (34)</td>
<td>5.0</td>
</tr>
<tr>
<td>Resident macrophage</td>
<td>42 ± 31 (50)</td>
<td>31 ± 15 (51)</td>
<td>16 ± 11 (36)</td>
<td>3.8</td>
</tr>
</tbody>
</table>

aExpressed as the number of gold particles per micrometer cell circumference (n/μm). These data were obtained at the indicated concentrations of the lectin in the incubation medium. The number of cells photographed for each cell category is indicated between parentheses.

bExpressed as percentage of the total number of cells in culture (mean of three experiments).

cVery few present (<0.2%).

morphometric analysis apparatus (MOP-AMO2, Kontron Messgeräte GmbH, FRG). These data are presented as mean ± the standard deviation (X ± SD). Significance of differences was determined with Student’s t-test.

The quantitative data on the expression of 5'N activity were obtained by counting, for each category of cells, the number of 5'N-positive and 5'N-negative cells in ultrathin sections. Cells with reaction product on the cell surface and/or in cell-surface invaginations were considered to be 5'N-positive. Since diffusion of reaction product was known to occur [2], an attempt was made to reduce any effect of this phenomenon on observations in clustered cells by only considering cells to be 5'N positive if reaction product was also present on a side of the cell not contiguous with another cell.

RESULTS

PO Activity

As found light microscopically too [15,19], colonies of mononuclear phagocytes developed in the bone marrow cultures. At the ultrastructural level the following cell types were identified: monoblasts, promonocytes, monocytes, and various types of macrophages distinguishable on the basis of PO activity patterns, i.e., exudate macrophages, resident macrophages, and cells devoid of PO activity (Fig. 1). Exudate-resident macrophages were seen infrequently, and granulocytes were seen only in the 2- and 4-day cultures where they occurred in proportions ranging from 7 to 11% of the total number of adherent cells (data not shown).

The contribution of each type of mononuclear phagocyte to the total adherent-cell population of the cultures was as follows (Fig. 2): monoblasts were recognized on days 4 and 7, both times in low percentages (<0.4%) (Fig. 2A). Promonocytes were observed predominantly on day 2 (17.9%) but also in the 4-, 7-, and 11-day
Fig. 1. A low magnification electron micrograph of cells in a 7-day bone marrow culture. In this cell population promonocytes (pro.), exudate macrophages (ex.), exudate-resident macrophages (ex.-res.), PO-negative macrophages (po-neg.), and resident macrophages (res.) can be recognized. \( \times 1,880 \).

cultures. After day 11 these cells were not present (Fig. 2B). Monocytes and exudate macrophages were most numerous on day 2 (29.8\%), after which the percentage declined and by day 14 these cells had disappeared (Fig. 2C). Exudate-resident macrophages occurred in the 2-, 4-, 7-, and 11-day cultures but infrequently (Fig. 2D).

The PO-negative cells were already observed in moderate numbers in the 2-day cultures. On day 4 they constituted the majority of the total cell population of the
cultures (71.3%), but after that the percentage dropped to moderate values on day 17 (Fig. 2E).

The frequency of resident macrophages showed an inverse relationship with the frequency of PO-negative cells. On day 2, resident macrophages were relatively scarce; after that the percentage gradually increased to a maximum, which was reached on or after day 11 of culture (72.1%) (Fig. 2F).

In these experiments the numbers of monoblasts were lower than previously reported [15,19]. This was due to the washing procedures which removed a large number of these relatively weakly adherent cells. In the fluid removed by washing, predominantly monoblasts, occasional promonocytes and granulocytes, but no monocytes or macrophages were encountered.
WGA Binding

The WGA binding studies were essentially performed on the day 7 of culture, because by that time all types of mononuclear phagocytes were present. Three WGA binding experiments were performed. In the first two the WGA concentration in the incubation medium was 0.1 mg/ml. To determine whether the binding studies were done at saturation, the WGA concentration was chosen ten times lower for the third experiment.

The results of the gold label quantifications for the various cell types are shown in Table 1 (experiments I–III; see also Figs. 3–7). In the first experiment a very high level of WGA binding was found in monoblasts, promonocytes, monocytes and/or exudate macrophages, and exudate-resident macrophages (Figs. 3–6). This level was significantly lower in the PO-negative cells and resident macrophages (P < 0.05; Fig. 7).

In the second experiment WGA binding was very high in the promonocytes, moderate in the group of monocytes and/or exudate macrophages and exudate-resident macrophages, and relatively low in the group of PO-negative cells and resident macrophages. These levels of WGA binding differed significantly from each other (P < 0.05). In this experiment no monoblasts were seen.

In the third experiment, despite the lower WGA concentration, WGA binding was still very high in the promonocytes, moderate in the group of monocytes and/or exudate macrophages, relatively low in the PO-negative cells, and low in the resident macrophages. These levels differed significantly from each other (P < 0.05). Monoblasts and exudate-resident macrophages were not encountered.

5’Nucleotidase

The distribution of 5’N activity over the various categories of mononuclear phagocytes is represented in Figure 8 and in the two righthand columns in Table 1. In general, the monoblasts, promonocytes, and the majority of the monocytes and exudate macrophages did not show 5’N activity at any timepoint (Figs. 9, 10). In the 7- and 11-day cultures, however, there were a few (< 1%) monocytes and exudate macrophages, all of which showed reaction product on the cell surface.

Exudate-resident macrophages were 5’N-positive, and the amount of reaction product on these cells was similar to that observed in the population of resident macrophages present at the same timepoints.

The majority of the PO-negative cells were devoid of 5’N activity in the 2- and 4-day cultures (Figs. 8, 11). A few 5’N-positive PO-negative cells were seen, but the amount of reaction product was small. On day 7 the majority of the PO-negative cells were 5’N-positive (51.9%, Table 1; Figs. 8, 12) and beyond this timepoint, up to 17 days, the 5’N-positive resident macrophage accounted for more than 70% of the number of cells in the cultures (Figs. 8, 13). 5’N-negative resident macrophages were seen regularly, but the frequency was low.

DISCUSSION

We found that the various types of mononuclear phagocytes that develop in liquid culture of murine bone marrow, as defined by morphology and peroxidatic (PO) activity patterns, differ in the expression of wheat-germ agglutinin (WGA) binding sites and 5’nucleotidase (5’N) activity. WGA binding was high in the pro-
Fig. 3. Monoblast from a 7-day bone marrow culture incubated for the demonstration of PO activity and WGA binding sites. PO activity is present in the nuclear envelope, rough endoplasmic reticulum, and a few large granules. A Golgi system is not discernible. This cell shows a high level of WGA binding, as indicated by numerous gold particles on the cell surface. ×17,300.

Fig. 4. Promonocyte from a 7-day bone marrow culture. PO activity is present in the nuclear envelope, rough endoplasmic reticulum, Golgi system, and granules. The cell shows a high level of WGA binding. ×17,000.

Fig. 5. Monocyte from a 7-day bone marrow culture. PO activity is only present in granules. The level of WGA binding is high. ×16,200.
Fig. 6. Exudate-resident macrophage from a 7-day bone marrow culture. PO activity is observed in the nuclear envelope, rough endoplasmic reticulum, and granules. This cell shows high WGA binding. ×10,200.

Fig. 7. A PO-negative macrophage (below) and a resident macrophage (upper left) from a 7-day bone marrow culture. Both cell types show intermediate to low number of gold particles on the cell surface. ×8,900.
monocytes, slightly lower in the exudate macrophages, and much lower in the PO-negative macrophages and the resident macrophages. The numbers of monoblasts and exudate-resident macrophages were too low to permit definitive conclusions, but WGA binding seemed to be high in these cells as well.

Since WGA binding by promonocytes, monocytes, and exudate macrophages was roughly equal, it was suspected that at the relatively high WGA concentration used, steric hindrance could hamper binding of the gold particles in the second incubation step. When a lower WGA concentration was used, the promonocytes showed a higher level of WGA binding than the monocytes and exudate macrophages. Thus, the expression of WGA binding sites seems to diminish during the differentiation of promonocytes to exudate macrophages. This assumption is supported by results of a previous study, which showed that blood monocytes express more WGA binding sites than monocytes and macrophages from the peritoneal cavity and that peritoneal exudate macrophages lose WGA binding sites with increasing time after intraperitoneal injection of a stimulus [12]. Under the latter experimental conditions,
Figs. 9–11. Cells from a 7-day bone marrow culture, which were characterized by PO cytochemistry and tested to determine the presence of 5'N activity, which proved to be absent.

Fig. 9. Promonocyte. ×16,800.

Fig. 10. Monocyte. ×12,600.

Fig. 11. PO-negative macrophage. ×8,800.
Figs. 12, 13. Cells from a 7-day bone marrow culture, which were characterized by PO cytochemistry and tested to determine the presence of 5'N activity. These cells were 5'N-positive, as shown by the presence of reaction product on the surface and in surface invaginations.

Fig. 12. PO-negative macrophage. ×12,000.

Fig. 13. Resident macrophage. ×8,800.
blood monocytes that have entered the peritoneal cavity undergo a process of differentia­tion [1,17,19,20,22]. In all three experiments the number of WGA binding sites differed considerably between monoblasts, promonocytes, monocytes, and exudate macrophages on the one hand and PO-negative and resident macrophages on the other.

The present study showed a distinct difference in 5'N activity between the group of monoblasts, promonocytes, monocytes, and/or exudate macrophages which were predominantly 5'N-negative and the group of PO-negative macrophages and resident macrophages which expressed 5'N activity. The assumption that the expression of 5'N activity is an indicator of cell differentiation is mainly supported by the finding that the PO-negative macrophages are 5'N-negative at 2 and 4 days of culture, but 5'N-positive beyond these timepoints. However, the question of whether monocytes can acquire 5'N activity upon differentiation needs further investigation. The 5'N activity of some of the monocytes and exudate macrophages in the 7-day cultures may have been due to a diffusion of reaction product from 5'N-positive cells like observed earlier [2,13].

A previous study [17] indicated that the differentiation of mononuclear phagocytes occurs in the following sequence: monoblast → promonocyte → monocyte → exudate macrophage → exudate-resident macrophage → resident macrophage. The present cytochemical data (see Fig. 2) confirm the first part of this developmental sequence, according to which monoblasts differentiate into monocytes and exudate macrophages. We have doubts as to whether the exudate-resident macrophage is of importance as a transitional form between the monocyte and the resident macrophage. If so, it should have been observed frequently especially at 4 and 7 days, because at these timepoints the monocytes had decreased in number whereas the number of resident macrophages had increased considerably. The exudate-resident macrophages might constitute a heterogeneous population comprising monocytes and exudate macrophages, which in response to the in vitro conditions have acquired PO activity in the RER and NE [3,4,8,22], and resident macrophages, which have internalized PO-positive material [8,10,13].

Another population that might represent transitional forms between monocytes and resident macrophages concerns the PO-negative macrophages. The nature of the latter cell type was previously not clear [12,13,18]. In the present study the PO-negative macrophages occurred in large numbers at a time when the numbers of resident macrophages had started to increase significantly. During their differentiation the large majority of the PO-negative macrophages apparently acquire 5'N activity on their plasma membrane (Fig. 8). These cells subsequently acquired PO activity in the RER and NE. The assumption that PO-negative macrophages are precursor cells of resident macrophages is in line with previous data, from which it could be deduced that the PO-negative macrophages are related to resident macrophages [9–12,17]. An interesting question, then, is whether these PO-negative macrophages are monocyte-derived. Although previous studies [10] provided contradictory evidence, this question can only be answered positively if the various cell types in these bone marrow cultures are known with certainty to have a common precursor, i.e., to belong to the same clone. This question deserves further investigation. In this connection, Bursuker and Goldman [5–7] have claimed colonies with 5'N activity and colonies without 5'N activity to occur in bone marrow cultures, suggesting that monocytes and exudate macrophages on the one hand and resident macrophages on the other derive from
different progenitor cells. In light microscopical experiments we found all colonies of mononuclear phagocytes to be 5′N-positive [19]. In the present study 5′N-negative and 5′N-positive cells were seen in one and the same electron microscopical section. Under the present experimental conditions, however, it is impossible to say whether this area included only one type of colony deriving from one progenitor cell.

In sum, mononuclear phagocytes generated in vitro were found to be heterogeneous with respect to WGA binding and 5′N activity. This heterogeneity might be due to the fact that there are two macrophage lineages. If, however, clonal growth of mononuclear phagocytes occurs, it is more likely that there is a single macrophage lineage in which monoblasts differentiate via PO-negative macrophages into resident macrophages. Further studies are needed to explore these possibilities, for instance by the study of single colonies under conditions giving rise to clonal growth.

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