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SHORT COMMUNICATIONS

The different inhibitory effects of phenylbutazone on soluble and particle stimulation of human neutrophil oxidative burst

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Polymorphonuclear leukocytes (PMNs) play an important role in host defence [1, 2] and also cause tissue damage in the inflammatory loci [3, 4] owing to their ability to migrate towards the injured and infected tissues [5, 6], to adhere to micro-organisms, to ingest them in phagosomes [7] and to kill them in various ways involving activation of their oxidative metabolism [8] and release of lysosomal enzymes [9]. Activation of PMN oxidative metabolism is characterized by strong cyanide-insensitive oxygen consumption [10] and by concomitant production of active oxygen derivatives such as superoxide anion (O_2^-) [11] and hydrogen peroxide (H_2O_2) [12]. The extent to which the PMN metabolism is activated can be precisely determined *in vitro* by measuring the particle engulfment rate, the O_2 consumption and the H_2O_2 production by isolated PMNs. These functional tests are also good tools for investigating the effect of drugs on PMN activities. Phenylbutazone (PBZ), a usual clinical non-steroidal anti-inflammatory drug, was recently shown to modulate the PMN-oriented migration stimulated by the synthetic peptide *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), but did not affect the PMN migration induced by the complement derived C_{5a} [13]. PBZ was also demonstrated to inhibit FMLP binding to PMN but not C_{5a} binding, suggesting that it interacts specifically with FMLP membrane receptors [14, 15]. The chemoattractant FMLP, like other particulate or soluble agents, activates the PMN oxygen metabolism; such activation has been suggested to occur through different, still undefined, mechanisms [16] all involving membrane perturbation. In order to determine whether PBZ acts on one or several of these mechanisms, we tested its effects on the PMN oxidative system induced by different stimuli. The results showed that the effects of PBZ on the PMN oxygen metabolism varied with the different stimuli.

Materials and methods

Phenylbutazone (a gift from Ciba–Geigy, Basel, Switzerland) was prepared extemporaneously as previously described [17]. Phorbol myristate acetate (PMA), FMPL and zymosan A were obtained from Sigma Chemical Co. (St Louis, MO). Dextran T 500 was from Pharmacia (Uppsala, Sweden). Soluene 350 and Dimilume R 30 were purchased from Packard Becker B.V. (Breda, The Netherlands). Catalase was from Boehringer (Mannheim, F.R.G.). Human leukocytes were isolated from 10 IU/ml heparinized venous blood by sedimentation of the erythrocytes using dextran as previously described [18]. The final pellet was resuspended in 0.1 M Krebs–Ringer phosphate buffer (KRP), pH 7.4. The rate of *Klebsiella* ingestion by PMN was measured as previously described [17]. Heat-killed ^{14}C-labelled *Klebsiella* were opsonized in AB serum and selected by differential centrifugation as in [17]. A suspension of 10^8 *Klebsiella* (500 μl) was mixed with 10^6 PMNs (500 μl) and the ingestion process was stopped at zero, 5 and 10 min. Results are expressed as the mean number of bacteria associated per PMN per 10 min of incubation. Cyanide-insensitive O_2 consumption by resting and stimulated PMNs was measured polarographically as previously described [19]. The stimulating agents were opsonized zymosan (STZ), PMA and FMLP at final concentrations of 0.67 mg/ml, 1 μg/ml and 5×10^{-7} M, respectively. PMA was initially dissolved in DMSO at 1 mg/ml, aliquoted and stored at a temperature of −80°, and FMLP was stocked in 0.15 M NaOH at a concentration of 10^{-3} M. Appropriate dilutions were made in KRP. The experiment in the presence of PBZ (added in the oxygraph chamber) was run concomitantly with the control in two Gilson's oxygraphs. For calculation, the O_2 concentration in the medium was considered to be 200 μM at 37°. Results are expressed as nmole O_2 consumed per 10^6 PMNs per min. PMN production of H_2O_2 was measured polarographically under the same conditions as O_2 uptake [17, 19] and was expressed as nmole of H_2O_2 produced by 10^6 PMNs per min. For each PMN function test, the effect of PBZ was studied in the drug's presence after incubating the cells with it for 15 min at 37°. All measurements were made in duplicate or triplicate. Means, standard deviations and correlation coefficients were calculated on a Compucorp 445 'statistician'. Paired or unpaired Student's *t*-tests were used to assess differences between control and assay values.

Results

Effect of PBZ on PMN ingestion of Klebsiella. The rate of PMN-associated labelled *Klebsiella*, in the absence of PBZ, was 7.13 ± 2.03 micro-organisms per PMN per 10 min of incubation. Therapeutic amounts of PBZ (100 and 150 μg/ml) did not significantly alter the PMN uptake of bacteria. High concentrations of PBZ (300 and 400 μg/ml) inhibited the PMN bacterial uptake (28 and 24%, respectively; $P < 0.01$). The values obtained after only 5 min of incubation indicated that high drug concentrations altered the ingestion process in the same proportion as after 10 min of incubation. The inhibitory effect of high concentrations of PBZ on the PMN-associated particles confirms earlier reports about the engulfment of other micro-organisms such as *E. coli* [20], *Staphylococcus* [21] and *Candida albicans* [22].

Effect of PBZ on oxygen uptake. In the absence of PBZ, the O_2 uptake by resting PMNs was 1.1 ± 0.33 nmole O_2 per 10^6 PMNs per min (mean ± 1 S.D. of 29 experiments). Various amounts of PBZ ranging from 10 to 600 μg/ml did not significantly reduce resting O_2 uptake by PMNs. These results suggest that under our experimental conditions PBZ is not cytotoxic. To confirm this point, the cells were incubated at 37° for 40 min in the presence of large amounts of PBZ (400 and 600 μg/ml) and their viability, assessed by their ability to exclude trypan blue, was more than 95%. The effects of PBZ on the stimulated oxygen consumption are illustrated in Fig. 1. The inset shows the kinetic behaviour of oxygen decrease following the addition of the stimuli to the oxygraph chamber containing the PMNs. Opsonized zymosan induced a fast and large decrease in oxygen at a rate of 9.17 ± 1.05 nmole per 10^6 PMNs per min, which thereafter remained fairly constant for about 7 min. PMA cause a similar decrease in oxygen consumption (7.94 ± 1.66 nmole O_2 per 10^6 PMNs per min), which also remained constant for about 8 min. However, with PMA the decline only started after a lag time of about 2 min. Lastly, the synthetic peptide FMLP produced fast stimulation of O_2 uptake (6.88 ± 1.26 nmole O_2 per 10^6 PMNs per min) but this rate only remained constant for 2 min and then rapidly declined to the rate of resting O_2 uptake. The early decrease

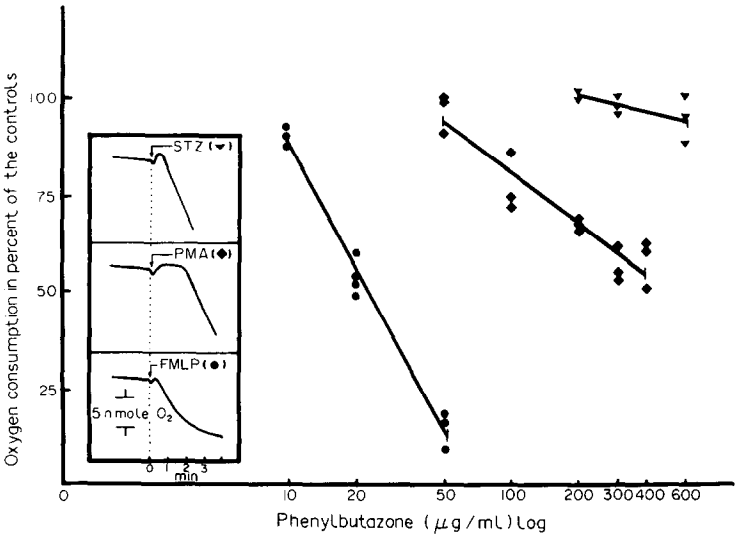

Fig. 1. Effects of PBZ on oxygen consumption by stimulated PMNs. Inset shows the beginning of the kinetics of O_2 consumption after PMN stimulation by 0.67 mg/ml STZ (▼), 1 μg/ml PMA (◆) and 5×10^{-7} M FMLP (●). Values for PMN O_2 uptake in the presence of PBZ are expressed as percentages of the control values. Mean control values (100%) for PMN O_2 consumption induced by STZ, PMA and FMLP, in nmole of O_2 consumed per 10^6 PMNs per min, were respectively 9.17 ± 1.05 (mean ± S.D. of 12 experiments), 7.94 ± 1.66 (15 experiments) and 6.88 ± 1.16. Regression parameters, calculated for each correlation coefficient (r) and slope (s), for the three stimulating agents were: $r = 0.451$, $s = -13.71$ for STZ; $r = 0.928$, $s = -43.73$ for PMA and $r = 0.980$, $s = -107.51$ for FMLP.

in the rate of FMLP-induced O_2 uptake was not due to a deficiency of the stimulating agent, because larger amounts of FMLP did not lengthen the stimulation period but shortened it, and further addition of FMLP to the medium had no stimulating effect. These differences in the triggering and duration of O_2 consumption by PMNs induced by these various stimuli suggest that they might induce the PMN oxygen burst by different means. Figure 1 shows the effect of PBZ on the O_2 consumption of PMNs subjected to these stimuli. At concentrations below 600 μg/ml, PBZ did not significantly alter the PMN response to zymosan. Very high concentrations of the drug (2 and 4 mg/ml) were required to inhibit 50 and 90% of the control activity, respectively (results not shown). In other respects, when leukocytes were preincubated for 15 min at 37° with 2 or 4 mg of PBZ/ml, then washed, their O_2 consumption induced by zymosan was similar to that of the control. This result confirms that PBZ is not cytotoxic. The PMA-induced O_2 consumption was slightly reduced by therapeutic amounts of PBZ. An inhibition of 50% compared to the controls (IC_{50}) was obtained with about 400 μg of PBZ per ml. Besides inhibiting the rate of O_2 uptake, PBZ slightly enhanced the lag phase preceding the PMA-induced decline in O_2. PBZ was observed to inhibit strongly FMLP-stimulated O_2 consumption, judged by the 50% inhibiting concentration ($IC_{50} = 20$ μg/ml). The O_2 uptake by PMN, monitored in the presence of *Klebsiella pneumoniae* as the stimulating agent (at 100 bacteria per PMN), was slightly inhibited by PBZ, by about 14 and 36% in the presence of 200 and 600 μg of PBZ per ml, respectively. As for zymosan, the PMNs preincubated with an inhibiting concentration of PBZ and then washed showed similar O_2 uptake to untreated PMNs when stimulated by PMA or FMLP. These results suggest that PBZ acts reversibly on the PMNs.

Effects of PBZ on the H_2O_2 production by stimulated PMNs. The oxygen consumed by PMNs in the presence of cyanide is reduced to superoxide radical and to H_2O_2 by dismutation of the superoxide radicals [23] and possibly by a direct bivalent reduction of molecular oxygen [8]. The results concerning the effect of PBZ on PMN production of H_2O_2 are given in Table 1. H_2O_2 production was monitored 4 and 6 min after stimulation by STZ and PMA, respectively. In the presence of STZ, the control value was about 7.54 ± 1.59 nmole H_2O_2 produced per 10^6 PMNs per min, and not significantly altered by PBZ. These results are in good agreement with those observed for O_2 uptake and suggest that PBZ did not interfere with O_2^- conversion to H_2O_2. The control value for PMA-induced H_2O_2 production was about 5.14 ± 1.64 nmole H_2O_2 per 10^6 PMNs per min and was decreased by PBZ to a greater extent than that observed for O_2 uptake. The fact that in the presence of PBZ a reduced amount of O_2 was recovered as H_2O_2 from O_2 taken by PMNs after PMA stimulation calls for further investigation. The production of H_2O_2 by FMLP-stimulated PMNs was measured in the presence of catalase added to the incubation medium before PMN activation by FMLP because of the brief duration of the stimulation. In the absence of PBZ, H_2O_2 production was about 5.86 ± 0.78 nmole H_2O_2 per 10^6 PMNs per min. Small amounts of PBZ greatly reduced the FMLP-stimulated production of H_2O_2 to a similar extent to that observed for O_2 uptake. All these results indicate that, as in the case of O_2 consumption, the effects of PBZ on H_2O_2 production varied according to the type of PMN stimulation.

Discussion

The present findings indicate that therapeutic concentration of phenylbutazone did not reduce the rate of *Klebsiella* ingestion of PMN, resting and STZ-induced O_2 consumption by PMNs or the resulting production of H_2O_2. On the other hand, PBZ inhibited PMA-induced O_2 uptake and H_2O_2 production by PMNs. It also strongly diminished the activities of PMNs when stimulated by FMLP. These inhibitory effects of PBZ on PMN responses after stimulation by PMA and especially by FMLP seemed to be due to some action of PBZ on the cell plasma membrane rather than to an action on the intracellular components of the oxidative system. This hypothesis is supported by the fact that PMNs treated with inhibitory amounts of PBZ and

Table 1. Effects of phenylbutazone on the production of H_2O_2 by stimulated PMNs

PBZ (μg/ml)	Stimulated production of H_2O_2 (percent of control production) FMLP (5×10^{-7} M)	PMA (1 μg/ml)	STZ (0.67 μg/ml)
	100 ± 13	100 ± 32	100 ± 19
0	(5.86 ± 0.78)	(5.14 ± 1.64)	(7.54 ± 1.59)
10	87 ± 8		
20	39 ± 6		
50	18 ± 6		
100		59 ± 10	90 ± 13
200		49 ± 14	81 ± 14
300		34 ± 23	80 ± 13
400		9 ± 15	—
600		—	79 ± 16

Production of H_2O_2 was continuously monitored by addition of catalase (approximately 6×10^3 units/ml) in the oxygraph chamber. When FMLP were used, catalase was added in the medium before PMN stimulation. When STZ and PMA were tested, catalase was added 4 and 6 min, respectively, after PMN stimulation started. Control values (100%) are the mean ± S.D. of 12, 13 and 9 experiments performed in the presence of STZ, PMA and FMLP, respectively. The corresponding production of H_2O_2 in nmole per 10^6 PMNs per min is indicated in parentheses. In the presence of PBZ, the values reported are the mean ± S.D. of the percentages calculated from the controls (three determinations).

then washed exhibited similar responses to those of the appropriate controls in the presence of FMLP or PMNs. The same observation was also reported earlier for the oriented migration of PBZ-treated and washed cells [13]. When particles were used as stimulating agents, the PMN responses induced were not affected by therapeutic amounts of PBZ. This was the case for the rate of *Klebsiella* ingestion by PMNs and was also previously shown for the phagocytosis of other bacteria [20–22], or of latex beads [24]. Similar results were observed for the STZ-induced O_2 uptake and H_2O_2 production by PMN. In contrast, the soluble stimuli PMA and FMLP induced responses which were more affected by PBZ. The highly inhibitory effect of small amounts of PBZ (IC_{50} = 20 μg/ml) on the O_2 uptake and H_2O_2 production by FMLP-stimulated PMNs was not unexpected, because similar inhibition by PBZ (IC_{50} = 10 μg/ml) of FMLP-induced optimal migration had been reported earlier [13]. The differences in the effects of PBZ on the PMN O_2 uptake stimulated by various agents argue in favour of the activation of the oxidative system through different initial pathways. The differences in the kinetic behaviour, the shortness of the burst in the presence of FMLP, and the existence of a lag time in the presence of PMA before O_2 uptake starts (Fig. 1) all seem to indicate that these two agents, known to bind to the PMN membrane, trigger the oxidative burst by different mechanisms.

In summary, therapeutic amounts of PBZ alter the O_2 uptake and H_2O_2 production by PMNs stimulated by soluble components like FMLP and PMA but do not significantly affect the rate of particle engulfment by PMNs or the O_2 uptake and H_2O_2 production induced by particle components. This suggests that the oxidative burst can be triggered in PMNs by different pathways, one of which is inhibited by PBZ, and that at low concentrations, this drug could be used as a probe for further analysis of soluble triggering mechanisms.

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REFERENCES

1. T. P. Stossel, *New Engl. J. Med.* **290**, 717; 774; 833 (1974).
2. R. L. Baehner, *Clin. Haemat.* **4**, 609 (1975).
3. J. M. McCord, *Science* **185**, 529 (1974).
4. M. L. Salin and J. M. McCord, *J. clin. Invest.* **56**, 1319 (1976).
5. H. J. Senn and W. F. Jungi, *Semin. Haemat.* **12**, 27 (1975).
6. J. I. Gallin and S. M. Wolff, *Clin. Haemat.* **4**, 567 (1975).
7. T. P. Stossel, *Semin. Haemat.* **12**, 83 (1975).
8. S. J. Klebanoff, *Semin. Haemat.* **12**, 117 (1975).
9. T. P. Stossel, T. D. Pollard, R. J. Mason and M. Vaughan, *J. clin. Invest.* **50**, 1745 (1971).
10. A. J. Sbarra and M. L. Karnowsky, *J. biol. Chem.* **234**, 1355 (1959).
11. B. M. Babior, R. S. Kipnes and J. T. Curnutte, *J. clin. Invest.* **52**, 741 (1973).
12. G. Y. N. Iyer, M. F. Islam and J. H. Quastel, *Nature, Lond.* **192**, 535 (1961).
13. A. Périanin, M. T. Labro and J. Hakim, *Biochem. Pharmac.* **31**, 3071 (1982).
14. C. Dahinden and J. Fehr, *J. clin. Invest.* **66**, 884 (1980).
15. D. R. Nelson, J. M. Gracyk, V. D. Fiegel, M. J. Herron and D. E. Chenoweth, *Blood* **58**, 752 (1981).
16. R. S. Weening, D. Roos, C. M. R. Weemaes, J. W. T. Homan-Muller and L. M. J. Van Schaik, *J. Lab. clin. Med.* **88**, 757 (1976).

17. M. Torres, D. de Prost, J. Hakim and M. A. Gougerot, *Eur. J. clin. Invest.* **9**, 209 (1979).
18. J. Hakim, E. Cramer, P. Boivin, H. Troube and J. Boucherot, *Eur. J. clin. Invest.* **5**, 215 (1975).
19. E. Feliu, M. A. Gougerot, J. Hakim, E. Cramer, C. Auclair, B. Rueff and P. Boivin, *Eur. J. clin. Invest.* **7**, 571 (1977).
20. R. R. Strauss, B. B. Paul and J. S. Sbarra, *J. Bact.* **96**, 1982 (1968).
21. C. O. Solberg, *Acta path. microbiol. scand. Sect. B* **82**, 258 (1974).
22. J. A. Whittaker, H. R. Hugues and M. Khurshid, *Br. J. Haemat.* **29**, 273 (1975).
23. J. A. Fee and J. S. Valentine, in *Superoxide and Superoxides Dismutases* (Eds. A. M. Michelson, J. M. McCord and I. Fridovich), p. 19. Academic Press, New York (1977).
24. P. Herzer and E. M. Lemmel, *Immunobiology* **157**, 78 (1980).

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Chemiluminescence of the *in situ* rat liver after acute ethanol intoxication—effect of (+)-cyanidanol-3

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The aerobic catabolism of several exogenous compounds in mammalian cells is associated with the formation of reactive oxygen species such as superoxide radical, hydroxyl radical, hydrogen peroxide and singlet oxygen [1]. These species are likely to exert toxic effects in biological systems, including lipid peroxidation, enzyme inactivation, mutagenicity and carcinogenicity [1–3]. The cytotoxicity of lipoperoxidation depends on the free radical-induced oxidative breakdown of polyunsaturated fatty acids [1–3]. Since these fatty acids constitute the major components of cell membranes, an enhanced lipid peroxidation would alter essential membrane functions and lead to cell injury [1–3].

Acute ethanol ingestion has been shown to produce an increased lipid peroxidation in the liver as assessed by different experimental procedures. Several invasive and destructive techniques such as malondialdehyde production [4–6], conjugated lipid diene formation [7, 8], and chemiluminescence [9] have been used in liver homogenates and in microsomal and mitochondrial preparations. However, the estimations of ethanol-induced lipid peroxidation by malondialdehyde formation [10–12] and by electron spin resonance spectroscopy [13] have produced conflicting results. An enhancement of hepatic lipid peroxidation by acute ethanol treatment has also been reported by measuring the *in vivo* exhalation of hydrocarbons such as ethane [14, 15], *n*-pentane [15, 16], propane, *n*-butane and isobutane [15].

Although hydrocarbon exhalation constitutes a truly non-invasive assay for detecting lipid peroxidation, it lacks organ specificity. Recently, this problem was overcome by the use of perfused rat liver, in which the addition of 44 mM ethanol markedly increased the production of ethane [17]. In view of these observations and of the role that lipid peroxidation could play in alcoholic liver disease in man [18], we report in this paper the effect of acute ethanol intoxication on the rate of lipid peroxidation in the liver *in vivo*, evaluated by measuring the low-level chemiluminescence of the intact organ [19]. This technique constitutes a specific assay which is non-invasive for the tissue and is related to the steady-state level of oxidative free radicals involved in the process of lipoperoxidation [19]. The results with the chemiluminescence measurements are compared with those obtained by the determination of malondialdehyde and diene conjugates, and the effect of the antioxidant flavonoid (+)-cyanidanol-3 [20] on the ethanol-induced chemiluminescent response of the liver was studied.

Male Long–Evans or Wistar rats (200–220 g) fasted overnight (16–18 hr) were given 5 g of ethanol/kg [as a 30% (v/v) solution in saline] or isovolumetric amounts of saline intraperitoneally. Experiments with (+)-cyanidanol-3 (Zyma S.A., Nyon, Switzerland, obtained through Ciba-Geigy, Chile) were performed in a separate group of animals that received 400 mg/kg of (+)-cyanidanol-3 (as a 80 mg/ml solution in saline) or isovolumetric saline subcutaneously, 1 hr before the administration of ethanol. Studies were carried out after 6 hr of ethanol treatment, and the animals were kept in a warm environment (28–30°).

Liver chemiluminescence was measured with a Johnson Foundation photon counter (Johnson Research Foundation, University of Pennsylvania, Philadelphia, PA, U.S.A.) specially adapted for organ chemiluminescence as described by Boveris *et al.* [19]. An EMI 9658 red-sensitive phototube with an applied potential of −1·3 kV was used. Details of the apparatus are depicted in Fig. 1. Rats were anesthetized with Nembutal (50 mg/kg in control rats and 15 mg/kg in animals intoxicated with ethanol, i.p.), and the liver was exposed. The abdominal cavity and skin were covered with aluminium foil in which a window specially cut for each rat allowed exposition of only the liver (Fig. 1). Chemiluminescence is expressed as counts per second (cps)/cm^2 of liver surface. The thiobarbituric acid assay for malondialdehyde production [21] and the determination of diene conjugates [22] in liver homogenates were carried out in a separate group of animals. Proteins were measured as described by Lowry *et al.* [23]. Significance between mean values was assessed by Student's *t*-test for unpaired results.

A typical determination of the spontaneous chemiluminescence of the *in situ* liver of a fasted rat that had been given ethanol for 6 hr is presented in Fig. 2. The *in situ* liver of an alcohol-intoxicated rat showed an enhanced light signal (about 60%) compared to that of a control rat. Furthermore, this significant increase in the low-level chemiluminescence of the *in situ* rat liver caused by acute ethanol administration was found to occur in two different strains of animals, when compared to the corresponding control rats which were given saline (Table 1). Since this organ-specific assay is associated with the steady-state level of oxidative free radicals that could lead to the peroxidation of polyunsaturated fatty acids [19], the results support the