The Activated Phagocyte of Polycythemia Vera

By M. R. Cooper, L. A. DeChatelet, C. E. McCall, and C. L. Spurr

Leukocytes isolated from patients with polycythemia vera (PCV), a panmyelopathy, have increased metabolic activity during the resting and phagocytizing states. Phagocytes from patients with PCV were studied by counting particle ingestion, measuring hexose monophosphate shunt (HMP) activity by the conversion of glucose-1-14C to 14CO2, and determining O2 consumption and nitroblue tetrazolium (NBT) reduction. Phagocytosis of polystyrene particles was increased. Resting glucose-1-14C activity was 6.9 ± 2.7 nmoles of glucose oxidized per hour per 5 × 10^6 phagocytes in normal and 16.4 ± 7.6 nmoles in polycythemia vera phagocytes. Hexose monophosphate shunt following phagocytosis increased to 50.6 ± 10.4 nmoles in normal and 91.7 ± 17.0 in polycythemia vera phagocytes (p < 0.005). Oxygen consumption was 3.6 ± 0.2 μl/hr/5 × 10^6 phagocytes in resting and 11.4 μl/hr/5 × 10^6 phagocytes in stimulated controls as compared to 4.8 ± 0.2 μl in resting and 18.4 μl in phagocytizing PCV cells (p < 0.001). The reduction of NBT by leukocytes was increased in all resting polycythemia vera phagocytes as compared to control phagocytes. The cells from some patients had increases in the amount of NBT reduced during phagocytosis. Phagocytes from four severely infected patients had increases in HMP activity in both resting and phagocytizing cells similar to those found in noninfected PCV phagocytes. Although the increased metabolic activity associated with phagocytosis can be explained by increased particle ingestion, the increased activity of resting PCV cells suggests other metabolic abnormalities perhaps related to the age of the phagocyte.

POLYCYTHEMIA VERA, a panmyelopathy, is characterized by an absolute erythrocytosis and it is often associated with a leukocytosis and thrombocytosis. Previous studies have indicated that numerous abnormalities exist in polycythemia vera. Wagner found an increased concentration of glycogen and an elevated rate of glycogenolysis in leukocytes from patients with polycythemia vera. Luganova and Seitz reported that the rate of glycogen turnover in polycythemia vera leukocytes was 1.5 times greater than in normal cells. Increased activities of uridine diphosphoglucose pyrophosphorylase, phosphoglucomutase, glucose-6-phosphate dehydrogenase, alkaline phosphatase, and an increased periodic acid Schiff reaction occur in

From the Departments of Medicine and Biochemistry, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, N. C.

Submitted August 30, 1971; revised April 18, 1972; accepted May 6, 1972.


M. R. Cooper, M.D.: Associate Professor, Department of Medicine, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, N. C.
L. R. DeChatelet, Ph.D.: Assistant Professor, Department of Biochemistry, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, N. C.
C. E. McCall, M.D.: Associate Professor, Department of Medicine, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, N. C.
C. L. Spurr, M.D.: Professor, Department of Medicine, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, N. C.
PCV leukocytes. Brandt found an increased phagocytic index of leukocytes from patients with polycythemia vera.

Phagocytosis and bactericidal activity of polymorphonuclear leukocytes are associated with profound changes in metabolism. Particle engulfment depends on glycolysis and occurs under either aerobic or anaerobic conditions. Phagocytosis is associated with an increase in oxygen consumption, hexose monophosphate shunt activity, hydrogen peroxide production, lipid synthesis, and a decrease in pH to 6-6.5 in the phagolysosome.

The results of this investigation demonstrate that leukocytes of polycythemia vera patients have an altered metabolism. Some of these findings are similar to those observed in leukocytes from certain patients with severe bacterial infection.

MATERIALS AND METHODS

Selection of Patients

Patients were diagnosed as having polycythemia vera according to the criteria of Wasserman and Bassen. All patients had splenomegaly, increased red cell mass (male, greater than 36 ml/kg; female, greater than 32 ml/kg), and normal arterial oxygen saturation (equal to or greater than 92%). Leukocyte alkaline phosphatase was increased by both histochemical and quantitative techniques. Variable degrees of leukocytosis and thrombocytosis were observed. No patient was infected during the study.

Isolation of Leukocytes

Leukocytes were isolated, largely free of platelets and red cells, by sedimentation and gentle centrifugation from 50 ml of heparinized venous blood. The blood was divided into 8 ml aliquots and 2 ml of plasma gel was added to each vial. The tubes were mixed by inversion and the red cells were sedimented at room temperature for 30 min. The white cells were washed with 0.9% saline. They were then suspended in 6 ml of cold distilled water for 30 sec to lyse red blood cells and isotonicity was restored with 2 ml of 3.5% saline. The remaining white cells were suspended in phosphate-buffered saline, counted in a white cell counting chamber, and brought to the desired concentration by adding phosphate-buffered saline to the cell suspension. Viability was determined by ability of the cells to exclude 1% trypan blue. Differential counts were performed on smears of the isolated white cell suspension and on Wright-stained blood smears. Phagocytes were defined as segmented neutrophils, band neutrophils, eosinophils, and monocytes. Appropriate corrections were made for the differential counts so that each study was based on an equal number of phagocytes. Approximately 95% of the cells isolated from our polycythemia vera patients were segmented neutrophils. The appropriate corrections made by differential counts from each group did not account for more than 2% to 3% of the cells; it is unlikely that these small differences could significantly alter the results.

Metabolic Studies

Quantitative Nitroblue Tetrazolium Test: Quantitation of the reduction of nitroblue tetrazolium to the insoluble blue formazan was done by the method of Baehner and Nathan.

Hexose Monophosphate Shunt Activity: Glucose utilization via the hexose monophosphate shunt and the Krebs cycle was estimated by a modification of the methods of Beck, and Sbarra and Karnovsky, employing glucose differentially labeled in the C-1 or C-6 position. Each flask contained 1.0 ml of Eagle's Medium, 199 0.2 ml of heat-inactivated, pooled AB serum, and 0.10 ml of either glucose-1-14C or glucose-6-14C (0.05 μCi). The isotopes were obtained from the New England Nuclear Corp., Boston, Mass. Specific activity of the glucose-1-14C was 52.2 mCi/mole and the specific activity of the glucose-
6-14C was 46.5 mCi/m mole. Phagocytosis was initiated in appropriate flasks by the addition of 0.15 ml of latex particles (0.81 μ diameter) at a ratio of about 100 particles/phagocyte; control flasks received an equal volume of phosphate-buffered saline. Reaction was initiated by the addition of 1.0 ml of cell suspension (containing 5 X 10^6 cells in phosphate-buffered saline) and allowed to proceed for 1 hr at 37°C. 14CO2 was collected in 0.5 ml of hydroxide of hyamine and counted in a liquid scintillation spectrometer as previously described.20

Respiration

Oxygen consumption was measured using a Clark-oxygen electrode according to a previously reported method.81

Iodination

Iodination was quantitated by a modification of the method of Pinkus and Klebanoff.22 Leukocyte suspensions were adjusted to a concentration of 6 X 10^7 phagocytes/ml. 0.1 ml of this suspension was incubated with 0.1 ml of fresh human AB serum, 0.10 ml glucose (1.50 mg), 0.10 ml Na125I (0.25 μCi/0.1 ml), 6 X 10^8 zymosan particles, and 0.10 ml of calcium-free phosphate-buffered saline. Each tube contained a total volume of 0.6 ml. Each ingredient was pipetted into 15 X 75 mm siliconized, flat-bottomed test tubes in the following sequence: serum, glucose, 125I, phosphate-buffered saline, polymorphonuclear leukocytes, and particles. During pipetting, the solutions were kept on ice.

Following gentle mixing, the test tubes were incubated for 60 min at 37°C. The reaction was stopped by the addition of 2 ml cold 5% trichloroacetic acid. The trichloroacetic acid precipitate was collected by centrifugation, washed 4 X with 5% trichloroacetic acid, and the white precipitate was suspended in 5 ml of distilled water. Standards were made by adding 0.1 ml of 125I (0.25 μCi/0.1 ml), to 2.5 ml distilled water. The tubes were counted in a gamma well scintillation counter for 1 min. The counts for the duplicate tubes were averaged, and the blank average (minus cells) was subtracted from its corresponding experimental (with zymosan) or control average (minus zymosan) to give counts attributable only to phagocytes.

Enzyme Studies

For the in vitro measurement of the enzyme activities, cells were suspended in phosphate-buffered saline and disrupted by sonication, as previously described.18 Cellular debris was removed by centrifugation at 27,000 g 15 min and enzyme activities were then determined immediately. The protein concentration of sonicates was estimated by the biuret method.23 Enzymes quantitated by slight modifications of previously reported methods included leukocyte 6-phosphogluconate dehydrogenase,24 glucose-6-phosphate dehydrogenase,25 acid and alkaline phosphatase,28 myeloperoxidase,27 glutathione reductase,28 pyruvate kinase,29 and hexokinase.30

Table 1. Krebs Cycle and Hexose Monophosphate Shunt Activity

<table>
<thead>
<tr>
<th></th>
<th>Control N=20</th>
<th>Infection N=4</th>
<th>Polycythemia Vera N=9</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-1-14C with particles</td>
<td>X = 50.8 ± 10.4†</td>
<td>X = 107.2 ± 16.3</td>
<td>X = 91.7 ± 17.0</td>
</tr>
<tr>
<td></td>
<td>p = 0.005</td>
<td></td>
<td>p = 0.005</td>
</tr>
<tr>
<td>G-1-14C without particles</td>
<td>X = 6.9 ± 2.7</td>
<td>X = 19.6 ± 6.6</td>
<td>X = 18.4 ± 7.6</td>
</tr>
<tr>
<td></td>
<td>p = 0.005</td>
<td></td>
<td>p = 0.005</td>
</tr>
<tr>
<td>G-6-14C with particles</td>
<td>X = 5.0 ± 2.2</td>
<td>X = 5.2 ± 3.8</td>
<td>X = 3.9 ± 1.8</td>
</tr>
<tr>
<td>G-6-14C without particles</td>
<td>X = 2.1 ± 0.8</td>
<td>X = 3.3 ± 1.5</td>
<td>X = 1.8 ± 2.7</td>
</tr>
</tbody>
</table>

* Results expressed as nano moles glucose oxidized per hour per 5 X 10^6 phagocytes.
† Standard deviation.
Table 2. Krebs Cycle and Hexose Monophosphate Shunt Activity in Patient (P.M.) With Acute Shigellosis

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>G-1-(^{14})C with particles</td>
<td>104.4*</td>
<td>46.9</td>
</tr>
<tr>
<td>G-1-(^{14})C without particles</td>
<td>18.1</td>
<td>5.1</td>
</tr>
<tr>
<td>G-6-(^{14})C with particles</td>
<td>3.1</td>
<td>3.9</td>
</tr>
<tr>
<td>G-6-(^{14})C without particles</td>
<td>1.1</td>
<td>2.7</td>
</tr>
<tr>
<td>NBT reduction (OD 515 nm)</td>
<td>0.240</td>
<td>0.141</td>
</tr>
</tbody>
</table>

* Nano moles glucose oxidized per hour per 5 \(\times\) 10^6 phagocytes.

Index of Phagocytosis

Phagocytes (1 \(\times\) 10^7) were incubated with polystyrene particles (0.81 \(\mu\) diameter) at a ratio of 100 particles per phagocyte for 15 min. Five hundred phagocytes were then counted and the number of particles in each cell was estimated. The index was calculated on the basis of 0-5 for each individual phagocyte. Thus, the phagocytic score could range from 0 to 2500. The degree of clumping on each slide was subjectively determined and scored by a scale of 0-4.

RESULTS

Table 1 gives Krebs cycle and hexose monophosphate shunt activity of phagocytes in 20 normal individuals, 9 patients with polycythemia vera, and 4 selected patients with acute bacterial infections. There was an increased resting glucose-1-\(^{14}\)C oxidation in both infected and polycythemia vera leukocytes as compared to normal phagocytes. Similarly, there was a marked increase in oxidation of glucose-1-\(^{14}\)C in both infected and polycythemia vera phagocytes during ingestion of particles. A patient with acute shigellosis was studied during the acute infection and following effective therapy. A marked increase in glucose-1-\(^{14}\)C oxidation was observed during the acute febrile illness which returned to normal 6 days later during the convalescent period (Table 2).

Table 3 reports the results of the phagocytic index and the degree of clumping in controls as compared with the severely infected patients and patients with polycythemia vera. The phagocytic index and the degree of clumping was increased in both infected and polycythemia vera patients. Similar results were obtained in the patient with acute shigellosis who showed

Table 3. Phagocytosis of Polystyrene Particles by Phagocytes

<table>
<thead>
<tr>
<th></th>
<th>Normal N=10</th>
<th>Severely Infected Patients (Bacterial) N=4</th>
<th>Polycythemia Vera N=7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phagocytic Index*</td>
<td>X = 1568 SD 326</td>
<td>X = 2214 SD 233 ((p = &lt;0.001))</td>
<td>X = 2104 SD 259 ((p = &lt;0.001))</td>
</tr>
<tr>
<td>Clumping†</td>
<td>X = 2.6 SD 0.5</td>
<td>X = 3.6 SD 0.5 ((p = &lt;0.01))</td>
<td>X = 4.3 SD 0.5 ((p = &lt;0.01))</td>
</tr>
</tbody>
</table>

* Five hundred phagocytes were counted; particles counted in each cell and scored 0-5 (Potential score 0-2500).
† Clumping scored on a 0 to 5 scale.
Table 4. Leukocyte Respiration in Polycythemia Vera

<table>
<thead>
<tr>
<th></th>
<th>Resting</th>
<th>Phagocytizing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (N = 6)</td>
<td>3.6 ± 0.2*</td>
<td>11.4 ± 0.9</td>
</tr>
<tr>
<td>Infected (N = 20)</td>
<td>4.1 ± 0.4</td>
<td>19.0 ± 0.9</td>
</tr>
<tr>
<td>(p &lt; 0.5)</td>
<td>(p &lt; 0.001)</td>
<td></td>
</tr>
<tr>
<td>Polycythemia vera</td>
<td>4.8 ± 0.2</td>
<td>18.4 ± 0.8</td>
</tr>
<tr>
<td>(p &lt; 0.001)</td>
<td>(p &lt; 0.001)</td>
<td></td>
</tr>
</tbody>
</table>

Phagocytosis of 0.81 μ latex particles in a ratio of 100 particles to one phagocyte.

* Standard error of the mean.

an increased phagocytic index during the acute infection which returned to normal during the convalescent period (data not shown). This finding correlates with the degree of leukocyte hexose monophosphate shunt activity demonstrated in this patient. Convalescent studies were not available from the other three patients with severe bacterial infections due to the lethality of their illnesses.

Phagocytes from patients with infections or polycythemia vera had increased oxygen consumption during phagocytosis as compared to normal cells (Table 4). In addition, resting oxygen consumption was increased in PCV leukocytes as compared to control and infected cells. Although the resting oxygen consumption was slightly increased in infected patients, the variation in this group was so great that the difference was not statistically significant.

Total reduction of NBT dye was increased in PCV leukocytes (Table 5). Most of this increase could be accounted for by an increase in the spontaneous (resting) reduction of NBT, rather than an increased increment upon phagocytosis (data not shown). Included for comparison are three patients with chronic granulomatous disease, 18 infected patients, and one patient with a complete deficiency of leukocyte glucose-6-phosphate dehydrogenase.

One patient with polycythemia vera had an increased hexose monophosphate shunt activity, increased resting and total nitroblue tetrizolium reduction, and a markedly increased oxygen consumption during phagocytosis.

Table 5. Total Nitroblue Tetrizolium Reduction

<table>
<thead>
<tr>
<th></th>
<th>X = 0.305*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SD = 0.072</td>
</tr>
<tr>
<td>Normal (N = 12)</td>
<td></td>
</tr>
<tr>
<td>Chronic granulomatous disease (N = 2)</td>
<td>X = 0.010</td>
</tr>
<tr>
<td></td>
<td>SD = 0.002</td>
</tr>
<tr>
<td>0%/G-6-PD (N = 1)</td>
<td>X = 0.007</td>
</tr>
<tr>
<td></td>
<td>SD = 0.002</td>
</tr>
<tr>
<td>Infected (N = 18)</td>
<td>X = 0.320</td>
</tr>
<tr>
<td></td>
<td>SD = 0.081</td>
</tr>
<tr>
<td>Polycythemia vera (N = 12)</td>
<td>X = 0.399</td>
</tr>
<tr>
<td></td>
<td>SD = 0.094</td>
</tr>
<tr>
<td></td>
<td>(p &lt; 0.001)</td>
</tr>
</tbody>
</table>

* Absorbance 515 nm/2.5 × 10⁶ phagocytes. Cells incubated in a shaking water bath for 15 min at 37°C, pH 7.4, in the presence of polystyrene particles (0.81 μ).
ACTIVATED PHAGOCYTE

Table 6. Leukocyte Enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Normal Range</th>
<th>Polycythemia Vera</th>
</tr>
</thead>
</table>
| Alkaline phosphatase                   | 0.5–1.5 \( \mu M/ hr/\) mg protein | \( X = 6.0 \)  
|                                        | SD = 2.3              |                   |
| Acid phosphatase                       | 2.5–3.0 \( \mu M/ hr/\) mg protein | \( X = 2.62 \)  
|                                        | SD = 0.12             |                   |
| Glucose-6-phosphate dehydrogenase      | 0.20–0.30 \( \mu M/min/mg protein \) | \( X = 0.28 \)  
|                                        | SD = 0.02             |                   |
| 6-phosphogluconic acid dehydrogenase   | 0.006–0.12 \( \mu M/min/mg protein \) | \( X = 0.094 \)  
|                                        | SD = 0.40             |                   |
| Pyruvate kinase                        | 2–3 \( \mu M/min/mg protein \) | \( X = 2.59 \)  
|                                        | SD = 0.40             |                   |
| Glutathione reductase                  | 0.02–0.05 \( \mu M/min/mg protein \) | \( X = 0.031 \)  
|                                        | SD = 0.008            |                   |
| Hexokinase                             | 0.0139–0.0210 \( \mu M/min/mg protein \) | \( X = 0.0177 \)  
|                                        | SD = 0.008            |                   |
| Myeloperoxidase                        | 0.3–0.6 \( \mu M/min/mg protein \) | \( X = 0.335 \)  
|                                        | SD = 0.02             |                   |

However, the resting oxygen consumption was similar to the control group, in contrast to other patients with polycythemia vera. This would suggest that some variation may occur in the metabolism of leukocytes from patients with this disease. We have not had the opportunity to study a patient with PCV during an acute bacterial infection.

Iodination of zymosan particles by phagocytes from polycythemia vera was similar to that seen in our control population (data not shown).

Leukocyte enzymes (Table 6): acid phosphatase, glucose-6-phosphate dehydrogenase, 6-phosphogluconic acid dehydrogenase, pyruvate kinase, glutathione reductase, hexokinase, and myeloperoxidase were normal in patients with PCV. Alkaline phosphatase was markedly elevated.

**DISCUSSION**

The conversion of glucose-\( ^{14}C \) to \( ^{14}CO_2 \), a measure of hexose monophosphate shunt activity, was increased in both resting and phagocytizing leukocytes obtained from patients with PCV. Although an increase in hexose monophosphate shunt activity is associated with intracellular bactericidal activity, its causal relationship to intracellular killing is not known.

Two diseases with decreased metabolic activity and impaired bactericidal capabilities of leukocytes have been described in which hexose monophosphate shunt activity is deficient. The first, chronic granulomatous disease of children, is characterized by defective killing of non-hydrogen peroxide-producing bacteria.\(^{20,31}\) The other disease entity is associated with the complete absence of leukocyte glucose-6-phosphate dehydrogenase.\(^{32}\) Other studies of a patient with 5% of the normal glucose-6-phosphate dehydrogenase activity in polymorphonuclear leukocytes have shown a decrease in the reduced pyridine nucleotide pool with functional defects in phagocytic and bactericidal activity similar to those observed in the patient with complete deficiency of this
Although bactericidal and metabolic characteristics of the phagocyte were impaired, the defects were not as severe as those in the original patient with complete absence of glucose-6-phosphate dehydrogenase activity. These data suggest that integrity of the hexose monophosphate shunt is necessary for adequate bactericidal activity. Less severe defects in metabolic activity have been found in leukocytes isolated from patients with systemic lupus erythematosus and hypogammaglobulinemia.

Increased metabolic activity of leukocytes has been less clearly defined in the literature. Such altered metabolic activity associated with phagocytosis has been observed in leukocytes of the newborn, pregnant females, and infected patients. Park et al. found increased oxygen consumption, nitroblue tetrazolium reduction, and hexose monophosphate shunt activity in resting newborn leukocytes. The mothers of these infants showed similar findings. However, the newborn and maternal leukocytes had normal increments in oxygen consumption, hexose monophosphate shunt activity, and nitroblue tetrazolium reduction during phagocytosis. Mitchell et al. found increased hexose monophosphate shunt activity in phagocytizing leukocytes from non-infected pregnant females.

Leukocytes from our patients with polycythemia vera had both an increased resting and phagocytizing reduction of nitroblue tetrazolium. Further studies have shown that some patients with polycythemia vera may demonstrate a false-positive histochemical test for the reduction of NBT (unpublished observations), similar to that observed in the newborn. The reduction of nitroblue tetrazolium to the insoluble blue formazan is thought to represent NADH oxidase activity. We recently studied a patient with a complete deficiency of leukocyte glucose-6-phosphate dehydrogenase with normal NADH oxidase levels but with an inability to reduce NBT during phagocytosis. Bellanti et al. have reported a direct correlation between NBT reduction and glucose-6-phosphate dehydrogenase activity. Whether NBT reduction is a measure of NADH oxidase activity or a reflection of over-all pyridine nucleotide oxidation is not clear.

A relationship between the age of the neutrophilic leukocyte and its phagocytic activity has been suggested. Brandt pulse-labeled rabbit cells with tritiated thymidine and studied the phagocytic activity of labeled leukocytes at various time intervals. A higher phagocytic index was found in the labeled neutrophil that first appeared in the peripheral blood on day 3, as compared to unlabeled cells in the same preparation. By the fourth day, unlabeled neutrophils had increased phagocytic activity as compared to older labeled cells. These data suggest that young neutrophils have an increased ability to phagocytize. Mauer and Jarrold using 32DFP-labeled granulocytes, found an increased production of neutrophils in polycythemia vera; Walker et al., in similar studies, concluded that there was an increased production of granulocytes in polycythemia vera.

Young erythrocytes and especially reticulocytes are more resistant to osmotic and oxidative stresses than older cells, and young cells contain greater enzyme levels and increased metabolic activity as compared to older cells. Thus,
the age of the neutrophil in polycythemia vera may partially explain the observed increases in metabolic activity.

Our findings indicate that polycythemia vera leukocytes can be characterized by an increased phagocytic index, increased clumping, and increased resting and phagocytizing hexose monophosphate shunt activity. In addition, oxygen consumption is increased in both resting and phagocytizing polycythemia vera leukocytes, and NBT reduction is elevated in the resting cells. Some of these abnormalities in leukocyte function may be related to the age of the phagocyte. The metabolism of cells from patients with acute bacterial infection is similar in some respects.

ACKNOWLEDGMENT

The excellent technical assistance of Miss Hazel Hooper, Mrs. Cindy Shannon, Mrs. Barbara Miller, Mrs. Sue Cousart, and Mrs. Pamela Shirley is gratefully acknowledged.

REFERENCES

The Activated Phagocyte of Polycythemia Vera

M. R. Cooper, L. R. DeChatelet, C. E. McCall and C. L. Spurr