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INFLUENCE OF D-PENICILLAMINE ON METABOLIC AND FUNCTIONAL ACTIVITIES OF NEUTROPHIL GRANULOCYTES

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Influence of D-Penicillamine (DPA) on metabolic and functional activities of neutrophil granulocytes was investigated in vitro by measuring superoxide anion production and beta-glucuronidase release as well as by determining phagocytic and intracellular killing activities of cells. Preincubation with DPA in the concentration range of 0.5-5.0 mM resulted in 28-53 % increase in superoxide anion production by granulocytes stimulated with 10^{-7} M FMLP. DPA in the same concentration range resulted in 145-371 % rise in the FMLP-stimulated beta-glucuronidase release. However, uptake and subsequent killing of viable Staphylococcus aureus was not influenced by incubation of granulocytes with various concentrations of DPA (0.5-5.0 mM). From these results we conclude that DPA may influence the superoxide anion production and beta-glucuronidase release in granulocytes without altering the phagocytic and intracellular killing activities of these cells. We suppose that the unchanged antibacterial activity of neutrophil granulocytes is resulted by the two opposite DPA effects: acting extracellularly reduces free radical level while the direct membrane effect results in enhancing metabolic activity in granulocytes.

INTRODUCTION

D-Penicillamine (DPA) has been used for the treatment of various diseases related to the toxic effect of oxygen metabolites not only in adults suffering from rheumatoid arthritis or Wilson's disease /18/ but also in premature infants. A favourable effect of this drug has been observed for the prevention and treatment of retinopathy of prematurity (ROP) /11, 12/. By initiation of DPA in the treatment of jaundice of neonates a significant decrease in the number of blood exchange transfusions has been achieved /13/. DPA has

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been found to inhibit lipid peroxidation induced by oxygen metabolites (superoxide anion, hydrogen peroxide, hydroxyl radical, singlet oxygen) in the red blood cell membrane /15, 16/. However, these toxic oxygen metabolites are known to play an important role in the intracellular killing of ingested bacteria by neutrophil granulocytes, as well /6, 7/. It is postulated that phagocytic cells attached to the inflamed site, release reactive oxygen species, which may also play role as mediators of inflammation, causing biomembrane destruction, denaturation, aggregation or depolymerization of biopolymers, cell death, as well as attracting and activating more leukocytes /6/. For that very reason the effect of radical scavanger drugs in a living organism is most complex. Vitamin E which is widely administered to premature infants for the prevention of ROP has been found to increase risk of sepsis and necrotizing enterocolitis /10/. Recently, Engle et al. in vitro studies found that pharmacologic concentrations of Vitamin E depressed superoxide production induced by phorbol myristate acetate in polymorphonuclear leukocytes /9/. Although controlled clinical trials revealed no increase in the number of bacterial infections during and after DPA treatment, the effect of this drug on metabolic and functional activities of human neutrophils has not been completely assayed.

From earlier results obtained in red blood cells we supposed that DPA has not only free radical scavenger- but also membrane effect. This may be connected with degranulation during which the granules fuse with the plasma membrane and discharge their contents both into the extracellular space and into the phagocytic vacuole.

Because the phagocytic cells' antimicrobal capacity is closely connected with their metabolic (toxic oxygen metabolites' production and degranulation) and functional activity (phagocytosis and intracellular killing of bacteria), this study was initiated to investigate the effect of DPA on superoxide anion generation and beta-glucuronidase release as well as on phagocytic and intracellular killing activation of human peripheral blood granulocytes.

MATERIALS AND METHODS

Isolation of granulocytes: Granulocyte enriched cell suspensions were prepared from heparinized (10 U/ml) venous blood of healthy adult donors, using standard separation techniques /4/. Blood was obtained to the institution informed consent policy. Cells were suspended in Krebs-Ringer phosphate dextrose (KRPD) buffer (pH=7.28). Contaminating erythrocytes were removed by hypotonic lysis. After separation more than 95 % of the granulocytes remained viable as checked by Tripan blue exclusion.

D-Penicillamine: DPA (Knoll, Ludwigshafen, West Germany) was freshly dissolved and diluted for stock solution. Three different concentrations of DPA (0.5, 2.0 and 5.0 mM) were chosen to test various effects on granulocytes. Concentrations of 0.5 and 2 mM were equivalent with the minimum and maximum serum level of the drug achieved in prematures by administration of 300 mg/kg/die of DPA /3, 2/. The 5 mM was a supposed toxic concentration of the drug.

Measurement of superoxide anion production: Production of superoxide anion by granulocytes was assayed by continuous recording of the reduction of ferricytochrome c at 550 nm in a Specord model 40 spectrophotometer /3/. The test cuvette contained 2 million granulocytes and 80 uM horse heart ferricytochrome c (Type III, Sigma) in 1.5 ml KRPD. After 10 min of equilibration, 10^{-7} M N-formyl-methionyl-leucyl-phenylalanine (FMLP, Vega) was added as a stimulus. Superoxide anion production induced by FMLP was calculated on the basis of the difference between spontaneous and stimulated generation of this oxygen metabolite. Results were expressed as nmols of the maximum superoxide anion released by 1 million cells

Degranulation assay: In order to monitor effect of DPA on degranulation stimulated with FMLP (10^{-7} M) one of the azurophil granule-associated enzymes, beta-glucuronidase release was measured. The enzyme activity in the supernatant of 5 million cells was determined by using phenolphthalein glucuronic acid (Sigma) as substrate /5/. Briefly: 0.2 ml supernatant was diluted in 1.8 ml of 0.1 M sodium acetate buffer (pH=4.6) and 0.1 ml phenolphthalein glucuronic acid. The samples were vortexed and incubated for 18 h at 37°C after which the reaction was terminated with ice cold 0.2 M glycin buffer (pH=10.4). Total enzyme activity was determined after treatment of cells with Triton X-100 (TTX, 0.2 %; Rohm and Haas Co., Philadelphia, PA). The optical density of samples was measured photometrically at 540 nm. The percentage enzyme release of stimulated granulocytes was calculated as a ratio between difference of stimulated (with FMLP) and spontaneous (without FMLP), and total release (with TTX).

Bacteria: Staphylococcus aureus (type 42D) was cultured overnight at 37°C in Nutrient broth (Oxoid, London), harvested by centrifugation for 10 min at 1500 x g, washed twice in KRPD and resuspended to a final concentration of 10^7 bacteria/ml.

Phagocytosis assay: Granulocytes and S. aureus (42D) were incubated at a cell to bacteria ratio of 1:1 and at a cell concentration of 5 x 10^6/ml in the presence of 10 % normal human serum. Phagocytosis of S. aureus by granulocytes was measured as the decrease in the number of viable extracellular bacteria during 120 min incubation of the phagocytic mixture at 37°C under slow rotation (4 rpm). Aliquots of the mixtures were taken at 0, 60 and 120 min of incubation and the number of viable extracellular bacteria was determined by colony counts after differential centrifugation of phagocytic cells and bacteria /14/.

Bacterial killing assay: 5 x 10^6/ml granulocytes were incubated with S. aureus (42D) at a cell to bacteria ratio of 1:1 at 37°C under slow rotation (4 rpm) in the presence of 10 % normal human serum. Intracellular killing of bacteria was determined as the decrease in the number of viable intracellular and extracellular bacteria after 120 min of incubation. Samples of the mixture were taken at 0, 60, and 120 min to determine the number of viable bacteria after lysis of the cells in ice cold (4°C) distilled water containing 0.01 % bovine albumin /14/.

Statistical methods: Data analysis was performed using standard statistical methods. In spite of the standardized separation technique and well controlled experimental conditions the granulocytes' activity varied in different experiments. In order to compare DPA effects obtained with different granulocyte populations results were expressed as the percentage of untreated control using paired Student's t-test. The significance to the differences was defined at the $p < 0.05$ level /1/.

RESULTS

Superoxide anion production: Generation of superoxide anion by neutrophils stimulated with FMLP (10^{-7}M) as a function of different concentrations of DPA is shown in Figure 1. Before assaying superoxide anion production granulocytes were incubated in the presence of various concentrations of DPA for 10 min at 37°C after which the cells were washed and suspended in KRPD. For control, superoxide anion production by granulocytes incubated in the absence of DPA under the same conditions was measured. Preincubation of cells with 0.5 mM DPA resulted in 28% increase in the superoxide anion production

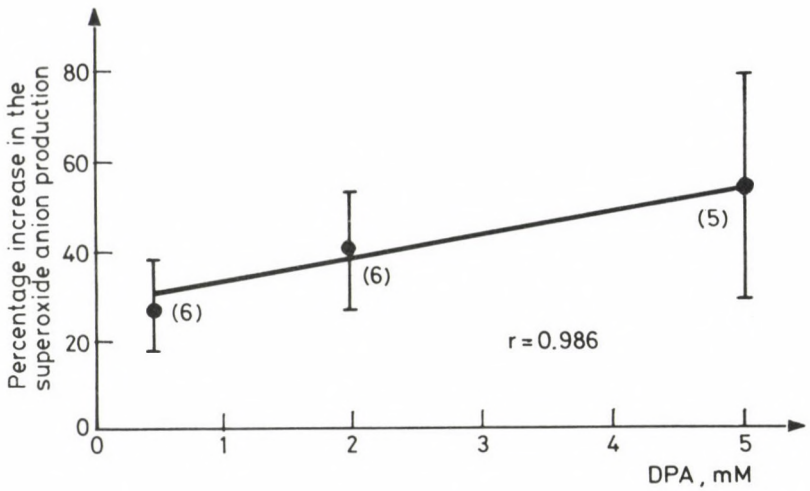

Fig. 1. Effect of DPA on the superoxide anion production of neutrophil granulocytes. 10^6 cells were preincubated with DPA of various concentrations for 10 min at 37°C. FMLP stimulated O_2 generation was measured by recording ferricytochrome c reduction. Each point represents the mean ± S.E. The number of experiments are indicated in brackets. r = correlation coefficient.

compared to the untreated control. At 2.5 mM DPA, 41 % increase was found while preincubation with 5 mM DPA produced 53 % rise. The rise was directly proportional to the DPA concentration of the examined range (r=0.986). No alteration was observed in the spontaneous superoxide anion production by the drug. (Data not shown.)

Beta-glucuronidase release: In order to assay the efficacy of the same stimuli in triggering the release of azurophil granules, release of beta-glucuronidase by neutrophils was measured. The enzyme's activities as a function of different concentrations of DPA are shown in Figure 2. Preincubation of cells with DPA in concentrations of 0.5; 2.0 and 5.0 mM for 10 min at 37°C increased the FMLP-stimulated enzyme release by 145; 156 and 371 % compared to the untreated control,

respectively. The rise was directly proportional to the DPA concentration in this range (r=0.958).

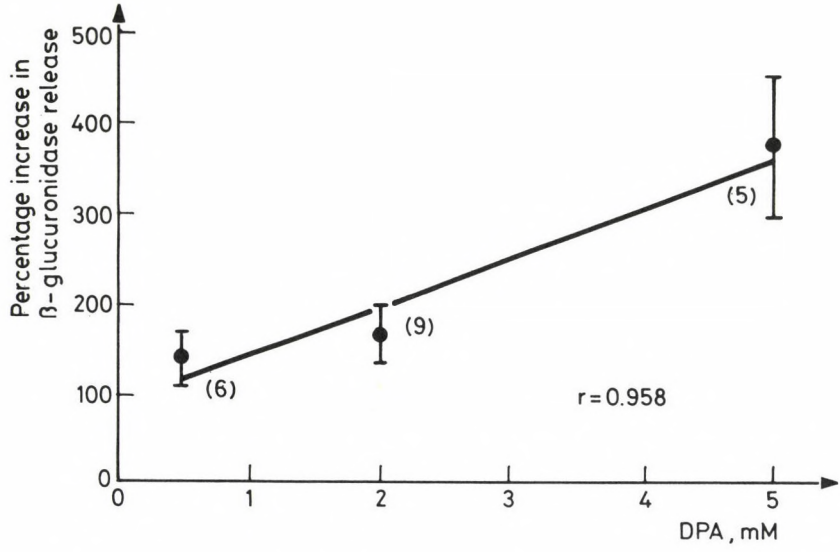

Fig. 2. Effect of DPA on β-glucuronidase release by neutrophil granulocytes. 5 x 10^6 cells were incubated with DPA of various concentrations for 10 min at 37°C. FMLP stimulated enzyme release was measured by using phenolphthalein glucuronic acid as a substrate. Each point represents the mean ± S.E. The number of experiments are indicated in brackets. r = correlation coefficient.

Phagocytosis: D-Penicillamine did not alter the phagocytic activity of granulocytes as is shown in Figure 3. DPA in the examined concentrations was added at 0 min of incubation of cells with bacteria. After 60 min of incubation the number of viable extracellular bacteria decreased to below 10 % of the initial number and further reduction was observed during the next 60 min incubation. The differences between phagocytic activity of control and DPA treated cells were not statistically significant. No inhibition in control experiments by DPA on bacterial proliferation was observed (data not shown.)

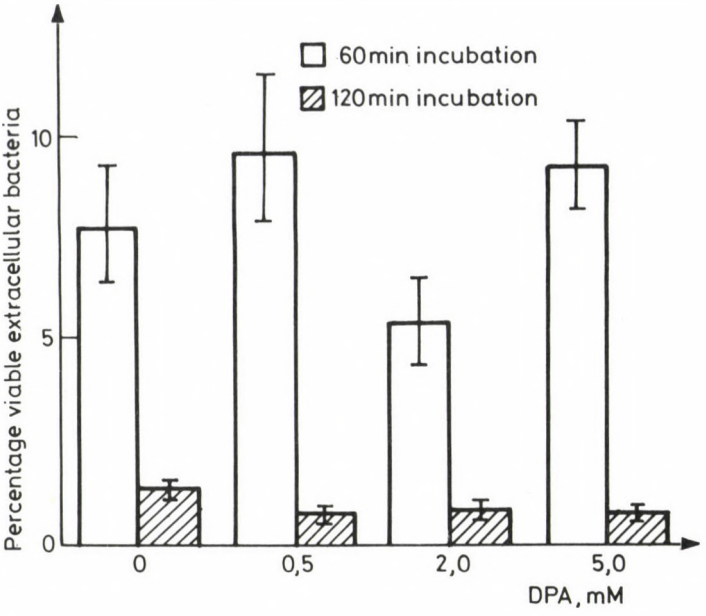

Fig. 3. Effect of DPA on the phagocytosis of S.aureus by neutrophil granulocytes. DPA of various concentrations was added to the mixture of cells and bacteria at 0 min. Samples were taken at 0; 60 and 120 min of incubation to determine the number of viable extracellular bacterial count. Data are expressed as the mean ± S.E. of at least 5 experiments.

Intracellular killing: In the bacterial killing test there was no alteration in the neutrophil function as it is shown in Figure 4. DPA in various concentrations was added at 0 min of incubation of granulocytes with bacteria and was permanently present during the test. After 60 min of incubation the number of viable bacteria decreased to 25 % of the initial number. 5 mM DPA slightly increased the killing activity of the neutrophils during this incubation period but the difference was not statistically significant. Further decrease in the viable bacterial count was observed at 120 min of incubation. It decreased to about 15 % of the initial number and no significant difference between DPA treated cells and the control was found.

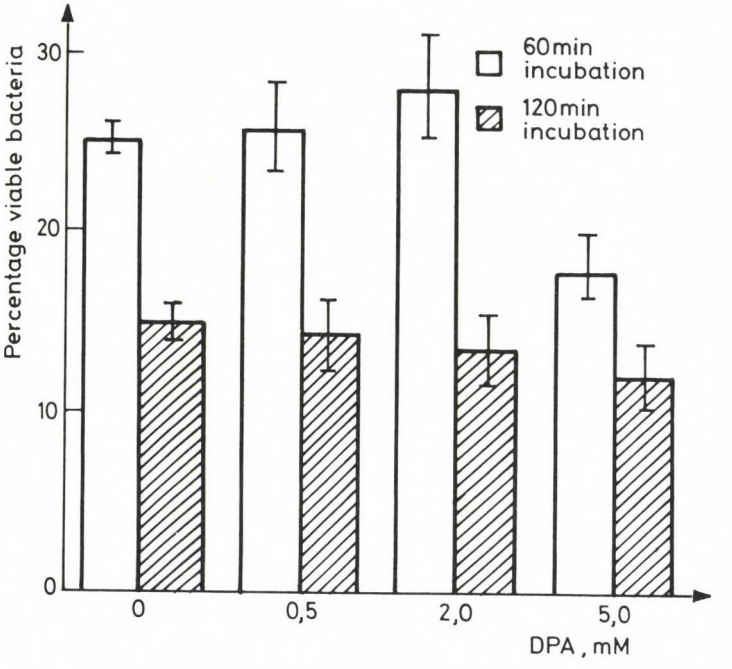

Fig. 4. Effect of DPA on the intracellular killing of S.aureus by neutrophil granulocytes. DPA of various concentrations was added to the mixture of cells and bacteria at 0 min. Samples were taken at 0; 60 and 120 min of incubation to determine the viable bacterial count after lysis of granulocytes with distilled water. Data are expressed as the mean ± S.E. of at least 10 experiments.

DISCUSSION

Polymorphonuclear neutrophil granulocytes play a central role in host defense mechanisms against bacterial pathogens /19/. The stimulated phagocytic cells (polymorphonuclear neutrophil- and eosinophil leukocytes, macrophages, monocytes) consume a large amount of oxygen in a non-mitochondrial reaction involving the one-electron reduction of oxygen to superoxide anion by NADPH-oxidase, a membrane-bound multicomponent electron transfer chain. The process is known as "respiratory burst" /2/. Superoxide then serves as the

precursor for the microbicidal compounds generated in both the myeloperoxidase-dependent and -independent systems. In the myeloperoxidase-dependent process, superoxide anion dismutates to form H_2O_2, which is then used by myeloperoxidase to oxidize chloride anion to hypochlorite ion. HOCl then participates in the generation of a derivative group of oxidants known as the chloramines. In the latter, O_2^- undergoes a metal-catalyzed reaction with hydrogen peroxide (Fenton- or Haber-Weiss reactions) to yield the highly reactive hydroxyl radical /6, 7/. However, during intracellular killing large amounts of toxic oxygen metabolites release into the extracellular space causing surrounding tissue damage. The most pronounced toxic effects induced by reactive oxygen species are: lipid peroxidation, destruction of biomembranes, nucleic acid damage, enzyme inhibition and protein degradation. Consequently, these toxic oxygen metabolites generated by granulocytes take part not only in the intracellular microbicidal process of host defense mechanisms against invading microbes, but are also involved in the inflammatory process /6, 20/.

Neutrophils are specifically constructed to use both the NADPH oxidase system and the granule constituents in a cooperative and concerted manner, and it is an intermixing of these two components that allows the neutrophil to realize its ultimate destructive potential /3, 20/.

Radical scavanger drugs which reduce O_2^- and consequently the toxic oxygen metabolites' level have negative influence on the antibacterial activity of phagocytic cells /9, 17/.

We supposed that D-Penicillamine which has been shown to inhibit lipid peroxidation induced by oxygen metabolites in red blood cell membrane modulates the metabolic activity of neutrophils in connection with their respiratory burst.

In this study it was found that superoxide anion generation was slightly and beta-glucuronidase release markedly increased by preincubation with DPA at concentrations of 0.5-5 mM and the rise was directly proportional to the concentration in the examined range. On the other hand, none of the examined DPA concentrations influenced the phagocytic or killing activity of neutrophils. Over the 120 min incubation period no significant

alteration of the cell functions was observed compared to the untreated control. In other words the bactericidal capacity of neutrophils treated with DPA was similar to that of the untreated control cells. From these results we conclude that DPA may influence the superoxide anion production and beta-glucuronidase release in granulocytes without altering their phagocytic and intracellular killing activities. We suppose that the unchanged antibacterial activity of neutrophil granulocytes is resulted by the two opposite effects of DPA: acting extracellularly reduces free radical level, while the direct membrane effect results enhancing metabolic activity in granulocytes.

REFERENCES

1. Armitage P: Statistical methods in medical research. Oxford and Edinburgh: Blackwell Scientific Publication 1971

2. Babior BM: The respiratory burst of phagocytes. J Clin Invest 73: 599, 1984

3. Babior BM, Kipnes RS, Curnutte ST: The production by leukocytes of superoxide, a potential bactericidal agent. J Clin Invest 52: 741, 1973

4. Boyum A: Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest 21: (suppl 97) 77, 1968

5. Brittinger G, Hirschhorn R, Douglas SD, Weissmann G: Studies on lysosomes. J Cell Biol 37: 394, 1968

6. Byczkowski JZ, Gessner T: Biological role of superoxide ion-radical. Int J Biochem 20: 569, 1988

7. Curnette JT, Babior BM: Chronic granulomatous disease. In: Harris H, Hirschhorn K (eds) Advances in Human Genetics, Vol. 16. Plenum Publishing Co, New York, p 229, 1987

8. Dewald B, Baggiolini M: Methods for assessing exocytosis by neutrophil leukocytes. In: Di Sabato G, Everse J (eds) Methods in enzymology Vol 132 Academic Press Inc Orlando, Florida, p 267, 1986

9. Engle WA, Yoder MC, Baurley JL, Yu P: Vitamin E decreases superoxide anion production by polymorphonuclear leukocytes. Pediatr Res 23: 245, 1988

10. Johnson L, Bowen FW Jr, Abbasi S: Relationship of prolonged pharmacologic serum levels of vitamin E to incidence of sepsis and necrotizing enterocolitis in infants with birth weight 1500 grams or less. Pediatrics 75: 619, 1985

11. Karmazsin L, Lakatos L, Balla Gy, Makay A, Hatvani I: Experimental data on the prevention of retrolental fibroplasia by D-penicillamine. Acta Paed Acad Sci Hung 21: 131, 1980

12. Lakatos L, Lakatos Zs, Hatvani I, Oroszlán Gy: Controlled trial of use of D-Penicillamine to prevent retinopathy of prematurity in very low-birth-weight infants. In: Stern L, Oh W, Friis-Hansen B (eds): Physiologic fondations of perinatal care. Elsevier, p 9, 1987

13. Lakatos L, Kövér B, Oroszlán Gy, Vekerdy Zs: D-Penicillamine therapy in ABO hemolytic disease of the newborn infant. Eur J Pediat 123: 133, 1976

14. Maródi L, Leijh PCJ, van Furth R: A micromethod for the separate evaluation of phagocytosis and intracellular killing of Staphylococcus aureus by granulocytes and monocytes. J Immunol Methods 57: 353, 1983

15. Matkovics B, Lakatos L, Szabó L, Karmazsin L: Effect of D-Penicillamine on some oxidative enzymes of rat organs in vivo. Experientia 37: 6, 1981

16. Oroszlán Gy, Lakatos L, Szabó L, Matkovics B, Karmazsin L: Hemoxygenase activity is decreased by D-Penicillamine in neonates. Experientia 39: 888, 1983

17. Rajkovic IA, Williams R: Enhancement of neutrophyl response by SH-containing compounds: modulation of superoxide and hydrogen peroxide production. Biochem Pharmacol 33: 1249, 1984

18. Sternlieb I, Bennet B, Scheinberg IH: Penicillamine induced Goodpasture's syndrome in Wilson's disease. Ann Intern Med 82: 673, 1975

19. Van Oss CJ: Phagocytosis: An overview. In: Di Sabato G, Everse J (eds) Methods in enzymology Vol. 132 Academic Press Inc. Orlando, Florida p 3, 1986

20. Weiss SJ: Tissue destruction by neutrophils. N Eng J Med 320: 365, 1989

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