THE DIGESTIVE CAPACITY OF PYRIDOXINE-DEFICIENT PHAGOCYTES *IN VITRO*

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PLATE XVIII

PYRIDOXINE deficiency depresses the ingestive capacity of phagocytes (Cottingham and Mills, 1943; van Bijsterveld, 1971), but nothing is known of the effect of pyridoxine deficiency on their digestive capacity. Some of the mechanisms of intracellular killing have been studied in detail (Hirsch, 1965; Zeya and Spitznagel, 1966) and recently attention has been drawn to the importance of phagocyte myeloperoxidase in intracellular digestion (McRipley and Sbarra, 1967; Klebanoff, 1968; Lehrer and Cline, 1969). Myeloperoxidase is derived from protoporphyrin IX, the initial reaction in the pathway being pyridoxal phosphate-dependent (Schulman and Richert, 1957; Richert and Schulman, 1959).

The over-all bactericidal power of pyridoxine-deficient and normal guinea-pigs was studied *in vitro* using phagocytes in homologous serum. In addition, since the relative contribution of intracellular digestion and bactericidal serum factors cannot be separated in such a system, the digestive power of phagocytes was studied separately in pooled normal serum from which a number of possible bactericidal components had been removed. The amount of histochemically detectable myeloperoxidase in the polymorphonuclear leucocytes of pyridoxine-deficient and normal animals was also compared.

MATERIALS AND METHODS

The diet, the techniques for collection and treatment of the intraperitoneal phagocytes, bacteria and serum, the method for total counts and the conditions during incubation for the *in-vitro* experiments were given in a previous report (van Bijsterveld). Experiments were carried out after 50 days of dieting in the pyridoxine-deficient group. *Moraxella nonliquefaciens* strain 21 was used as test organism.

The *in-vitro* systems contained 20 per cent. serum and 5×10^6 phagocytes from pyridoxine-deficient or normal animals and 2.2×10^6 bacteria per ml—a cell-to-bacterium (c/b) ratio of 2.25. Bactericidal capacity was expressed as a reduction factor (RF), i.e., the ratio of viable organisms recovered from systems without phagocytes and those containing phagocytes of normal or deficient animals, using the method of Miles, Misra and Irwin (1938) for viable counts.

Pooled guinea-pig serum was adsorbed on bentonite (5 mg dry weight bentonite per ml serum) at 0°C to eliminate lysozyme and bentonite adsorbable factor (BAF, Glynn and Milne, 1967) according to the technique of Myrvik and Weiser (1955). Residual lysozyme

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was assayed by the agar diffusion method developed according to the principles of Fleming (1922), Goldsworthy and Florey (1930), Boasson (1938) and Smolelis and Hartsell (1949). The substrate was *Micrococcus lysodeikticus* (Difco) in a concentration of 50 mg per ml in a 1-mm agarose layer. One cycle of adsorption was sufficient to remove all lysozyme. The haemolytic titre of complement decreased after this treatment from 40 to 35. Serum adsorbed with bentonite was symbolised by "L—, BAF—".

Antibody to *Moraxella nonliquefaciens* was eliminated by repeated absorption at 0°C, according to the method of Wardlaw (1962), with 50 mg dry weight of *M. nonliquefaciens* strain 21 per ml guinea-pig serum. Residual antibody was tested with the indirect fluorescent antibody technique with fluorescein isothiocyanate-conjugated gamma-globulin from rabbit anti-guinea-pig-serum, according to the technique of Lewis *et al.* (1964). Complete elimination of antibody was obtained with two cycles of adsorption. The haemolytic titre of complement decreased from 40 to 5 after this treatment. Serum after adsorption of antibody was symbolised by "C±, Ab—".

Complement was destroyed by heating the serum at 56°C for 20 min. and residual complement was assayed by the haemolytic system. Heated serum was symbolised by "C—".

The effect of treated serum on phagocytosis was studied by counting the average number of ingested bacteria and the proportion of cells participating in phagocytosis, after 1 hr of incubation at 37°C. The polymorphonuclear leucocyte was used as indicator cell with cell-to-bacterium (c/b) ratios of 0.44 and 2.25; 400 cells were counted in each experiment as in van Bijsterveld.

Phagocytes from nine pyridoxine-deficient and nine normal animals were used and the experiments were carried out on a single day. After incubation for 3 hr, the phagocytes were disrupted by means of a motor-driven glass pestle and viable counts of the bacteria were made.

To evaluate the intracellular antimicrobial component, phagocytes from pyridoxine-deficient and normal guinea-pigs were examined in pooled treated normal guinea-pig serum. In order to follow the time-course of the intracellular digestive capacity the mechanical disruption of the phagocytes was omitted and viable counts were made directly from the phagocyte-bacterial suspension. To make this possible the ingestion velocity was increased by centrifuging the phagocyte-bacteria mixtures immediately after preparation at room temperature for 5 min. at 450*g*. After resuspension the systems were incubated at 37°C and viable counts were made after 1, 2 and 3 hr.

Histochemically demonstrable myeloperoxidase was measured by counting granules in the leucocytes, stained according to the method of Sato and Sekiya (1963), of groups of 24 pyridoxine-deficient and control animals.

The Wilcoxon two-sample technique and χ^2 contingency tables were used to compare the reduction factors, the distribution of the number of bacteria in the phagocytes and the amount of myeloperoxidase in the polymorphonuclear leucocytes of deficient and normal animals. The P values given are those of two-tailed tests.

RESULTS

The over-all bactericidal power *in vitro* of phagocytes in homologous serum is shown in table I. The average reduction of the number of viable bacteria after 3 hours' incubation was 89 per cent. for the pyridoxine-deficient system (RF = 8.92)* and 98 per cent. for the normal system (RF = 57.34). This difference was significant (P = 0.01). There was no significant difference between the bactericidal power of the serum of normal and deficient animals. The average killing after 3 hours' incubation with serum alone for all normal

* The percentage reduction in the number of viable organisms is given by $\left(1 - \frac{1}{\text{RF}}\right) \times 100$.

PYRIDOXINE-DEFICIENT PHAGOCYTOSIS

FIG. 1.—Polymorphonuclear leucocytes from a pyridoxine-deficient guinea-pig, stained for myeloperoxidase. The cytoplasm of the leucocytes is devoid of myeloperoxidase granules. $\times 1600$.

FIG. 2.—Polymorphonuclear leucocytes from a normal guinea-pig, stained for myeloperoxidase. The nuclei of the leucocytes are partially obscured by the closely packed myeloperoxidase granules. $\times 1600$.

and deficient systems was 62 per cent. (RF = 2.60); the 95 per cent. confidence interval was 46–70 per cent.

The bactericidal power of a serum pool, treated in various ways to eliminate antibody, complement and BAF is shown in the first column of table II.

TABLE I

Reduction factors of Moraxella nonliquefaciens strain 21 exposed to phagocytes from deficient and normal animals in homologous serum after 3 hr at 37°C

Experiment no.	Reduction factors in serum plus phagocytes from	
	deficient animals	normal animals
1	3.57	8.49
2	4.38	4.58
3	4.37	300.00
4	5.37	4.31
5	4.28	90.00
6	3.88	21.77
7	6.14	10.67
8	45.00	7.00
9	3.26	69.23
Average	8.92	57.34

TABLE II

The effects of normal pooled guinea-pig serum, and serum after heating or absorption with bentonite and Moraxella on killing and phagocytosis of Moraxella by normal guinea-pig phagocytes

Serum components	Reduction factor of serum alone	Average number of			
		ingested bacteria at a cell-to-bacterium ratio of		cells phagocytosing at a cell-to-bacterium ratio of	
		0.44	2.25	0.44	2.25
C+, Ab+, L+, BAF+	1.43	2.01	0.48	0.71	0.27
C±, Ab−, L−, BAF−	0.76	1.92	0.51	0.79	0.34
C−, Ab+, L+, BAF+	0.86	0.89	0.15	0.46	0.13

Elimination of complement by heating reduced the RF below unity. With this treatment, however, phagocytosis was impaired (table II). In contrast, with preservation of complement but loss of BAF, lysozyme and antibodies, the RF was reduced to less than 1, but ingestion by the phagocytes was hardly impaired and the proportion of cells participating in phagocytosis was relatively high.

For optimum discrimination the number of cells with more than one ingested organism must be low. At a cell-to-bacterium (c/b) ratio of 0.44, the proportion of phagocytes ingesting more than one organism was 54 per cent., but at a c/b ratio of 2.25 only 9 per cent., and this was the ratio used.

TABLE III

Reduction factors of M. nonliquefaciens strain 21 after 1, 2 and 3 hours' incubation at 37°C with phagocytes from pyridoxine-deficient and control animals in the presence of pooled normal guinea-pig serum adsorbed on bentonite and Moraxella

Experiment no.	Reduction factor after incubation for					
	1 hr		2 hr		3 hr	
	deficient animals	normal animals	deficient animals	normal animals	deficient animals	normal animals
1	1.70	1.78	1.65	1.70	2.48	1.69
2	1.47	1.72	1.34	2.24	2.12	2.40
3	1.74	2.23	1.93	2.33	2.00	1.80
4	1.96	2.11	2.14	2.31	1.82	2.91
5	2.09	3.80	2.62	2.92	2.09	2.20
6	1.84	2.61	1.88	1.32	1.90	1.42
7	2.91	2.20	2.88	2.66	3.02	2.46
8	2.06	2.12	2.40	2.26	1.78	2.20
9	1.65	2.06	2.95	2.02	2.36	1.68
Average	1.94	2.29	2.20	2.20	2.17	2.08

TABLE IV

Distribution of myeloperoxidase granules in polymorphonuclear leucocytes from groups of 24 deficient and normal animals

Animals	Percentage of polymorphonuclear leucocytes having myeloperoxidase granules numbering			
	0	1-15	16-30	30 or more
Pyridoxine-deficient	14	38	38	10
Normal	4	19	34	43

In table III the reduction factors for the in-vitro normal and deficient phagocyte-bacterial systems in the C±, Ab—, L—, BAF— serum after 1, 2 and 3 hours' incubation are given. The digestive capacity of the deficient system was 15 per cent. lower than that of the normal system after the 1st hr of incubation. There was an indication of significance in this difference ($P = 0.06$). No difference in digestive capacity was found after 2 and 3 hours' incubation.

This relatively small decrease in digestive capacity of phagocytes in the pyridoxine-deficient animals was correlated with lack of myeloperoxidase. Table IV shows that the leucocytes of the deficient animals carried decidedly less myeloperoxidase and this difference was highly significant ($P < 0.0005$). Figures 1 and 2 show the appearance of polymorphonuclear leucocytes from a deficient and a normal animal, stained for myeloperoxidase. The decrease in myeloperoxidase in the deficient animals was rather abrupt and occurred after an average of 46 days of dieting (95 per cent. confidence limit 42–50 per cent.). Large fluctuations were found in the number of staining granules between animals of each group as well as considerable fluctuations within each animal when leucocytes were examined at weekly intervals.

DISCUSSION

The over-all bactericidal power of phagocytes and homologous serum was greater in the normal systems. This was, however, not necessarily an expression of greater antimicrobial activity of the cells in the normal system, since it might well result from differences in serum factors conditioning the organisms before they were digested by the phagocytes.

In order to evaluate the antimicrobial capacity of phagocytes of deficient and normal animals, all in-vitro phagocyte-bacterial systems were supplied with the same serum from which various bactericidal factors had been eliminated. Since differences in digestive capacity of the phagocytes of the systems might be in rate rather than amount (as they proved to be) time-consuming mechanical disruption had to be eliminated and maximal phagocytosis induced as soon as possible.

To ensure that any differences found were a reflection of differences in antimicrobial capacity rather than differences in distribution of the number of the organisms in the phagocytes, the relatively high c/b ratio of 2.25 was used.

Myeloperoxidase is an important part of the antimicrobial system of the phagocyte. The prosthetic group of myeloperoxidase is derived from protoporphyrin IX, which is ultimately derived from the parent tetrapyrrole porphyrin. The initial reaction in the pathway of the synthesis of tetrapyrroles is the condensation of glycine with succinyl CoA, yielding δ -amino-levulinic acid (Wittenberg and Shemin, 1950; Shemin and Wittenberg, 1951). This reaction is pyridoxal phosphate-dependent (Schulman and Richert, 1957; Richert and Schulman, 1959). It is presumably through the formation of δ -amino-levulinic acid and ultimately of myeloperoxidase that pyridoxine deficiency interferes with phagocytic destruction of bacteria.

SUMMARY

In-vitro killing of *Moraxella nonliquefaciens* by phagocytes in homologous serum systems of the guinea-pig was decreased in pyridoxine deficiency. The decreased rate of intracellular killing was correlated with a decrease in histochemically detectable myeloperoxidase.

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