Hexose Monophosphate Shunt Activity and Oxygen Consumption During Phagocytosis: Temporal Sequence[1] (36690)

LAWRENCE R. DECHATELET, PATSY WANG, AND CHARLES E. MCCALL

The Departments of Biochemistry and Medicine, The Bowman Gray School of Medicine, Winston-Salem, North Carolina 27103

During the process of phagocytosis by polymorphonuclear leukocytes (PMN), there occur profound changes in the metabolism of the cells, including increases in oxygen consumption, hexose monophosphate shunt (HMS) activity, and hydrogen peroxide production (1). The importance of these changes to the bactericidal capability of the neutrophil is deduced primarily from data obtained from cells of patients with chronic granulomatous disease of childhood. In this particular syndrome, the normal increments in O_2 consumption, H_2O_2 production, and HMS activity are lacking (2). This is accompanied by defective killing of ingested bacteria.

The exact mechanism of these changes is a subject of considerable controversy. The involvement of NADH oxidase (3), NADPH oxidase (4), and D-amino acid oxidase (5) has been suggested to explain the increases in O_2 consumption and H_2O_2 production. Similarly the exact relationship between the hexose monophosphate shunt increase and the bactericidal activity of the cell is not established. Most workers feel that the initiating event centers about activation of an oxidase (6), and the increase in HMS activity is a secondary phenomenon which follows the generation of hydrogen peroxide. Strauss *et al.* (7) on the other hand, have postulated a reaction sequence which is initiated by the activation of glutathione reductase. In this scheme, the activation of the HMS occurs before the changes in O_2 consumption or H_2O_2 production.

In the present report, we have attempted to clarify the temporal relationships of these events by careful quantitative measurements of particle uptake, O_2 consumption, and HMS activity at various time intervals following phagocytosis.

Materials and Methods. Polymorphonuclear leukocytes were isolated from normal venous blood as previously described (8). The cells were counted by conventional means and suspended in Hanks' balanced salt solution (HBSS) containing 10% fresh AB serum to a concentration of 5×10^6 cells/ml. The isolation prodedure typically yielded a suspension containing greater than 90% PMN; cell viability was always greater than 90% measured by the exclusion of 1% trypan blue dye.

Phagocytosis assay. The kinetics of the phagocytic process were determined by measuring the uptake of radiolabeled bacteria according to the procedure of Root, Rosenthal and Balestra (9). A stock culture of *Staphylococcus aureus* was grown overnight on trypticase soy broth containing 100 μCi of uniformly ^{14}C-labeled amino acids (Calatomic, San Diego, CA). The bacteria were heated in a boiling water bath for 20 min, washed 2 $\times$ with 0.9% saline, and suspended in HBSS to a standard optical density (0.075 absorbance at 525 nm). Phagocytosis was initiated by the addition of 1.0 ml of labeled bacteria to a flask containing 1.0 ml of cell suspension and 1.0 ml of HBSS. The reaction was terminated at varying time intervals by the addition of 1.0 ml 0.04 *M* NaF with cooling in an ice bath. Noningested bacteria were separated from the leukocytes by centrifugation at 4° at 100*g* for 10 min followed by 3 washes with 2.0 ml of 10% fetal calf serum in HBSS containing 0.01 *M* NaF. The resulting leukocyte pellets were dried overnight at

[1] Supported in part by grants AI-10732 and CA-12197 from the National Institutes of Health.

55°, and digested the following day with 0.50 ml of 0.2 *N* NaOH for 4 hr at 60°. The solutions were then neutralized with 0.20 ml of 3% acetic acid, 0.50 ml of distilled water was added, and 1.0 ml aliquots were counted in 10 ml of Aquasol (New England Nuclear Corp., Boston, MA) in a Tri-carb scintillation spectrometer (Packard Instrument Company, Inc., Downers Grove, IL).

Hexose monophosphate shunt activity. Glucose utilization via the HMS was determined by a modification of a previously described method (10), employing glucose differentially labeled in the C-1 or C-6 position. Each flask contained 0.90 ml of HBSS, 0.10 ml of isotope (containing 0.20 μCi) and 1.0 ml of the same bacterial suspension used in the phagocytosis assay. Glucose-1-^{14}C (sp act 52.2 mCi/mmole) and glucose-6-^{14}C (sp act 46.5 mCi/mmole) were obtained from the New England Nuclear Corp., Boston, MA. Reaction was initiated by the addition of 1.0 ml of cell suspension and stopped at varying periods of time by the addition of 1.0 ml of 5% trichloroacetic acid. $^{14}CO_2$ released during the course of the incubation was trapped in hydroxide of hyamine and counted as previously described (10).

Respiration. Oxygen consumption was measured with a Clark oxygen electrode (Yellow Springs Instrument Company, Yellow Springs, OH) according to a previously reported method (10). Each chamber of the instrument contained 1.0 ml of HBSS and 1.0 ml of labeled *S. aureus*. Reaction was initiated by the addition of 1.0 ml of cell suspension.

All assays (phagocytosis, HMS, and O_2 consumption) were run under exactly the same conditions. The same cell suspension and bacteria were used in each assay, and the assays were all performed simultaneously.

Results and Discussion. The time course of uptake of radiolabeled bacteria is illustrated in Fig. 1A. In this system, phagocytosis is complete within 10 min after the initiation of the reaction. This phagocytic curve is almost identical to that published by Root, Rosenthal and Balestra (9) who demonstrated complete uptake within 12 min. Similarly the high value at zero time presumably representing absorption or "trapping" of the labeled bacteria was encountered by Root, Rosenthal and Balestra (9). The slight decrease in cell associated bacteria at longer time intervals has likewise been reported previously (11).

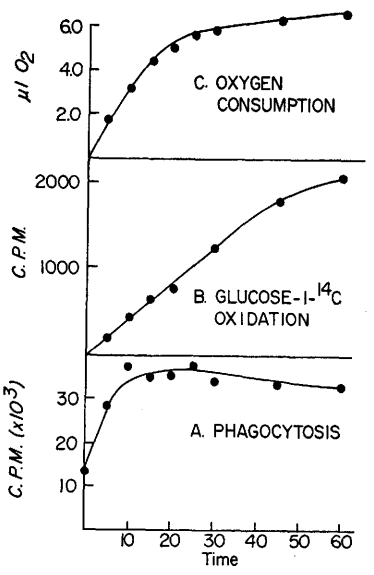

FIG. 1A. The uptake of ^{14}C-labeled *S. aureus* by PMN as a function of incubation time. (B) The oxidation of glucose-1-^{14}C by phagocytizing PMN as a function of incubation time. (C) The oxygen consumption of phagocytizing PMN as a function of incubation time.

In contrast to this very rapid uptake of labeled bacteria, the hexose monophosphate shunt increase is linear for 30–45 min (Fig. 1B). Similar findings have been reported by Stossel *et al.* (12), using emulsions as the ingested material; and by Michell *et al.* (13), using PMN monolayers. These latter investigators demonstrated a short lag period before a linear rate of glucose-1-^{14}C oxidation was observed. We have seen this lag period in some experiments, but the effect was not consistently observed (data not shown).

The time course of oxygen consumption (Fig. 1C) is intermediate between that of phagocytosis and the HMS. The rate of oxygen consumption begins to decrease at 15 min and has slowed to resting levels by 30 min time. Because the Clark oxygen electrode involves vigorous agitation of the cells we were concerned that the decrease in O_2

uptake might reflect a loss of viability. Studies were conducted with trypan blue under the exact conditions used here. Better than 90% of the cells were viable at the start of the experiment and this remained essentially constant during the entire hour (85% viability was found at 60 min). Hence, the present results cannot be explained by a loss in viability.

Similar results were obtained with other methods of initiating phagocytosis (data not shown). When PMN were allowed to phagocytize an emulsion containing Oil Red O according to the technique of Stossell *et al.* (12), phagocytosis was again completed within 10 min, the O_2 consumption remained linear for somewhat longer (about 20 min), and the HMS continued to increase in a linear fashion for at least 30 min. The same qualitative results were observed when cells were challenged with latex spherules. Particle uptake (assessed by microscopic examination) was complete in a very short period of time; the O_2 uptake decreased somewhat later, followed by the HMS activity. Although the time sequence of events was similar with the different types of particles, the magnitude of the effects varied considerably with the type of particle used. Ingestion of bacteria stimulated both O_2 consumption and HMS activity to a greater extent than did ingestion of either paraffin oil emulsion or latex particles. This is in agreement with the work of Mandell (14).

Under the conditions of the present experiment, particle ingestion by PMN is very rapid. At a time when phagocytosis is virtually complete, oxygen consumption is increasing in a linear fashion. This implies that the act of phagocytosis sets into action a series of metabolic events which are not reliant upon continued particle uptake. Similarly, at a time when the O_2 consumption is beginning to slow down, the HMS activity continues at a linear rate of increase. These data are consistent with a mechanism in which the initiating event involves an increase in oxygen consumption with the HMS stimulation following in time. The data do not support mechanisms in which the HMS stimulation precedes the increase in O_2 consumption.

Summary. Under conditions in which ingestion of labeled bacteria by PMN is accomplished within 10 min, oxygen consumption continues to increase in a linear fashion up to 20 min, while the HMS increase continues for at least 30 min. These results support a mechanism in which the O_2 increase precedes the HMS activation.

The authors thank Mrs. Pam Shirley and Mrs. Cindy Shannon for their excellent technical assistance.

1. Karnovsky, M. L., Sem. Hemat. **5**, 156 (1968).
2. Holmes, B., Page, A. R., and Good, R. A., J. Clin. Invest. **46**, 1422 (1967).
3. Cagan, R. H., and Karnovsky, M. L., Nature (London) **204**, 255 (1964).
4. Iyer, G. Y. N., and Quastel, J. H., Can. J. Biochem. Physiol. **41**, 427 (1963).
5. Cline, M. J., and Lehrer, R. I., Proc. Nat. Acad. Sci. U.S.A. **62**, 756 (1969).
6. Baehner, R. L., Gilman, N., and Karnovsky, M. L., J. Clin. Invest. **49**, 692 (1970).
7. Strauss, R. R., Paul, B. B., Jacobs, A. A., and Sbarra, A. J., Arch. Biochem. Biophys. **135**, 265 (1969).
8. DeChatelet, L. R., and Cooper, M. R., Biochem. Med. **4**, 61 (1970).
9. Root, R. K., Rosenthal, A. S., and Balestra, D. J., J. Clin. Invest. **51**, 649 (1972).
10. DeChatelet, L. R., Cooper, M. R., and McCall, C. E., Infec. Immunity **3**, 66 (1971).
11. Suzuki, J. B., Booth, R. R., and Greg, N., J. Infec. Dis. **123**, 93 (1971).
12. Stossel, T. P., Mason, R. J., Hartwig, J., and Vaughan, M., J. Clin. Invest. **51**, 615 (1972).
13. Michell, R. H., Pancake, S. J., Noseworthy, J., and Karnovsky, M. L., J. Cell Biol. **40**, 216 (1969).
14. Mandell, G. L., Proc. Soc. Exp. Biol. Med. **137**, 1228 (1971).

Received April 26, 1972. P.S.E.B.M., 1972, Vol. 140.