Effects of omeprazole on neutrophil chemotaxis, super oxide production, degranulation, and translocation of cytochrome b−245

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Abstract
The effects of omeprazole on polymorphonuclear neutrophil (PMN) chemotaxis, superoxide generation, degranulation and translocation of cytochrome b−245 were investigated. Omeprazole (10−6−5×10−3 mol/l) reduced chemotaxis under agarose in a dose dependent manner, and the effect was irreversible. Superoxide anion generation was inhibited 50% at a concentration of 2.5×10−5 mol/l and completely abolished at 5×10−3 mol/l. Acid degraded omeprazole also inhibited O2− generation. Omeprazole did not scavenge O2− generated in a cell free xanthin-xanthine oxidase system. Degranulation by PMNs was inhibited only by omeprazole in concentrations above 10−4 mol/l. Translocation of cytochrome b−245, essential for generation of O2−, was not affected by omeprazole. In conclusion, the anti-ulcer agent omeprazole in concentrations obtained during intravenous administration may inhibit the function of PMNs in vitro.

Omeprazole (5-methoxy-2-((4-methoxy-3,5-dimethyl-2-pyridyl)(methyl)sulphanyl)-1H-benimidazol) is a new anti-pulcer agent presently under extensive investigation.1,4 Omeprazole is an inhibitor of the H+K+ ATPase in the secretory membrane of the parietal cell3 and accumulates by a mechanism that is dependent on the acid environment.1 Only few other cells generate a low pH and one of these is the polymorphonuclear neutrophil granulocyte (PMN).

Activation of PMNs by chemotactic factors or during phagocytosis is followed by metabolic activation, secretion of H+ into the phagolysosome, and alkalinisation of the internal pH.1−4 The response to activation depends on a H+Na+ exchange, phosphorylation of a membrane associated complex,10 and a membrane bound H+ATPase.11,12 Translocation of a cytochrome b−245 from granules to the membrane initiates nicotinamide-adenine-dinucleotide phosphate (NADPH)-oxidase activity and the production of super oxide anion (O2−) and oxygen radicals assumed to be important for intracellular bacterial killing and tissue damage.11

It is possible that omeprazole can accumulate in PMNs and affect their function. The present investigation describes the effects of omeprazole on neutrophil function (chemotaxis, degranulation), production of O2−, and translocation of cytochrome b−245 from the granules to the cell membrane.

Methods
Omeprazole was donated by B Wallmark (Hässle, Möln达尔, Sweden). Dextrane T500 and Percoll were purchased from Pharmacia Fine Chemicals, formyl-methionine-leucine-phenylalanine (FMLP), zymosan A, human and bovine serum albumin, ferricytochrome c (gr VI), cytochalasin B, superoxide dismutase, xanthine, xanthine oxidase, phenol-phenalenine-β-glucuronic acid, p-nitrophenyl phosphate (NADH), sodium pyruvate, sodium hydroxysulfite, piperezine-N′N′bis(2-ethane-sulfonic acid) (Pipes), EGTA, Mycobacterium lysodeikticus cell walls, human lysozyme, Coomassie brilliant blue G-250 were purchased from Sigma Chemical Co. Polyethylene glycol 400 was purchased from Fisher Chemical Co, agarose (Seakem ME) from Marine Colloids, Isolymp from Gallard and Schlesinger, and medium 199 and Hepes from Grand Island Biological Co.

ISOLATION OF PMNS
Buffy coats from healthy donors were mixed with dextrane to sedent the erythrocytes. The cells were collected from the leukocyte rich plasma by centrifugation (100 g; 10 minutes; 4°C), resuspended in NaCl 0.15 mol/l, and PMNs were purified on Isolymp gradients (360 g; 35 minutes). Residual erythrocytes were removed by hypotonic lysis (distilled H2O; 30 seconds). The leukocytes were washed thrice and were resuspended in Ca+++Mg+++containing buffer (Na: 119 mmol/l; K: 4.7 mmol/l; Ca: 0.75 mmol/l; Mg: 1.2 mmol/l; NaH-PO4H2PO4: 15 mmol/l; glucose: 5.5 mmol/l; pH 7.3) or in medium 199 (pH 7.3) with Hepes 25 mmol/l and human serum albumin 0.5 mg/ml. The cell suspensions contained more than 95% PMNs and more than 97% excluded trypan blue.

SOLUTIONS OF OMEPRAZOLE
Omeprazole was dissolved in polyethylene glycol 400 (final concentration: 50 mmol/l), aliquoted, and stored at −70°C. Omeprazole was thawed immediately before use, diluted with bicarbonate 0.75 mg/ml to 25 mmol/l, followed by further dilution with Ca+++Mg++ containing buffer (pH 7.3), and was used within 30 minutes.

Acid degradation was done by the addition of hydrochloric acid (0.01 mol/l) 0.1 ml to 0.9 ml of 10−5−10−3 mol/l omeprazole (37°C; 15 minutes). The pH was adjusted to 7.3 with Hepes buffer and the degraded omeprazole was kept on ice until used.
Concentration Migration ZAS: zymosan

ASSESSMENT OF PMN FUNCTION

Migration
Migration and chemotaxis were studied by the agarose technique. Agarose (1% in medium 199; NaHCO₃: 400 μg/ml; Hepes 25 mmol/l; human serum albumin 0.5%) containing omeprazole or solvent was layered on microscopic slides. Six rows of three wells were punched (d: 2.5 mm). Chemotaxant (FMLP 10⁻¹⁷ mol/l; zymosan activated serum (ZAS)) or medium 199 was applied to the two lateral wells, and after 45 minutes. PMNs (5×10⁵, 10 μl) in medium 199 was applied to the central well. The slides were incubated in a humidified atmosphere (90 minutes, 37°C, CO₂ 5%). The slides were then fixed in glutaraldehyde (2-5%); 18 hours, agarsore was removed, and they were dried. The distance migrated was measured after magnification (×60) and the chemotactic response was given by the ratio between stimulated and unstimulated migration. Samples were measured in triplicate (interslide variation: 9%; intraslide variation 7%; no of experiments 12).

Generation of O₂⁻
Ferricytochrome c reduction was used to measure the generation of O₂⁻. PMNs in Ca²⁺ Mg²⁺ buffer were incubated with omeprazole (30 minutes, 37°C) followed by the addition of cytochalasine B (2 μg/ml). After five minutes the PMNs were isolated by centrifugation, resuspended in buffer without drug, and transferred to a cuvette with ferricytochrome c (0.2 mmol) in Ca²⁺ Mg²⁺ buffer to a final volume of 1 ml. Incubations with appropriate concentrations of polyethylene glycol 400 served as controls. After the addition of FMLP (10⁻⁷ mol/l) the change in absorbance was followed in a dual beam spectrophotometer (Cary 15) with a reference cuvette containing ferricytochrome c, superoxide-dismutase (50 units/ml), and PMNs. The generation of O₂⁻ was calculated from the change in absorbance using a coefficient of 21 mM⁻¹ cm⁻¹. The results are given as the percentage of PMNs incubated with solvent.

Assessment of O₂⁻ generation in a cell free system
Scavenging of O₂⁻ by omeprazole was assessed in a xanthine-xanthine oxidase system. Reaction mixtures contained ferricytochrome c (25 μmol), xanthine (1 μmol), and xanthine oxidase (0.2 units) in phosphate buffer (0.12 mol/l, pH 7.4), omeprazole, or polyethylene glycol 400. The reaction was initiated with xanthine-oxidase and the change in absorbance at 550 nm was followed in a dual beam spectrophotometer with a reference cuvette containing superoxide dismutase (100 units). The results are given as the percentage of incubations with solvents.

Degranulation
The release of glucuronidase (EC 3.2.3.31) and lysozyme (EC 3.2.1.17) by PMNs activated with FMLP was used to measure degranulation. PMNs (1.3×10⁶/ml) in Ca²⁺ Mg²⁺ buffer were incubated with omeprazole or solvent (37°C; 30 minutes), cytochalasine B (2 μg/ml) was added, and after five minutes FMLP (10⁻⁷ mol/l). The reaction was stopped after 10 minutes by placing samples on ice. After centrifugation (1000 g, 10 minutes 4°C), the supernatant was assayed for enzymatic activity. Results are given as the percentage of activity released from unstimulated cells after sonication (Triton-x100 0-25%; 3×15 seconds, Branson Inst.).

Glucuronidase was determined by the release of phenolphthalein from phenolphthalein-β-glucuronate (1 mmol/l in acetate buffer 0.1 mol/l; pH 4.4). Lysozyme was measured as the decrease in absorbance at 450 nm of M lysodeikticus cell walls (0-2 mg/ml in phosphate buffer 0.062 mol/l; pH 6.2). Release of lactate dehydrogenase was followed as a marker of cell damage by the change in absorbance at 340 nm with sodium pyruvate as substrate.

SUBCELLULAR FRACTIONATION

Incubation and activation of PMNs
PMNs in Ca²⁺ Mg²⁺ buffer (5×10⁶ cells) were incubated (30 minutes, 37°C) with omeprazole (2.5×10⁻⁴ mol/l) or solvent followed by activation with FMLP (10⁻⁷ mol/l; 15 minutes). Unstimulated PMNs were kept as control. The reaction was stopped with ice cold buffer; PMNs were recovered by centrifugation and resuspended in relaxation buffer (KCl 100.
mmol/l, NaCl 3 mmol/l, MgCl₂ 3·5 mmol/l, Pipes 10 mmol/l, pH 7·3).

Disruption of PMNs
PMNs in 8–10 ml relaxation buffer were pressurised with N₂ (300 psi; 30 minutes; 4°C) in a nitrogen bomb (Artisan, Waltham MA, USA) under stirring for the first 15 minutes. The cavitate was collected dropwise into EGTA (pH 7·4 final concentration 1 mmol/l). Unbroken cells (25–35%) and nuclei were removed by centrifugation (500 g, 15 minutes; 4°C).

Fractionation on Percoll gradients
Fractionation was performed essentially as previously described. The supernatant from the cavitate was layered on a discontinuous gradient of 9 ml Percoll (density 1·05 g/ml) layered on 9 ml with density 1·12 g/ml. After centrifugation (49000 g, 20 minutes; 4°C; Beckman TI-60) three bands were visible and could be recovered by pipetting. Percoll was removed by centrifugation (210000 g; Beckman TI-50) and the material was resuspended in phosphate buffered saline.

Spectroscopy
Absorption spectra (440–600 nm; Cary 15) were measured after addition of triton x-100 (0·2%). The samples were divided and solid sodium hydrosulphite (1–2 mg) was added to the sample cuvette. Cytochrome b–245 was quantitated from the 559 nm peak using an absorption coefficient of 21·6 M⁻¹ cm⁻¹, and myeloperoxidase from the 472 nm peak using an absorption coefficient of 75 M⁻¹ cm⁻¹.

Miscellaneous
Alkaline phosphatase (EC 3.1.3.1) was measured with p-nitrophenyl-phosphate as substrate (1 mg/ml) in 50 mM sodium barbital buffer with MgCl₂, pH 10·5. Protein was measured as described by Bradford with bovine serum albumin as standard.

Results
CHEMOTACTIC FUNCTION
Preincubation with omeprazole reduced the FMLP chemotactic response in a concentration dependent fashion (Fig 1). Omeprazole reduced the distance migrated when both FMLP as well as ZAS were used as chemoattractants. Incubation with omeprazole (5×10⁻⁵ and 10⁻⁴ mol/l; 30 minutes) and resuspension in media without omeprazole reduced the chemotactic index to 61–65% and 53–63% of untreated control cells (n=3). When acid degraded omeprazole was used a similar inhibition was observed (Table 1).

TABLE II Distribution of marker proteins in fractions from Percoll gradients. Values given are percentages of total recovery from the gradients. (Values median (range) of four experiments)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>96 (89–100)</td>
<td>4 (0–11)</td>
<td>0</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>2 (0–3)</td>
<td>55 (43–76)</td>
<td>39 (22–49)</td>
</tr>
<tr>
<td>β-glucuronidase</td>
<td>6 (0–11)</td>
<td>24 (11–35)</td>
<td>70 (59–81)</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>1 (0–4)</td>
<td>31 (25–50)</td>
<td>58 (48–68)</td>
</tr>
</tbody>
</table>

Figure 2: Production of O₂⁻ by polymorphonuclear neutrophils preincubated with omeprazole in varying concentrations and activated with FMLP 10⁻⁴ mol/l. (Values mean (SD)).

Figure 3: Release of lysozyme, β-glucuronidase, and lactate dehydrogenase (LDH) from FMLP stimulated polymorphonuclear neutrophils preincubated with omeprazole in the concentrations indicated. (Values mean (SD) of seven experiments.)
Polyethylene glycol (Values (PEG400) or solvent PMNs cytochrome b-245 (fraction A) and B) of mean inactive with Preincubation DEGRANULATION activation (alkaline phosphatase) low (control: Percoll TRANSLOCATION cell damage of lactate dehydrogenase) gives 5,6-di methylbenzimidazole (n= 3)). Generation of O₂⁻ was also measured after incubation of PMNs with acid degraded omeprazole, imidazole, and 5,6-di-methyl-benzimidazole. Only acid degraded omeprazole (10⁻⁵–10⁻⁴ mol/l) was found to inhibit O₂⁻ generation by 92% of control incubations. Imidazole or 5,6-di methylbenzimidazole had no effect in concentrations from 10⁻⁵ to 5 x 10⁻⁴ mol/l.

DEGRANULATION OF PMNS Preincubation with omeprazole followed by activation of PMNs showed a diminished liberation of enzymatic activity into the medium in concentrations above 10⁻⁵ mol/l. The marker for cell damage (lactate dehydrogenase) remained low (control: 6% (range 5–8); omeprazole 5 x 10⁻⁴ mol/l: 5% (range 0–12) (Fig 3).

TRANSLOCATION OF CYTOCHROME b⁻二十四 Nitrogen cavitation of PMNs and centrifugation on Percoll gradients results in three distinct bands. Table II gives the distribution of marker proteins. Fraction A contains the marker for membranes (alkaline phosphatase) and fractions B and C markers for granules (β-glucuronidase, lysozyme, myeloperoxidase).

Spectroscopy of reduced versus non-reduced samples showed translocation of cytochrome b二十四 from the granule fraction to the membrane fraction after activation. Figure 4 depicts the distribution of cytochrome b in resting, activated, and omeprazole treated PMNs. Incubation with omeprazole or polyethylene glycol 400 did not change the distribution of cytochrome b二十四.

Discussion The present study found that omeprazole inhibits PMN chemotaxis, degranulation, and O₂⁻ generation in concentrations that can be observed after intravenous administration of omeprazole. Similar studies have not been reported before. Omeprazole inhibited the response of PMNs in a concentration dependent fashion, with an IC₅₀ of 2.5 x 10⁻⁵ mol/l. This is higher than the IC₅₀ for the parietal cells (IC₅₀: 5 x 10⁻⁶ mol/l). The differences in the IC₅₀ required to affect PMNs and the parietal cells make a clinical effect unlikely during peroral administration since the plasma concentrations are low. However, during intravenous administration concentrations of 1–2 x 10⁻⁵ mol/l are observed for a period of several hours. An effect in vivo is not unlikely considering the irreversibility of the in vitro effect observed in the present study. Omeprazole is a substituted benzimidazole with a high affinity for intracellular compartments with an acid pH. In the acid environment the drug degrades and the activity is associated with the degradation products. The mode of action is inhibition of the parietal cell H⁺, K⁺-ATPase, causing a diminished proton secretion into the secretory canaliculus.

The reasons for the present study were: (1) the affinity of omeprazole for acid environment and (2) the acidification of the phagolysosome by a proton secreting mechanism in the activated PMN. The mechanism of inhibition observed is probably an effect upon the PMN itself, since no effect could be demonstrated in a cell free system. Increased oxidative metabolism and generation of O₂⁻ occur as early events in the activation of PMN. Omeprazole and its acid degradation products had a similar effect and it is probably the degradation products that are active. The acid degradation used in the present study results in generation of sulphamid acid derivatives known to be active in the parietal cell.

Inhibition by omeprazole and its degradation products and lack of effect in a cell free system or a parietal cell model of related imidazoles indicate that a cellular mechanism is inhibited. The findings that a period of 30 minutes is required for maximal inhibition and that the effect is irreversible point to a cellular mechanism. Several mechanisms are possible.

Omeprazole has a high affinity for the glutathione-system and inhibition of the glutathione-system diminishes production of O₂⁻ by PMNs. Inhibition of the NADPH- oxidase or blocking the formation of the oxidase in the membrane itself may also have reduced O₂⁻ generation.

Activation of PMNs is associated with formation of a dormant membrane associated a NADPH-oxidase by translocation of cytochrome b二十四 from the granules to the membrane. This translocation was not affected by omeprazole. A direct effect on the oxidase itself or on assembly of the oxidase in the membrane cannot be excluded. Imidazole in a high concentration (30–50 mmol/l) inhibits the oxidase but imidazole and 5,6-dimethyl-benzimidazole in low concentrations did not inhibit O₂⁻ generation.

Omeprazole may also inhibit ATPase in the PMN membrane. A quainab sensitive ATPase has been described for the PMN membrane by...
some\textsuperscript{27} but the existence is disputed.\textsuperscript{11} During activation of PMNs an electroneutral proton secretion is activated. This includes an Na\textsuperscript{+}H\textsuperscript{+} ATPase and there is evidence that this proton pump requires assembly of several components at the membrane.\textsuperscript{4,5,12} The present study raises the possibility of common features between the proton pump in the parietal cell and in PMNs. It is noteworthy in this context that omeprazole inhibits another phagocyte – viz the osteoclast in vitro at an IC\textsubscript{50} of 10\textsuperscript{−5} mol/l.\textsuperscript{28}

In conclusion, a new anti-ulcer drug, omeprazole, has been shown to inhibit the functions of PMNs in concentrations that are reached during intravenous administration. Whether these findings have clinical importance remains to be studied in vivo. A beneficial implication of this study is that omeprazole may have an anti-inflammatory activity.

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