Phagocytosis and Leucocyte Enzymes in Protein-Calorie Malnutrition

By RATNAM J. SELVARAJ and K. SEETHARAM BHAT
National Institute of Nutrition, Indian Council of Medical Research,
Jamai Osmania, Hyderabad-7, India

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1. Enzymes pertinent to bactericidal activities of leucocytes were assayed in children suffering from protein-calorie malnutrition. 2. Leucocytes obtained from malnourished and control children contained similar activities for glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Granule-bound NADPH oxidase activity was low in leucocytes isolated from malnourished patients and failed to show the phagocytic stimulation which is normally seen in control leucocytes. Further, leucocytes obtained from malnourished patients did not release the acid phosphatase from lysosomes during phagocytosis, unlike those from controls. 3. Treatment of the malnourishment with a diet high in calories and protein resulted in significant increase in the activities of glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and NADPH oxidase and in releasing the acid phosphatase from the lysosomes into the supernatant fraction during phagocytosis. 4. The significance of these enzyme changes are discussed in relation to the increased susceptibility of these patients to infection.

Recently we have demonstrated that leucocytes isolated from patients suffering from protein-calorie malnutrition show altered metabolic and bactericidal activities. The decreased resistance to infection in malnourishment is probably due to the decreased phagocytic activity of leucocytes. There is a marked decrease in the phagocytic stimulation of hexose monophosphate shunt activity in these leucocytes and this probably accounts for the decreased bactericidal activity observed *in vitro* in these cases (Selvaraj & Seetharam Bhat, 1972).

The various metabolic events that take place during phagocytosis in leucocytes and their importance have been recently reviewed by Sbarra *et al.* (1971). In the present study, some of the enzymes that are involved in these metabolic activities have been studied in an attempt to pin-point the defect in leucocytes isolated from malnourished patients at the enzyme(s) level.

Materials and Methods

Venous blood was taken from 12 children suffering from protein-calorie malnutrition and 9 control children and leucocytes were isolated as described below.

Blood (3 ml) was taken in a 20 ml siliconized syringe containing 0.3 ml of 20% dextran (mol.wt. 20000) and 3 ml of calcium-free Krebs-Ringer phosphate solution and the syringe was allowed to stand in a vertical position at 5°C for 45–60 min after mixing the contents thoroughly. The resulting buffy coat leucocytes were removed by extrusion through a bent needle into siliconized test-tubes. The cells were

washed twice in Krebs-Ringer phosphate solution by light centrifugation and total leucocyte counts were made. Leucocytes were also isolated from seven of these patients after treatment for 3–4 weeks with a diet providing 880J (200 cal) and 3 g of protein/day per kg body weight. Incubations were carried out in a Dubnoff metabolic shaker at 37°C for 20 min in siliconized glass tubes with 5×10^6 leucocytes in 1 ml of Krebs-Ringer phosphate medium containing heat-inactivated (56°C, 30 min) 20% autologous plasma. Phagocytosis was induced by the introduction of heat-killed *Escherichia coli* at a phagocyte/particle ratio of 1:100. The reaction was stopped by the addition of ice-cold 0.25 M-sucrose and the leucocytes were separated by light centrifugation. The leucocytes were suspended in 0.25 M-sucrose to give a final concentration of 5×10^6 cells/ml and homogenized for 2 min at 4°C with a Potter-Elvehjem homogenizer to disrupt the cells and release the granules. The homogenate was divided into two portions. One portion was exposed to freezing and thawing for six times to release the granule-bound enzymes and was used to assay the total enzyme activities. The other portion was centrifuged at 12000g for 30 min in the cold to isolate the granules. The pellet was resuspended to its original volume in 0.25 M-sucrose and assayed for the different enzymes after freezing and thawing. The supernatant was used for assaying the soluble enzymes.

Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were assayed by measuring the native fluorescence of the NADPH formed (Theorell & Bonnichsen, 1951). These enzymes were assayed in the total homogenate only,

since no activity is present in the granules. Reaction mixture for the determination of glucose 6-phosphate dehydrogenase contained, in 0.3 ml: homogenate containing $1-3 \times 10^5$ cells; $3 \mu\text{mol}$ of MgCl_2 ; $3 \mu\text{mol}$ of KCl ; $0.75 \mu\text{mol}$ of nicotinamide; $1.5 \mu\text{mol}$ of glucose 6-phosphate; and $12.5 \mu\text{mol}$ of glycylglycine buffer, pH 8.7. The reaction was started by the addition of $0.03 \mu\text{mol}$ of NADP and was followed by the increase in fluorescence at 460 nm for the first 5 min. The content of the 6-phosphogluconate dehydrogenase reaction mixture was similar except that the substrate was $0.75 \mu\text{mol}$ of 6-phosphogluconate. NADPH oxidase was assayed in the extracts of the granules by measuring the decrease in E_{340} in a Beckman DU spectrophotometer (Iyer & Quastel, 1963) for the first 5 min. All the NADPH oxidase activity sedimented with the granules and no activity could be detected in the supernatant. The reaction mixture contained in 0.5 ml: extract of the granules from homogenate containing $5-10 \times 10^5$ leucocytes; $0.25 \mu\text{mol}$ of MnCl_2 ; and $5 \mu\text{mol}$ of sodium phosphate buffer, pH 5.5. The reaction was started by the addition of $0.05 \mu\text{mol}$ of NADPH. Acid phosphatase activity was measured in the total homogenate, granular and supernatant fractions spectrophotometrically at 410 nm by the formation of *p*-nitrophenol from *p*-nitrophenyl phosphate. The reaction mixture contained in 0.4 ml: $1-10 \times 10^5$ -cell homogenate; $4 \mu\text{mol}$ of *p*-nitrophenyl phosphate and $20 \mu\text{mol}$ of citrate buffer, pH 5.0. After incubation at 37°C for 1 h, the reaction was stopped by adding a mixture of $40 \mu\text{mol}$ of EDTA and 2mmol of NaOH in 0.1 ml.

Results

The results of the experiments are presented in Tables 1-3.

Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities in leucocyte

homogenates of both control and malnourished patients and the effect of treatment are shown in Table 1. Phagocytosis did not affect these enzyme activities. There were no significant differences in the activities of these two enzymes between malnourished and control children. Treatment resulted in increase in the activities of glucose 6-phosphate and 6-phosphogluconate dehydrogenases in leucocytes isolated from malnourished subjects. However, the values after treatment were not significantly different from the controls.

Table 2 includes the results obtained for NADPH oxidase activity in leucocyte granules. During phagocytosis there was a marked stimulation of this enzyme in control children. The enzyme activity was significantly lower ($P < 0.02$) in malnourished patients and the phagocytic stimulation was also absent. After treatment there was no significant increase in NADPH oxidase activity in the resting leucocytes; the activities observed were comparable with those seen in leucocytes isolated from control children. As the patients recovered from the disease there was an increase in NADPH oxidase activity in the phagocytosing cells, and the leucocytes isolated from malnourished patients showed a significant phagocytic stimulation ($P < 0.01$) which was absent before treatment.

Table 3 contains the results obtained for acid phosphatase assays of leucocyte fractions. Acid phosphatase activity of the whole-cell homogenate in malnourished children was similar to that observed in controls. The total enzyme activity was not influenced by particle uptake. However, there was a significant decrease ($P < 0.01$) in the granular fraction and a significant increase ($P < 0.001$) in the supernatant fraction during phagocytosis in the controls. These changes were not observed in leucocytes isolated from malnourished patients. Although the total enzyme activity did not alter as a result of treatment, the enzyme was released from the granules

Table 1. Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities in leucocytes

Details of the assay systems are given in the Materials and Methods section. The enzyme activities are given as nmol of NADPH formed/min per 10^5 leucocytes (means $\pm$ S.E.M. of the number of observations in parentheses). *P* values were determined by one-tailed pair test. NS, not significant.

	Glucose 6-phosphate dehydrogenase		6-Phosphogluconate dehydrogenase	
	Resting	During phagocytosis	Resting	During phagocytosis
Control (9)	0.63 ± 0.16	0.57 ± 0.13	0.85 ± 0.17	0.71 ± 0.15
Malnourished before treatment (12)	0.58 ± 0.10	0.52 ± 0.07	0.80 ± 0.12	0.71 ± 0.11
<i>P</i> , for control versus malnourished	NS	NS	NS	NS
Malnourished, after treatment (7)	0.84 ± 0.07	0.80 ± 0.07	1.29 ± 0.10	1.22 ± 0.11
<i>P</i> , for effect of treatment	<0.05	<0.01	<0.05	<0.05

Table 2. *NADPH oxidase activity in leucocyte granules*

Details of the enzyme assay are given in the Materials and Methods section. Enzyme activities are expressed as nmol of NADPH oxidized/min per 10^5 leucocytes (means $\pm$ S.E.M. of the number of observations in parentheses). *P* values were determined by one-tailed pair test. NS, Not significant.

	Resting	During phagocytosis	Phagocytic effect	
			% of resting activity	<i>P</i> value
Control (9)	0.15 $\pm$ 0.03	0.25 $\pm$ 0.05	197 $\pm$ 48	<0.05
Malnourished, before treatment (12)	0.07 $\pm$ 0.01	0.07 $\pm$ 0.01	100 $\pm$ 12	NS
<i>P</i> , for control versus malnourished	<0.02	<0.001	<0.01	
Malnourished, after treatment (7)	0.09 $\pm$ 0.02	0.15 $\pm$ 0.04	173 $\pm$ 19	<0.01
<i>P</i> , for effect of treatment	<0.07	<0.001	<0.05	

and appeared in the supernatant fraction to a large extent. Stained slides of leucocytes from malnourished children during phagocytosis did not show any detectable alterations in bacterial uptake, on microscopic examination.

Discussion

The vital protective role of the leucocytes in defence against bacterial infection is well recognized. Recently much interest has arisen regarding the biochemical events that occur during particle uptake and destruction by polymorphonuclear neutrophils (Karnovsky, 1968; Reed & Tepperman, 1969; Schultz, 1970; Sbarra *et al.*, 1971). Stimulation of glycolysis during phagocytosis provides necessary energy for particle uptake (Sbarra & Karnovsky, 1959) and it also lowers the intracellular pH (Dubos, 1954) and releases the lysosomal enzymes (Cohn & Hirsch, 1960).

On the other hand, the oxidative metabolic changes like stimulation in oxygen uptake and hexose monophosphate shunt activity have been reported to be intimately associated with the intracellular bactericidal property of the phagocyte (McRipley & Sbarra, 1967*a,b*; Good *et al.*, 1968; Klebanoff, 1968), and are not required for ingestion of bacteria (Selvaraj & Sbarra, 1966; Good *et al.*, 1968). The stimulated oxidation of glucose through the hexose monophosphate shunt results in an increase in the production of NADPH. This is oxidized by a particle-bound enzyme, NADPH oxidase, with the formation of hydrogen peroxide, which has been shown to increase in leucocytes during phagocytosis (Iyer & Quastel, 1963; Rossi & Zatti, 1964; Zatti & Rossi, 1966; Paul & Sbarra, 1968). Hydrogen peroxide in combination with the lysosomal enzyme, myeloperoxidase, and a halide ion form a potent bactericidal system in leucocytes (Klebanoff, 1968). Deficiency of any of the enzyme(s) involved in hydrogen peroxide produc-

tion would result in decreased bactericidal activity. Recently a clinical condition, chronic granulomatous disease in children, has been described where oxidative metabolism of leucocytes is seriously hampered. The patients invariably die of bacterial infection, although the leucocytes contain bacteria that had been ingested by phagocytosis (Good *et al.*, 1968).

Our previous report has shown that leucocytes obtained from malnourished patients show only marginal stimulation of hexose monophosphate shunt during phagocytosis. This defect is chiefly due to alterations in the neutrophils and not due to serum factor(s) (Selvaraj & Seetharam Bhat, 1972). Of the various enzymes that may be implicated in this leucocytic defect, the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were found to be similar to those observed in controls (Table 1). Treatment of these patients resulted in significant increase in the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in whole-cell homogenates. This may be due to the induction of the enzyme, since feeding a diet high in carbohydrate with optimum amount of protein has been reported to induce glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities in rat liver (Johnson & Sassoon, 1967).

However, leucocytes from malnourished patients contained less NADPH oxidase in both resting cells and cells during phagocytosis. These cells also fail to show an increase in NADPH oxidase activity during phagocytosis (Table 2). This would result in decreased NADP formation, which is the rate-limiting factor in the operation of the hexose monophosphate shunt.

We have previously shown that the hexose monophosphate shunt activity is stimulated during phagocytosis to only 377 $\pm$ 146% in leucocytes from 19 malnourished children compared with the 734 $\pm$ 179% stimulation observed in 14 normal children (Selvaraj & Seetharam Bhat, 1972). This is probably

Table 3. *Acid phosphatase activity in leucocyte fractions*

Details of the enzyme assay system are given in the Materials and Methods section. Enzyme activities are expressed as nmol of *p*-nitrophenyl phosphate hydrolysed/60 min per 10^3 leucocytes (mean $\pm$ S.E.M. of the number of observations in parentheses). *P* values were determined by one-tailed pair test. NS, Not significant.

	Granules			Supernatant		
	Phagocytic effect			Phagocytic effect		
	Resting	During phagocytosis	% of resting value <i>P</i> value	Resting	During phagocytosis	% of resting value <i>P</i> value
Control (8)	28.6 $\pm$ 4.1	25.9 $\pm$ 3.8	72 $\pm$ 6 <0.01	2.7 $\pm$ 0.9	9.8 $\pm$ 1.8	489 $\pm$ 121 <0.001
Malnourished, before treatment (12)	24.4 $\pm$ 1.4	20.5 $\pm$ 1.2	100 $\pm$ 2 NS	3.9 $\pm$ 0.6	3.9 $\pm$ 0.6	106 $\pm$ 10 NS
<i>P</i> for control versus malnourished	NS	NS	<0.001	NS	<0.01	<0.001
Malnourished, after treatment (7)	34.0 $\pm$ 5.1	30.8 $\pm$ 5.4	71 $\pm$ 4 <0.001	3.2 $\pm$ 0.7	12.2 $\pm$ 1.9	553 $\pm$ 55 <0.001
<i>P</i> for effect of treatment	NS	NS	<0.001	NS	<0.01	<0.001

due to the failure of NADPH oxidase in leucocytes of these patients to respond to phagocytosis (Table 2), since particle uptake does not appear to be affected. Again this failure to show normal stimulation in NADPH oxidase during phagocytosis would result in the production of insufficient hydrogen peroxide which in turn might prevent the release of the lysosomal myeloperoxidase. This is further supported by the decreased release of acid phosphatase from lysosomes into the supernatant fraction during phagocytosis in these patients (Table 3). The net result is impairment in bactericidal potency. This conclusion had been established by our previous studies. Leucocytes from six well-nourished children showed a $91 \pm 3\%$ bactericidal rate in 15 min, whereas those from eight malnourished children showed only $15 \pm 10\%$ bactericidal activity (Selvaraj & Seetharam Bhat, 1972).

The deficiency of NADPH oxidase in leucocytes isolated from malnourished patients may be due to the direct effect of protein deficiency or increased cortisol concentrations in circulation which has been shown to occur in malnutrition (Leonard & MacWilliam, 1964; Rao *et al.*, 1968). Cortisol has been reported to inhibit NADH oxidase of human leucocytes (Yielding & Tomkins, 1959; Mandell *et al.*, 1970). The mechanisms involved in the observed decrease in NADPH oxidase activity in leucocytes isolated from malnourished patients need further study.

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