Glycogen Accumulation in Polymorphonuclear Leukocytes, and Other Intracellular Alterations That Occur during Inflammation

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ABSTRACT Neutrophils isolated from the blood were compared to those from inflammatory exudates in the peritoneal cavity of guinea pigs. Inflammatory neutrophils were shown to have 10-fold more glycogen than blood neutrophils. This was also reflected in the morphology of these cells. The large accumulations of glycogen in inflammatory neutrophils exists in ordered arrays of \( \beta \)-granules. Other morphological changes including accumulations of lipid droplets and a decrease in the number of lysosomal granules also accompany the change from blood neutrophils to inflammatory neutrophils. These results show that there are major metabolic differences in the two types of neutrophils.

Most studies on neutrophils are carried out with cells obtained either from the circulation of large mammals (e.g. humans) or from peritoneal exudates in small mammals (e.g. guinea pigs, rabbits). In many cases, inferences have been drawn about circulating neutrophils based upon studies with inflammatory exudate neutrophils, and vice versa. It is becoming apparent, however, that there are significant differences between these two types of neutrophils. Wright and Gallin (29) have shown that human exudate neutrophils exocytose the contents of specific, but not azurophil, granules; circulating neutrophils, on the other hand, do not exocytote either granule type, unless appropriately stimulated. Takamori and Yamashita (25) compared blood and peritoneal neutrophils from rabbits, and found that peritoneal cells were osmotically more fragile and produced more \( \text{O}_2^- \) anion than blood neutrophils, whereas blood neutrophils had higher levels of alkaline phosphodiesterase than those from the peritoneum.

In this report, we shall describe differences between circulating and elicited peritoneal neutrophils from the guinea pig. We have observed that the glycogen content increases, while the granule content decreases, as neutrophils change from circulating to elicited peritoneal cells; in addition, lipid droplets are common in elicited cells, whereas circulating cells are essentially devoid of these structures. These phenotypic changes, particularly with respect to glycogen accumulation, are discussed in terms of alterations of the cells environment as they leave the bloodstream and enter the peritoneal cavity.

MATERIALS AND METHODS
Sample Preparations

Neutrophils were obtained from male Hartley guinea pigs (600-1,000 g) (Charles River Breeding Laboratories, Inc., Wilmington, MA and Rockland Inc., Gilbertville, PA), which were maintained on chow pellets and water ad libitum.

Exudate neutrophils were routinely obtained following intraperitoneal injection of 30 ml of sterile 12% sodium caseinate (Difco Laboratories, Detroit, MI) in isotonic saline. Cells were collected by peritoneal lavage, at various times up to 18 h post injection, using the method of DePierre and Karnovsky (4). Neutrophils were also elicited by injection of 20 ml of sterile saline containing 50 \( \mu \)g/ml of lipopolysaccharide (from Escherichia coli, serotype No. 0127:B8, Sigma Chemical Co., St. Louis, MO).

Circulating neutrophils were obtained by cardiac puncture; blood was drawn into sterile syringes containing heparin (40 U/ml blood) (Elkin-Sinn, Inc., Cherry Hill, NJ). Leukocytes were separated from the blood by dextran sedimentation, as previously described (7). Both circulating and exudate neutrophils were washed in Hanks' balanced salt solution with HEPES buffer (HBSS). In order to test the effects of this isolation procedure, exudate neutrophils were incubated with heparin and dextran in the same manner as the circulating cells. This treatment does not alter either the glycogen levels within these cells or their ultrastructural appearance.

Both cell preparations were predominantly neutrophils, the casein-elicited peritoneal cells being >93% and the circulating cells >85% of this cell type, as determined in Giemsa-stained preparations. In each experiment, cell viability, as measured by trypan blue exclusion, was >95%.

Bone marrow was used in some experiments. This tissue was obtained by dissection from the femurs of guinea pigs immediately after sacrificing by ether asphyxiation.

Undiluted exudate fluid was obtained 18 h after injection of caseinate. The exudate fluid was centrifuged to remove cells. Aliquots of the exudate fluid (from
three different animals) were stored at -70°C and assayed for glycogen content within 24 h, or in one case, 5 d.

**Biochemical Measurements**

Glycogen levels were determined by the method described by Passonneau and Lauderdale (14). Briefly, pelleted cells were resuspended in 12.5 vol of 0.03 N HCl. The samples were homogenized and boiled for 8 min in order to extract the glycogen. Samples were then assayed by the enzymatic method, employing amylo-α-1, 4-α-l, 6-glucosidase, hexokinase, glucose 6-P-dehydrogenase, and NADP (all enzymes were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN). Cell-free exudate fluid was assayed in the same manner. Measurements were made on an Aminco Bowman spectrophotometer (SLM-Aminco, SLM Instruments Inc., Urbana, IL), with excitation at 350 nm and emission at 400 nm. Protein was determined by the method of Lowry et al. (11) with bovine serum albumin, essentially fatty acid-free (Sigma Chemical Co.), as the standard.

**Electron Microscopy**

Washed neutrophils were pelleted and resuspended by one of two fixation procedures: (a) 3% glutaraldehyde-0.1 M cacodylate (pH 7.3) with 5% sucrose, or (b) 3% glutaraldehyde-0.1 M cacodylate (pH 7.3) with 5% sucrose, 0.03% trinitrophenol, and 0.03% CaCl2 (8). Fixation was carried out at room temperature for 45–60 min. Cells were washed several times in the same cacodylate buffer, and then postfixed in either (a) buffered osmium, 2% OsO4-0.1 M cacodylate (pH 7.3), or (b) reduced osmium, 2% OsO4-0.1 M cacodylate (pH 7.3) containing 3% potassium ferrocyanide. This recipe for reduced osmium is a modification of the original one described by Karnovsky (9). During dehydration in ethanol (70% step), cells were pelleted into melted 2% agar. The agar was solidified, and portions containing cells were cut into small pieces. Subsequent steps could be carried out without the necessity of centrifugation. Following dehydration, the cells were infiltrated with, and embedded in, Epon 812. Small pieces of bone marrow were processed in the same manner, except they were not embedded in agar.

Neutrophils that were attached to glass cover slips were also examined. Washed cells were resuspended in HBBS at 1 × 10⁷ cells/ml, and allowed to settle onto and attach to cover slips for varying times up to 30 min at 37°C. The cover slips (22 mm diameter, no. 2 thickness) had been thoroughly cleaned by treating with 3 N HCl and 95% ethanol, mixed 1:1, then rinsed well in distilled water, 95% ethanol, and acetone. Cover slip preparations were fixed as described above. The cells on cover slips were processed for electron microscopy and embedded, as we have previously described, so that thin sections could be cut both parallel and perpendicular to the substratum (19).

For routine morphological examination, thin sections were cut on a diamond knife, picked up on bare copper grids, and then stained with uranyl acetate and lead citrate. Sections were examined in a Philips 200 electron microscope, operated at 60 kV.

**Glycogen Cytochemistry**

Glycogen was readily identified in cells treated with reduced osmium; however, the mechanism by which this treatment contrasts glycogen granules is not fully understood. Therefore, glycogen was also localized at the ultrastructural level by the more chemically defined periodic acid-thioaraborhodazide-silver proteinate (PA-TCH-SP) method of Thierry (26). The cytochemical reaction was carried out on thin sections on bare nickel grids. Controls consisted of (a) omitting the periodic acid oxidation step, or (b) preincubating samples with 0.5% alpha-amylase (Type VI-A, Sigma Chemical Co.) in phosphate buffer (0.1 M, pH 7.4) for 120 min at 37°C (1).

**Morphometry**

The "lysosomal" granule content of circulating and elicited neutrophils was compared. Cells prepared for routine ultrastructure were examined, and micrographs were made at X 8,500. Prints from these negatives were enlarged to X 20,000. Cells were selected so that comparable areas of cytoplasm were examined for each type of neutrophil. Only cells that had one or two lobes of the nucleus visible were selected. A semi-quantitative determination was made by counting the total granules per cell profile.

**RESULTS**

Analytical measurements show that the glycogen content of 18 h casein-elicited peritoneal neutrophils was approximately 10-fold higher than that in the circulating neutrophils of the guinea pig (Fig. 1). However, glycogen could not be detected in the cell-free exudate fluid. Glucose levels in that fluid were 67–76 mg%.

This chemically defined difference in glycogen content was reflected in the morphology of these cells. Cells postfixed in ferrocyanide-reduced osmium show differences between circulating and elicited neutrophils in the number of structures that appear to be glycogen granules (Fig. 2). This was further investigated using the more specific PA-TCH-SP cytochemical reaction for glycogen localization. Comparison of cells reacted with the PA-TCH-SP method confirms that there are large differences in the number of glycogen granules in circulating and elicited neutrophils (Fig. 3). In thin section, the size of aggregates of glycogen granules was fairly uniform from cell to cell in preparations of circulating neutrophils. The elicited neutrophils, on the other hand, were morphologically heterogeneous with respect to the amount of cytochemically detectable glycogen. However, all elicited cells examined had larger deposits of glycogen granules than found in circulating neutrophils. Increased levels of glycogen were evident as early as 4 h after initiation of the inflammatory response.

In cells that were fixed in suspension, the glycogen deposits were generally found to be distributed uniformly within the extranuclear portions. When elicited neutrophils were allowed to attach and spread on glass cover slips before fixation, the glycogen deposits were not uniformly distributed within the cytoplasm. Under these conditions, the glycogen was usually located in the apical region of the cells, away from the substratum (Fig. 4). This rearrangement of glycogen distribution was...
Figure 4 Portion of a casein-elicited neutrophil that was allowed to attach and spread on a glass cover slip for 15 min before fixation in glutaraldehyde and buffered osmium. Sections cut perpendicular to the substratum and then reacted for glycogen localization with the PA-TCH-SP method. Attached neutrophils form a close association with the substratum (the cell-cover slip interface is evident at the arrowheads). During attachment and spreading, glycogen (G) accumulates in the apical region of the cell. No counterstain. x 12,200. Inset: higher magnification showing the cell-cover slip interface (arrowheads) and the appearance of individual glycogen granules following the PA-TCH-SP procedure. No counterstain. x 42,000.

Rapid, being observed as early as 2 min after attachment to the substratum.

Maturing neutrophils from guinea pig bone marrow also have glycogen deposits. Based upon cytochemical observations, it appears that neutrophils within the bone marrow have about the same amount of glycogen as the circulating neutrophils (Fig. 5).

 Guinea pigs that were injected intraperitoneally with sterile saline containing lipopolysaccharide also exhibited an inflammatory response. In this case, the number of cells recovered from the peritoneal lavage was much less than with casein elicitation. Morphological and cytochemical examination of lipopolysaccharide-elicited neutrophils showed that they also had increased amounts of glycogen when compared with the circulating neutrophils (Fig. 6).

The glycogen in each type of neutrophil examined is in the form of β-granules. Often the individual β-granules of an aggregate exist in an ordered arrangement. These paracrystalline arrangements were most evident in the elicited neutrophils. Additionally, the arrays of glycogen granules were often in association with membranes of the ER system. The glycogen was usually on one cytoplasmic face of the ER, but not both (Fig. 7).

A semi-quantitative estimate of the “lysosomal” granule content of circulating and elicited neutrophils was made by counting granules in cell profiles of thin sections. The 18 h-elicited neutrophils have fewer “lysosomal” granules than the circulating neutrophils (Table I).

Another morphological difference that distinguishes the elicited peritoneal neutrophils from the circulating neutrophils is the presence of lipid droplets. Cells from all time points examined (4, 8, 12, and 18 h) during casein-induced inflammation have lipid droplets (Figs. 2 and 6). These structures were not observed in circulating neutrophils.

DISCUSSION

The circulating neutrophil is derived from hemopoietic tissue within the bone marrow. These cells are generally thought to be fully differentiated when they enter the circulation, and are no longer biosynthetically active. Recent evidence, however, shows that circulating neutrophils from humans can synthesize...
RNA and proteins, and that the rates of synthesis can be modulated by Concanavalin A, an agent that stimulates these cells (5).

There are other conditions that may lead to changes in the state of neutrophils. When neutrophils leave the circulation and move to sites of inflammation, they are exposed to different environmental conditions; these new conditions may induce metabolic changes within these cells. Hartman and Goretsky (6) report increased glycolytic activity of exudate guinea pig leukocytes when compared with blood leukocytes. In this paper we show that movement of neutrophils into the peritoneal cavity of guinea pigs, in response to casein or lipopolysaccharide, leads to a dramatic increase in the amount of glycogen contained within these cells. This result is consistent with an earlier observation of Scott and Cooper (23), who showed increases in glycogen levels in elicited neutrophils. Our quantitative measurements of glycogen content show a 10-fold increase in casein-elicited neutrophils over circulating cells. This quantitative difference was dramatically reflected in the morphological and cytochemical appearance of these cells. When peritoneal exudates were induced with lipopolysaccharide a similar pattern was observed in morphological and cytochemical experiments. The increase in glycogen with two different eliciting agents supports the concept that it is the movement out of the circulatory system into a different environment that is in some way turning on the biosynthetic activity leading to glycogen synthesis. Further support for this comes from the work of Wulff (30), who observed an increase in the periodic acid-Schiff staining for glycogen of human neutrophils in "skin window" preparations when compared with blood neutrophils. During this study hundreds of circulating neutrophils were examined in thin section; none of these had levels of glycogen approaching that observed in exudate cells. This would argue against the possibility that there is a population of circulating neutrophils relatively rich in glycogen that preferentially migrates into the peritoneum. Another possibility
that could account for accumulation of glycogen would be if glycogen granules were free in the exudate fluid and were either phagocytosed or pinocytosed by the cells. However, this appears to be unlikely since glycogen could not be detected in cell-free exudate fluid. Furthermore, glycogen deposits were not membrane-enclosed as would be expected if the glycogen had been phagocytosed or pinocytosed. Thus, it appears that movement to sites of inflammation induces glycogen synthesis in neutrophils.

Morphological and biochemical studies of glycogen particles isolated from neutrophils from the rabbit peritoneum (28) and human blood (24) show that in both species the glycogen particles are of the \( \beta \)-type. The glycogen particles present in guinea pig neutrophils (from bone marrow, the circulation, and the peritoneum) are also of the \( \beta \)-type, and are morphologically indistinguishable from glycogen in rabbit and human neutrophils.

The glycogen granules in bone marrow and circulating neutrophils fixed in suspension are essentially randomly distributed throughout the cytoplasm. The granules occur singly or in small clusters. The amount of glycogen, as estimated from thin sections, appears to be uniform from cell to cell in bone marrow and circulating neutrophils. The results presented for guinea pig bone marrow neutrophils are similar to those previously reported for human and rabbit bone marrow (13, 15). Elicited neutrophils (both casein and lipopolysaccharide-induced) are much more heterogeneous with respect to glycogen content when thin sections of individual cells are compared.
The glycogen granules within elicited cells primarily occur as aggregates. The aggregates are essentially uniformly distributed within the cytoplasm of cells fixed in suspension. When elicited neutrophils are allowed to attach and spread on glass cover slips, the glycogen aggregates assume an apical localization. The mechanism by which the rearrangement of glycogen occurs as the peritoneal neutrophils spread is not known; however, it may be through some passive process. During attachment and spreading, which are active processes, the relatively inert glycogen particles may be merely excluded from the portion of the cell involved in spreading on the substratum.

Of special interest is the demonstration of highly ordered arrays of glycogen particles in elicited guinea pig neutrophils. In favorable sections, the glycogen granules are arranged in paracrystalline patterns. Another interesting feature of glycogen distribution is that the paracrystalline patterns are often seen in association with cytoplasmic membranes. The glycogen is usually preferentially associated with only one cytoplasmic face of the cisternae. These cytoplasmic membranes, presumably small segments of ER, are also increased in number in the elicited cells. Although rare, other cases of regularly arranged glycogen particles have been reported (2, 3), in these cases, the glycogen granules are lined up side by side like beads on a string, rather than as paracrystalline arrays, as in the elicited guinea pig neutrophil. It is not known whether this ordered arrangement of glycogen in association with cytoplasmic membranes is the primary arrangement reflecting the pattern for glycogen synthesis, or is a secondary event arising post-synthesis. One possible interpretation is that the membranes, possibly membrane glycoproteins, serve as the initiation site ("primer") for new glycogen synthesis. The question of the association of glycogen, the glycogen synthetic machinery, and cytoplasmic membranes has been previously addressed. Luck (12) showed that glycogen synthetase was not associated with membranes of the endoplasmic reticulum in liver. Wanson and Drochmans (27) have shown that glycogen synthetase had a specific affinity for glycogen particles, and that small particles of glycogen, as well as phosphorylase and phosphorylase kinase, can associate with isolated sarcoplasmic reticulum vesicles. Whether membranes serve as sites of glycogen initiation or not, it is noteworthy that this intricate geometric arrangement can be demonstrated in the guinea pig neutrophil.

Glycogen metabolism and its regulation are important in neutrophils, because these cells rely primarily on glycolytic mechanisms for energy production (22). Furthermore, glycogen levels are responsive to phagocytic stimulus; the amount of relatively inert glycogen particles may be merely excluded from the portion of the cell involved in spreading on the substratum.
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