Phagocytosis in riboflavin- or pyridoxine-deficient rats

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The effect of riboflavin or pyridoxine deficiency on phagocytosis was examined in rats to elucidate the mechanism of impaired wound healing in these deficiencies reported earlier. Phagocytic ingestion by peritoneal leukocytes was 60.4% and 68%, respectively, in riboflavin- or pyridoxine-deficient rats compared with weight-matched control animals. Lactate production, which is a measure of glycolytic activity and exocytic degranulation, were also reduced to 74% and 65%, respectively, in riboflavin deficiency but were unaltered in pyridoxine deficiency. Food restriction per se increased production of superoxide anion by leukocytes by two fold, and it was further increased by 1.5 fold during particle uptake only in pyridoxine deficiency. However, postphagocytic digestive events, as judged by the activities of acid phosphatase and collagenase in the incised wound, were not affected in either deficiency. Thus, impaired phagocytosis other than reduced collagenization (reported earlier) may contribute to the adverse effects of vitamins B_2 or B_6 deficiencies on wound healing. (J. Nutr. Biochem. 5:189–192, 1994.)

Keywords: phagocytosis; glycolysis; superoxide; degranulation; riboflavin; pyridoxine

Introduction

Earlier studies from our laboratory have shown impaired skin wound healing in either riboflavin- or pyridoxine-deficient rats.[1–3] This was attributed to reduced collagen content and crosslinking without ruling out other sites of biochemical lesions in the complex cascade of events associated with wound healing. Injury to the skin results in acute inflammatory response characterized by vasodilation, edema, and accumulation of phagocytes at the site of injury. In a subsequent study, pyridoxine deficiency was found to enhance inflammatory response as judged by paw edema and lipid peroxidation, suggesting that this may also contribute to the impairment of wound healing in pyridoxine deficiency. A similar effect was not seen in riboflavin deficiency.[4] However, in riboflavin deficiency the activities of leukocytic NADPH oxidase, a key enzyme in phagocytosis and superoxide dismutase, were reduced.[4] The present study deals with yet another aspect of wound healing, phagocytosis. Phagocytes perform the important function of killing microorganisms and clearing up dead tissues at the wound site. Delayed clearance of tissue debris at the wound site due to reduced phagocytosis is known to affect wound healing.[5]

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Methods and materials

Male weanling rats of Wistar/NIN strain (inbred) were divided into four groups by randomized block design: ad libitum fed control, riboflavin-deficient, pyridoxine-deficient, and control weight-matched for the vitamin-deficient groups. The animals were housed individually in screen-bottomed cages at 25 ± 1° C and a 12-hour light-dark cycle. They were fed a semi-synthetic diet containing 70% sucrose, 20% vitamin-free casein (Sigma Chemical Co., St. Louis, MO, USA), 5% peanut oil, 4% salt mixture* and 1% vitamin mixture† for a period of 6 weeks. The composition of vitamin and salt mixture was as described earlier.[1,3] Riboflavin or pyridoxine deficiency was induced by omitting the respective vitamin from the diet. The weight-matched control rats received the same diet as the ad lib-fed control group, but in restricted amounts to keep their body weights at par with those of the deficient groups.

At the end of 6 weeks of feeding the respective diets, rats were injected with 1 mL of 10% sodium caseinate (Difco Lab, Detroit, MI USA) intraperitoneally. Sixteen hours after the injection, blood sample was drawn from the orbital plexus with the aid of a heparin-

*Composition of vitamin mixture (in mg): thiamin, 8.3; riboflavin, 13.3; nicotinic acid, 66.6; pyridoxine hydrochloride, 8.3; calcium pantothenate, 66.6; biotin, 0.66; folate, 3.3; vitamin B_{12}, 0.06; vitamin K, 83.3; inositol, 166.6; p-aminobenzoic acid, 166.6, 10,000 IU vitamin A, 1000 vitamin D, 20 mg vitamin E and 0.5 g choline chloride were added to 1 kg diet.
†Composition of salt mixture (in g): sodium chloride, 105.00; potassium chloride, 289.70; calcium phosphate, 354.20; calcium carbonate, 11.45; magnesium sulphate, 90.00; ferric ammonium citrate, 17.90; sodium fluoride, 0.57; copper sulphate, 0.20; alum, 0.09; potassium iodide, 0.05; zinc sulphate, 0.05; cobalt chloride, 0.05 and manganese sulphate, 0.20.

ized tube. The animals were sacrificed by cervical dislocation and peritoneal leukocytes were collected by saline lavage. A suspension of 10^7 cells/2 mL in Krebs-Ringer phosphate buffer, pH 7.4, was made in a siliconized test tube.

Phagocytic process by peritoneal leukocytes was examined by measuring the following parameters: phagocytic ingestion of emulsified paraffin oil containing oil Red O[6] (Edward Gurr Ltd., London, UK) glycolysis as assessed by lactate excretion,[7] superoxide anion production,[8] exocytic degranulation of specific granules of leukocytes as determined by extracellular release of alkaline phosphatase.[9]

For the measurement of phagocytic process, stimulation of leukocytes was carried out by treating with phorbol myristate acetate (PMA) (Sigma Chemical Co.). The leukocytes were incubated with 5 μg PMA/mL incubation medium for the measurement of glycolysis[7] and with 500 ng PMA/mL incubation medium for the determination of superoxide anion production[8] and exocytic degranulation.

To assess the efficacy of phagocytic process in digesting dead and necrotic tissue at the wound site, activities of acid phosphatase,[10] a lysosomal enzyme, and collagenase,[11] the enzyme involved in the digestion of major extracellular components, were determined in the incision wound. Wound infliction was done as described earlier.[1] Briefly, a 5-cm long midline incision through full-skin thickness was made on the dorsal side of the animals after feeding the respective diets for 5 weeks.[1] On day 3 after wounding, animals were sacrificed and the wounds were excised together with 2 to 3 mm of adjacent skin for the estimation of acid phosphatase and collagenase activities.

Erythrocyte glutathione reductase activation coefficient (EGR-AC) and erythrocyte aspartate amino-transferase activation coefficient (EAAT-AC) were measured as indices of riboflavin and pyridoxine status respectively.[12]

Data were analysed by the analysis of variance and Duncan's multiple range test.

Results

Riboflavin or pyridoxine deficiency was established by the increments in EGR-AC and EAAT-AC values, respectively *(Table 1)*. A common weight-matched control group was included for both the deficient groups because in our earlier studies as well as the present study the mean body weights of these two groups were not significantly different. To maintain the body weights of weight-matched control rats on par with the deficient animals their food intakes were reduced by 2 to 3 g/day, but the diet had sufficient amounts of vitamins and minerals to meet their requirement as evidenced by normal values for EGR-AC and EAAT-AC in the weight-matched control group.

Phagocytic ingestion was reduced by 40% in riboflavin deficiency and 33% in pyridoxine deficiency compared with both the control groups *(Table 2)*. Food restriction did not affect this parameter.

Lactate production, which is a measure of glycolytic activity, was significantly reduced in the leukocytes of riboflavin-deficient rats both under unstimulated and stimulated conditions *(Table 2)*. However, percent stimulation of glycolysis in the riboflavin-deficient group was similar to the other groups. Neither pyridoxine deficiency nor food restriction significantly altered glycolytic activity under unstimulated or stimulated conditions.

Data given in *Table 3* show that superoxide production under basal condition was elevated in all three experimental groups compared with the ad lib-fed control group. This trend was seen even under a stimulated state, though not significantly except in pyridoxine-deficient rats whose leukocytes had the highest superoxide levels under the stimulated state.

Exocytic degranulation of specific granules of leukocytes measured as extracellular release of alkaline phosphatase was significantly lower in riboflavin deficiency *(Table 3)*. However, alkaline phosphatase activity after stimulation with PMA was similar in all four groups. Percent degranulation tended to be lower in riboflavin deficiency *(Table 3)*.

Activities of acid phosphatase and collagenase in the incised wound were not affected by either of the deficiencies.

Discussion

The results of the study suggest that phagocytic activity is impaired both in riboflavin and pyridoxine deficiency. Glycolysis, which supplies the energy needed for particle uptake,[13] was decreased in the leukocytes isolated from riboflavin-deficient rats *(Table 2)*. Activity of hexokinase, which is one of the rate-limiting enzymes in the glycolytic pathway, and availability of NAD, which is also a regulatory factor in the control of glycolysis, have been reported to be reduced in the liver of riboflavin-deficient rats.[14–16] Hence, a reduction in ATP formation due to decreased rate of glycolysis may be responsible for the lower phagocytic activity of leukocytes of riboflavin-deficient rats.

In pyridoxine deficiency, though phagocytic activity was reduced, glycolysis was not affected *(Table 2)*. Phagocytosis stimulates numerous intracellular events including burst of oxygen consumption and production of reactive oxygen metabolites. Increased endogenous production of hydrogen peroxide during phagocytosis has been shown to subsequently attenuate phagocytosis by causing autooxidative damage to the cell membrane.[17] In pyridoxine deficiency there was increased superoxide anion production under a stimulated

Table 1 Effect of riboflavin or pyridoxine deficiency on body weight of rats

	Ad lib-fed control	Weight-matched control	Riboflavin deficient	Pyridoxine deficient
Initial body weight (g)	42.8 ± 2.51	42.6 ± 2.63	42.4 ± 2.71	42.8 ± 2.78
Final body weight (g)	192.3 ± 5.71[a]	89.8 ± 3.10[b]	85.9 ± 3.393[b]	92.7 ± 3.72[b]
EGR-AC	1.16 ± 0.10[b]	1.23 ± 0.06[b]	1.49 ± 0.08[a]	—
EAAT-AC	1.17 ± 0.06[b]	1.22 ± 0.08[b]	—	1.61 ± 0.09[a]

Values are mean ± SEM of 12 animals/group. Values not sharing a common superscript are significantly different ($P < 0.05$).

Table 2 Effect of riboflavin or pyridoxine deficiency on phagocytic ingestion and glycolysis

	Ad lib-fed control	Weight-matched control	Riboflavin deficient	Pyridoxine deficient
Phagocytic ingestion (μg of oil ingested/min/mg protein)	148.6 ± 13.1[a] (5)	144.5 ± 16.6[a] (5)	87.0 ± 10.2[b] (4)	97.9 ± 16.4[b] (4)
Lactate excretion (μmol/10^7 cells/hr)				
Unstimulated	0.72 ± 0.09[a] (5)	0.68 ± 0.05[a,b] (5)	0.51 ± 0.02[b] (4)	0.73 ± 0.06[a] (4)
Stimulated*	0.93 ± 0.11[a] (5)	0.88 ± 0.06[a] (5)	0.65 ± 0.05[b] (4)	0.88 ± 0.08[a] (4)
% Stimulation	131.6 ± 6.60 (5)	129.4 ± 9.30 (5)	127.9 ± 8.30 (4)	121.3 ± 3.84 (4)

Values are mean ± SEM. Numbers in parentheses indicate number of samples analyzed in each group. Values in a row not sharing a common superscript are significantly different.
*In the presence of 5 μg phorobol myristate acetate per mL incubation medium.

Table 3 Effect of riboflavin or pyridoxine deficiency on superoxide anion production and exocytic degranulation of specific granules of peritoneal leukocytes

	Ad lib-fed control	Weight-matched control	Riboflavin deficient	Pyridoxine deficient
O_2^- produced (nmol/min/mg protein)				
Unstimulated	0.086 ± 0.01[b]	0.169 ± 0.05[a]	0.175 ± 0.03[a]	0.159 ± 0.02[a]
Stimulated*	0.582 ± 0.09[b]	0.733 ± 0.12[b]	0.830 ± 0.12[a,b]	1.149 ± 0.11[a]
% Stimulation	694.2 ± 73.0[a]	460.0 ± 44.4[b]	492.1 ± 34.6[b]	706.2 ± 31.8[a]
Alkaline phosphatase†				
Released into the supernatant	2.45 ± 0.27[a]	2.52 ± 0.25[a]	1.66 ± 0.18[b]	2.09 ± 0.16[a]
Present in cell pellet	13.93 ± 1.14	15.36 ± 1.81	14.56 ± 1.45	15.46 ± 1.60
% Degranulation	17.57 ± 2.37	17.35 ± 2.37	12.01 ± 2.81	14.09 ± 1.92

Values are mean ± SEM of six samples per group. Values in a row not sharing a common superscript are significantly different ($P < 0.05$).
*In the presence of 500 ng of phorbol myristate acetate per mL incubation medium.
†Expressed as μmoles phosphate/min/mg protein.

condition *(Table 3)*. While the mechanism for this is not clear, it could lead to increased formation of hydrogen peroxide and thereby attenuate phagocytosis. Activities of catalase and glutathione peroxidase, which can dispose of hydrogen peroxide, have been reported to be reduced in pyridoxine deficiency.[18]

It is difficult to get a sufficient number of phagocytes from wounded skin for the measurement of phagocytosis, NADPH oxidase activity, and superoxide anion production, and hence these parameters were measured in peritoneal leukocytes. However, the observations are still applicable to phagocytes because during the inflammatory stage wounded tissue is mostly infiltrated with leukocytes that perform the phagocytic function.

The effect of riboflavin deficiency on skin wound healing is unlikely to have been mediated through its effects on pyridoxal 5′-phosphate synthesis, because studies reported by us[19,20] and Rasmussen et al.[21] show that for the same duration of deficiency, pyridoxal phosphate levels in tissues like blood, liver, and muscle are not affected though activity of hepatic pyridoxine 5′-phosphate oxidase is markedly reduced. This can be due to a slower turnover of the coenzyme as a result of binding to apoenzymes because activities of some pyridoxal 5′-phosphate-dependent enzymes like tyrosine amino transferase and serine dehydratase are significantly increased in the livers of riboflavin-deficient rats.[20]

Studies reported earlier have shown impaired skin wound healing in riboflavin and pyridoxine-deficient rats as judged by a longer period for epithelialization, reduced wound contraction, and tensile strength of wound. Collagen content and maturity of scar tissue were also markedly reduced.[1,2] Because phagocytes perform the important function of clearing up tissue debris and foreign bodies at wound site, a reduction in phagocytic rate may also contribute to the delay in wound healing in riboflavin as well as in pyridoxine deficiency.

References

1 Lakshmi, R., Lakshmi, A.V., and Bamji, M.S. (1989). Skin wound healing in riboflavin deficiency. *Biochem. Med. Metab. Biol.* **42,** 185–191

2 Lakshmi, A.V., Diwan, P.V., and Bamji, M.S. (1988). Wound healing in pyridoxine deficiency. *Nutr. Res.* **8,** 1203–1206

3 Lakshmi, R., Lakshmi, A.V., and Bamji, M.S. (1990). Mechanism of impaired skin collagen maturity in riboflavin or pyridoxine deficiency. *J. Biosci.* **15,** 289–295

4 Lakshmi, R., Lakshmi, A.V., Diwan, P.V., and Bamji, M.S. (1991). Effect of riboflavin or pyridoxine deficiency on inflammatory response. *Indian J. Biochem. Biophys.* **28,** 481–484

5 Shmuel Shoshan (1981). Wound healing in, *Int. Rev. Connect. Tissue Res.,* **9,** 1–26.

6 Stossel, T.P., Pollard, T.D., Mason, R.J., and Vaughan, M. (1971).

Isolation and properties of phagocytic vesicles from polymorphonuclear leukocytes. *J. Clin. Invest.* **50,** 1745–1757
7 Absolom, R.D. (1986). Basic methods for the study of phagocytosis. *Methods in Enzymology,* **132,** 145–146.
8 Absolom, R.D. (1986). Basic methods for the study of phagocytosis. *Methods in Enzymology,* **132,** 152.
9 Estensen, R.D., White, J.G., and Holmes, B. (1974). Specific degranulation of human polymorphonuclear leukocytes. *Nature (London),* **248,** 347–348
10 Alvarez, E.F. (1962). The kinetic and mechanism of the hydrolysis of phosphoric acid esters by potato acid phosphatase. *Biochem. Biophys. Acta* **59,** 663–672
11 Cawston, F.E. and Murphy, G. (1981). Mammalian collagenases. *Methods in Enzymology,* **8,** 711–715.
12 Bayoumi, R.A. and Rosalki, S.B. (1976). Evaluation of methods of coenzyme activation of erythrocyte enzymes for detection of deficiency of vitamin B_1, B_2 and B_6. *Clin. Chem.* **22,** 327–335
13 Selvaraj, R.J. and Sabarra, A.J. (1966). Relationship of glycolytic and oxidative metabolism to particle entry and destruction in phagocytosing cells. *Nature* **211,** 1272–1276
14 Eldjarin, L. and Bremer, J. (1962). The inhibitory effect at the hexokinase level of disulphides on glucose metabolism in human erythrocytes. *Biochem. J.* **84,** 286–291
15 Burch, H.B., Lowry, O.H., Bradley, M.E., and Max, P.F., Jr. (1970). Hepatic metabolites and cofactors in riboflavin deficiency and calorie restriction. *Am. J. Physiol.* **219,** 409–415
16 Rose, I.A. and Warms, J.V.B. (1966). Control of glycolysis in human red blood cells. *J. Biol. Chem.* **241,** 4848–4854
17 Baehner, R.I., Boxer, L.A., Allen, J.M., and Davis, J. (1971). Autooxidation as a basis for altered function by polymorphonuclear leukocytes. *Blood* **50,** 327–335
18 Selvam, R. and Ravichandran, V. (1991). Lipid peroxidation in liver of vitamin B-6 deficient rats. *J. Nutr. Biochem.* **2,** 245–250
19 Lakshmi, A.V. and Bamji, M.S. (1974). Tissue pyridoxal phosphate concentration and pyridoxamine phosphate oxidase activity in riboflavin deficiency in rats and man. *Br. J. Nutr.* **32,** 249–255
20 Lakshmi, A.V. and Bamji, M.S. (1975). Pyridoxal 5′-phosphate-dependent enzymes in riboflavin deficiency. *Indian J. Biochem. Biophys.* **12,** 136–138
21 Rasmussen, K.M., Barsa, P.M., and McCormick, D.B. (1979). Pyridoxamine (pyridoxine) 5′-phosphate oxidase activity in rat tissues during development of riboflavin or pyridoxine deficiency. *Proc. Soc. Exp. Biol. Med.* **161,** 527–530