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STUDIES ON PHAGOCYTOSIS—III.
TRICARBOXYLIC ACID CYCLE AND THE CYTOCHROME SYSTEM AS ENERGY SOURCES FOR PHAGOCYTOSIS IN RABBIT PERITONEAL EXUDATE POLYMORPHONUCLEAR LEUKOCYTES

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Abstract—The phagocytosis of starch granules by rabbit peritoneal exudate polymorphonuclear leukocytes measured by the uptake of ^{131}I-labeled human serum albumin was found to be suppressed by anaerobiosis, iodoacetate, malonate, cyanide and ATPase inhibitors. The suppressive effect of iodoacetate can be partially relieved by pyruvate. The results suggest that the tricarboxylic acid cycle and the cytochrome system play important roles in supplying energy for the phagocytic process.

It has been well established that phagocytosis by polymorphonuclear leukocytes (PMN) is accompanied by increases in respiration, glycolysis, and the hexose monophosphate shunt (HMP) activity [1–9]. However, the relationship between these metabolic changes and the phagocytic process per se is not yet clearly understood. Glycolysis is commonly regarded as the metabolic pathway in polymorphonuclear leukocytes that provides energy for the phagocytic process, and the oxidative metabolism via the tricarboxylic acid (TCA) cycle is believed to be of minor importance in these cells [2, 4, 10, 11]. This view has been questioned recently by Patriarca *et al.* [12], who made the observation that PMN phagocytosis *in vitro* is not influenced by glucose, but lactate production in PMN is higher in the presence of glucose than in its absence. It has also been reported that mild degrees of physical injury augment glycolysis in PMN and cause a decrease in respiration [13, 14]. The role of the tricarboxylic acid cycle and of the cytochrome system in supplying energy for PMN phagocytosis was re-investigated employing a recently developed quantitative and sensitive method for measuring phagocytosis which forms the subject of this report.

MATERIALS AND METHODS

Rabbits. New Zealand albino rabbits were used to provide PMN.

PMN. Rabbit peritoneal leukocytes were obtained according to the procedure of Cohn and Morse [15]. The cells were sedimented at 1000 rev/min (125 *g*) for 15 min in a Sorvall centrifuge at 4°. After removing the supernatant, the cells were gently resuspended in Krebs–Ringer phosphate buffer to a concentration of 5×10^7 cells/ml. The final leukocyte suspension was chilled in ice and used within 20 min. Repeated observations on stained smears showed that better than 98 per cent of the leukocytes were polymorphonuclear. All exudates employed were essentially free of red blood cells. In any given experiment, only cells from a single animal were used.

Starch. The starch used was isolated from the seed of *Saponaria vaccaria* or Montana "cow soapwort" [16]. It is made up of extremely small granules with diameters distributed between 0.5 and 1.6 μm.

Buffer. Krebs–Ringer phosphate buffer, pH 7.4, containing one-half of the usual amount of $CaCl_2$, was filtered through a treated Millipore filter [17] (Millipore Filter Corp., Bedford, MA) and used within 3 days of preparation.

Chemicals. Radio-iodinated human serum albumin ([^{131}I]HSA), (65–90 μCi/mg) was purchased from Abbott Laboratories (North Chicago, IL). [^{14}C-1]glucose (16.3 μCi/mg) was purchased from the Nuclear-Chicago Instrument and Chemical Co.

Measurement of phagocytosis. The degree of phagocytosis was determined by measuring the uptake of [^{131}I]HSA as previously described [18]. Briefly, a suspension of PMN, rabbit serum and drug solution or buffer was placed in a 25-ml serum vial. The mixture was incubated with shaking at 37° for 30 min. A suspension of starch, containing ^{131}I-labeled albumin and glucose, was then added to the serum vial. The incubation was resumed for an additional 30 min and then terminated by the addition of 2.0 ml of ice-cold sodium ethylenediamine tetra-acetic acid (NaEDTA) and the serum vial was then placed immediately on ice. The contents were transferred to a precooled centrifuge tube. After centrifugation, the supernatant was discarded and the pellet was washed three times with NaEDTA saline. The radioactivity associated with the final pellet was determined by liquid scintillation after treatment with hydroxide of hyamine. In any given experiment, only cells from a single animal were used.

Measurement of [^{14}C-1]*-glucose utilization.* [^{14}C-1]-glucose utilization by PMN was determined according to a previously reported procedure [18].

RESULTS

Effect of ATPase inhibitors on phagocytosis of starch granules by PMN. Each experiment was performed five times. Chlorpromazine and ouabain suppressed

Table 1. Effect of ATPase inhibitors on the [^{131}I]HSA uptake by rabbit peritoneal exudate PMN during phagocytosis

Compound	Concn (M)	Radioactivity* (cpm)	P†
Control	0	9829 ± 126	
Chlorpromazine	1×10^{-4}	3884 ± 151	<0.001
Quinine	1×10^{-4}	6032 ± 201	<0.001
Ouabain	1×10^{-4}	8061 ± 69	<0.001

* Background radioactivity, i.e. radioactivity associated with the sample at zero time, has been subtracted from these values. Results are given on the basis of 5×10^7 PMN for 30 min. Each mean value (±S.E.) represents the average of six determinations.

† Student's *t*-test.

($P < 0.001$) the uptake of [^{131}I]HSA by PMN at a concentration of 1×10^{-4} M (Table 1). Quinine, at a concentration of 1×10^{-4} M, suppressed phagocytosis as measured either by [^{131}I]HSA uptake or by visual counting of starch granules (Table 2). But the phagocytosis-stimulated conversion of [^{14}C-l]glucose to $^{14}CO_2$ was not affected by quinine ($P < 0.25$) (Table 2).

Effect of iodoacetate and pyruvate on phagocytosis of starch granules by PMN. Pyruvate, at a concentration of 5×10^{-3} M, had no effect on [^{131}I]HSA uptake or the conversion of [^{14}C-l]glucose to $^{14}CO_2$ by PMN under phagocytic conditions (in the presence of starch granules). Iodoacetate (1×10^{-4} M) suppressed the [^{131}I]HSA uptake during phagocytosis by approximately 50 per cent in apparent agreement with the results by visual counting of granules ingested. The phagocytosis-stimulated conversion of [^{14}C-l]glucose to $^{14}CO_2$ was completely inhibited by iodoacetate (1×10^{-4} M). Pyruvate (5×10^{-3} M) partially reversed (approximately 50 per cent) the suppressive effect of iodoacetate (1×10^{-4} M) on [^{131}I]HSA uptake, but not on its inhibitory effect on [^{14}C-l]glucose utilization (Table 3).

Effect of malonate and KCN on [^{131}I]HSA uptake by rabbit peritoneal PMN during phagocytosis. Malonate (1×10^{-2} M) and KCN (1×10^{-3} M) suppressed the [^{131}I]HSA uptake by PMN during phagocytosis ($P < 0.001$) (Tables 4 and 5).

Relative efficiency of [^{131}I]HSA uptake by PMN incubated in the presence of starch granules under atmosphere of oxygen, air or nitrogen. The phagocytosis of starch granules by rabbit peritoneal exudate PMN

Table 2. Effect of quinine on [^{131}I]HSA uptake and [^{14}C-l]glucose utilization by rabbit peritoneal exudate PMN in the absence or presence of starch granules and on the number of engulfed starch granules

Conditions	[^{131}I]HSA uptake* (cpm)	[^{14}C-l]glucose utilization ($^{14}CO_2$)* (cpm)	Distribution of engulfed particles† (particles/cell)					No. of expts.
			0	1–5	6–10	11–15	>15	
Leukocytes	1,486 ± 129	1,900 ± 49						6
Leukocytes + starch	9,829 ± 126	11,278 ± 119	0	4	30	61	6	6
Leukocytes + starch + quinine (1×10^{-4} M)	6,032 ± 201	11,720 ± 397	6	29	46	20	0	6

* Background radioactivity, i.e. radioactivity associated with sample at zero time, has been subtracted from these values. Results, expressed as the mean ±S.E., are given on the basis of 5×10^7 PMN for 30 min.

† Results are expressed as percentage of cells containing the designated number of particles.

Table 3. Effect of iodoacetate and pyruvate on phagocytosis of starch granules by rabbit peritoneal exudate PMN measured by [^{131}I]HSA uptake, [^{14}C-l]glucose utilization and visual counting of engulfed starch granules

Conditions	[^{131}I]HSA uptake* (cpm)	[^{14}C-l]glucose utilization ($C^{14}O^2$)* (cpm)	Distribution of engulfed particles† (particles/cell)					No. of expts.
			0	1–5	6–10	11–15	>15	
Leukocytes	581	769 ± 44						5
Leukocytes + pyruvate‡		886 ± 44						5
Leukocytes + iodoacetate§		149 ± 4						5
Leukocytes + iodoacetate§ + pyruvate‡		192 ± 6						
Leukocytes + starch	2327 ± 55	6209 ± 47	4	3	23	55	15	5
Leukocytes + starch + pyruvate‡	2407 ± 78	6405 ± 81	2	3	21	59	16	5
Leukocytes + starch + iodoacetate§	1355 ± 60	303 ± 9	4	17	52	24	2	5
Leukocytes + starch + iodoacetate§ + pyruvate‡	1975 ± 32	412 ± 11	3	12	42	36	7	5

* Background radioactivity, i.e. radioactivity associated with sample at zero time, has been subtracted from these values. Results, expressed as mean ±S.E., are given on the basis of 5×10^7 PMN for 30 min.

† Results are expressed as percentage of cells containing the designated number of particles.

‡ 5×10^{-3} M.

§ 1×10^{-4} M.

Table 4. Effect of malonate on [^{131}I]HSA uptake by rabbit peritoneal exudate PMN in the presence of starch granules

Conditions	Radioactivity* (cpm) Mean ± S. E.	P†
Leukocytes	1486 ± 129	
Leukocytes + starch	9829 ± 126	
Leukocytes + starch + malonate (1×10^{-2} M)	8077 ± 290	<0.001

* Background radioactivity, i.e. radioactivity associated with the sample at zero time, has been subtracted from these values. The results are given on the basis of 5×10^7 PMN for 30 min. Each mean value represents the average of five determinations.

† Student's *t*-test.

as measured by [^{131}I]HSA uptake was suppressed (50 per cent) under a nitrogen atmosphere and stimulated under an oxygen atmosphere (30 per cent) (Table 6).

DISCUSSION

It has been well established that phagocytosis by PMNs is accompanied by increases in respiration, glycolysis and HMP activity [1, 2, 4, 6, 9, 19, 20]. Only a small fraction of total leukocyte glucose (less than 10 per cent) is normally metabolized via the HMP pathway [21–25]). It has been suggested that the HMP pathway might be stimulated in PMN during phagocytosis in order to provide sufficient NADPH for lipid synthesis in PMN during phagocytosis [26], or the stimulation may be related to the degranulation phenomenon which occurs in polymorphonuclear leukocytes during particle ingestion [21, 27], but probably does not provide energy for the phagocytic process [21]. Oxidative metabolism via the tricarboxylic acid cycle is also believed to be unimportant in these cells [2, 4, 10, 11]. The energy required for PMN phagocytosis is generally accepted to be derived from glucose metabolism to lactate via glycolysis. This view has been questioned recently by Patriarca *et al.* [12], who made the observation that PMN phagocytosis *in vitro* is not influenced by the presence of glucose in the incubation medium, but lactate production in

Table 6. Relative efficiency of [^{131}I]HSA uptake by rabbit peritoneal exudate PMN in the presence of starch particles under atmosphere of oxygen, air or nitrogen

Atmosphere	Radioactivity* (cpm)	P†
Air	5388 ± 143	
Nitrogen	2670 ± 90	<0.001
Oxygen	6990 ± 105	<0.001

* Background radioactivity, i.e. radioactivity associated with the sample at zero time, has been subtracted from these values. Results are given on the basis of 5×10^7 PMN for 30 min. Each mean value ± S.E. represents the average of six determinations.

† Student's *t*-test.

PMN is higher in the presence of glucose than in its absence. It has also been reported that mild degrees of physical injury augment glycolysis in polymorphonuclear leukocytes and cause a decrease in their respiration [13, 14].

The view that glycolysis provides the energy for PMN phagocytosis is supported by the observations of some investigators that (a) glycolytic enzyme inhibitors such as iodoacetate or fluoride inhibit phagocytosis, (b) respiratory inhibitors such as cyanide and antimycin A do not inhibit particle uptake, and (c) phagocytosis by PMN appears to be equally efficient under aerobic or anaerobic conditions [1, 2, 28–30]. Most investigators are in agreement that compounds interfering with glycolysis exert a profound inhibitory effect on phagocytosis [1, 2, 21, 29–31]. Cohn and Morse [2] found that the phagocytosis of bacteria by rabbit peritoneal exudate polymorphonuclear leukocytes was effectively blocked by iodoacetate (2×10^{-4} M) and sodium fluoride (2×10^{-4} M). Karnovsky *et al.* [1, 29] showed that phagocytosis of latex particles or starch granules by guinea pig peritoneal exudate polymorphonuclear leukocytes was inhibited (approximately 80 per cent) by iodoacetate (1 to 3×10^{-4} M) and by sodium fluoride (2×10^{-2} M). These results were interpreted as indicative that a functioning glycolytic pathway is required for the performance of phagocytosis. It should be noted, however, that both iodoacetate and sodium fluoride are ATPase inhibitors. The suppression of phagocytosis by these compounds, at least in part, might be due to their action on ATPase. The inhibitory effect of sodium fluoride on Na^+ extrusion

Table 5. Effect of KCN on [^{131}I]HSA uptake by rabbit peritoneal exudate PMN in the presence of starch particles

Conditions	Radioactivity* (cpm)	P†	Distribution of engulfed particles (particles/cell)† 0	1–5	6–10	11–15	>15	No. of expts.
Leukocytes	800 ± 33							
Leukocytes + starch	5292 ± 83		0	4	30	61	6	6
Leukocytes + starch + KCN (1×10^{-3} M)	3945 ± 80	<0.001	3	7	53	36	2	6

* Background radioactivity, i.e. radioactivity associated with the sample at zero time, has been subtracted from these values. Results are given on the basis of 5×10^7 PMN for 30 min. Each mean value ± S.E. represents the average of six determinations.

† Student's *t*-test.

and K^+ uptake by swine and human erythrocytes, for example, has been shown to be due primarily to inhibition of NaK-ATPase rather than inhibition of glycolysis as was initially believed [32]. This possibility is enhanced by the finding that phagocytosis of starch granules by rabbit peritoneal exudate PMN was significantly suppressed by all three ATPase inhibitors tested (chlorpromazine, ouabain and quinine) (see Table 1).

McKinney *et al.* [33] reported that malonate does not affect the ability of human polymorphonuclear leukocytes to phagocytize bacteria, suggesting that the cells do not depend on the tricarboxylic acid cycle as a source of metabolic energy. Sbarra *et al.* [34] found that phagocytosis was suppressed by malonate, but only at an unusually high concentration of 0.1 M. Since a similar concentration of KCl or NaCl also inhibited phagocytosis, it was concluded that the increased osmotic pressure of the system was responsible for the suppression by malonate. In disagreement with these reports, phagocytosis of starch particles by rabbit PMN measured by the [^{131}I]HSA uptake was significantly suppressed by malonate at a concentration of 1×10^{-2} M (see Table 2), well below the minimum concentration required for cations to influence phagocytic function.

Opinions regarding the effects of inhibitors of cellular respiration on phagocytosis vary. Cohn and Morse [2], Sbarra and Karnovsky [1] and Stossel *et al.* [35] found no inhibition of phagocytosis by cyanide (1×10^{-3} to 8×10^{-4} M). On the other hand, in agreement with the present results (Table 3) that KCN is suppressive at 1×10^{-3} M, Greendyke *et al.* [36] and Gordon and King [37] found that cyanide suppressed phagocytosis at a concentration of 1×10^{-2} M. Cohn and Morse [2] and Greendyke *et al.* found distinct inhibition by 2,4-dinitrophenol (2×10^{-4} M), as did Sherstneva (1×10^{-3} M) [38], whereas Sbarra and Karnovsky [31] found no inhibition by this uncoupler of oxidative phosphorylation at a concentration of 1×10^{-4} M.

One of the crucial observations which led to the conclusion that glycolysis is the principal energy source for phagocytosis in PMN was that phagocytosis measured by microscopic counting of engulfed particles appeared to be equally efficient under aerobic or anaerobic conditions [1], implying that this important function of PMN is independent of available oxygen. In contrast, under the conditions employed in the present study, phagocytosis is clearly suppressed by nitrogen (50 per cent) and stimulated by oxygen (see Table 4).

The inhibition of phagocytosis by iodoacetate is well known. This compound blocks glycolysis presumably by blocking the action of triose phosphate dehydrogenase. A study on the effect of the addition of metabolic intermediates which cannot be formed in the presence of iodoacetate may provide insight on the underlying mechanism of phagocytosis inhibition. Pyruvate, at a concentration of 1×10^{-3} M, partially relieved the inhibition of phagocytosis by iodoacetate (2×10^{-4} M). To explain this phenomenon, Selvaraj and Sbarra [40] suggested that oxidative decarboxylation of pyruvate yields acetyl CoA which can act as an energy source—perhaps by reversal of acetic thiokinase to yield ATP. In view of the present findings that phagocytosis is suppressed by malonate, KCN, and by anaerobiosis, it would seem that the restoration of phagocytic function by pyruvate is best explained on the basis of substrate availability for oxidative phosphorylation via the tricarboxylic acid cycle. The results suggest that glycolysis is important not only in supplying energy (2 moles ATP/mole of glucose) for phagocytosis but also in providing pyruvate for energy-producing oxidative metabolism. This conclusion is in agreement with the observation (see Table 3) that under conditions where glycolysis (measured by the conversion of glucose-[1-^{14}C] to $^{14}CO_2$) was inhibited almost completely (95 per cent) by iodoacetate, the rate of phagocytosis was reduced by only 40 per cent. It appears other pathways provided sufficient amounts of substrate for the TCA cycle to permit phagocytosis to proceed at a reduced rate. The fact that the phagocytic function that was suppressed by iodoacetate was only partially restored by pyruvate is in accordance with the view that the suppressive action of iodoacetate is due in part to its effects on glycolysis and on ATPase.

There are many possible explanations for the apparent disagreement between current results and those of certain similar studies reported in the literature. The discrepancy may reflect differing species and/or source (blood, exudate) of PMN, isolation procedure, incubation conditions *in vitro*, particles employed, and method used for measuring phagocytic activity. Starch granules, instead of bacteria or latex particles used by other investigators, were employed in the present study. It has been shown that phagocytosis by poly-morphonuclear leukocytes and the effect of drugs thereon vary depending on the nature of particles offered to the phagocytes [41, 42]. Phagocytosis of starch granules, unlike that of bacteria, does not appear to require serum opsonins. To what extent the use of starch granules may have contributed to the discrepancy between the present results and those reported previously by other investigators has not yet been determined. In the present study, as in those of Cohn and Morse [2, 15, 23] rabbit peritoneal exudate PMN was employed. Karnovsky and his colleagues, who made major contributions to this field, used guinea pig peritoneal exudate PMN. The possibilities that the discrepancy between some of the results here reported and those of similar studies in the literature might be due to species difference in the leukocytes employed, particles used, and isolation procedures are currently under investigation.

The method used in the present study for measuring phagocytic activity was different from that used by other investigators in prior reports, which could also account for the discrepancy in results. Conventional measures of phagocytosis are not sensitive, accurate or reliable. Methods based on visual counts of engulfed particles or growing out bacteria are at best approximate and inadequate to determine less than massive depressions of uptake. The inadequacy of such methods has been emphasized in a number of papers [1, 28, 36, 43]. Metabolic changes in the phagocytes during phagocytosis, such as increased oxygen consumption, or increased hexose monophosphate shunt activity, have been utilized for evaluation of phagocytic activity [1, 9, 44]. Although these meta-

bolic changes have been shown to accompany phagocytosis, more recent reports [45–47] and the present data (see Tables 2 and 3) clearly indicated that such metabolic changes can be completely dissociated from phagocytosis. In other methods where starch granules [29] or bacteria [28] labeled with a radioisotope, or emulsions of paraffin oil [35] were employed, measurements suffered from an inability to distinguish adherent from ingested particles. The method used in the present study, although indirect, is sensitive, objective, quantitative and capable of distinguishing adherent from ingested particles [18]. The advantage of this method is illustrated by the work of Cannarozzi and Malawista [48]. Employing [^{131}I]HSA uptake as a measure of phagocytosis, these investigators showed a biphasic effect of cytochalasin B on phagocytosis not observed in their earlier studies [49, 50], according to these authors, probably because sufficient distinction was not made between adherent and intracellular particles.

The present findings of suppressive effect of an aerobiosis, malonate, cyanide, and the relief of iodoacetate suppression by pyruvate suggest that, at least for rabbit peritoneal exudate PMN, the tricarboxylic acid cycle and the cytochrome system play important roles in providing energy for the performance of phagocytosis. Glycolysis appears to be important not only in supplying energy for phagocytosis, but also in providing pyruvate for energy-producing oxidative metabolism.

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