Chronic Granulomatous Disease: Mode of Action of Sulfamethoxazole/Trimethoprim

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Dedicated to Professor W. H. Hitzig on his 60th birthday

Summary

Four possible modes of action for the clinically observed effectiveness of sulfamethoxazole/trimethoprim in chronic granulomatous disease were evaluated: (1) inhibition of bacterial catalase, (2) improvement of granulocyte oxygen metabolism, (3) synergism of the antibiotic with nonoxygen-dependent granulocyte killing mechanisms, and (4) a purely antibiotic effect based on uptake and concentration of the antibiotic by and within granulocytes.

While the first three mechanisms were excluded, the fourth mechanism is highly probable; sulfamethoxazole was found to reach granulocyte associated concentrations 1.7-fold and trimethoprim 4.1-fold of extracellular levels. Penicillin G, a known non-penetrating antibiotic, reached 0.3-fold, and tetracycline, a known penetrating agent, 7.1-fold the extracellular level. These findings indicate that sulfamethoxazole/trimethoprim is an antibiotic combination uniquely suited for the long-term prophylaxis of infections in patients with defects of intracellular phagocyte killing.

Speculation

Cell permeable antibiotics that concentrate within the acidic granules and vacuoles of phagocytes are likely to be lipid-soluble weak bases.

Chronic granulomatous disease (CGD) is an inherited disease characterized by recurrent and sometimes fatal infections due to a metabolic defect of the polymorphonuclear leukocytes (PMN). CGD-PMN are deficient in one or several metabolic steps necessary to generate oxygen radicals including hydrogen peroxide which, together with myeloperoxidase and chloride, forms a microbicidal complex in the phagocytic vacuole (21). This deficiency of oxygen radicals and hydrogen peroxide in CGD-PMN results in the survival of ingested catalase positive (19, 28, 34), but not of catalase negative bacteria (e.g., streptococci, lactobacilli, obligate anaerobic bacteria). Although both groups of organisms generate hydrogen peroxide, release of hydrogen peroxide into the vacuole and subsequent restoration of the microbicidal complex is possible only in the absence of bacterial catalase. The complex can also be restored by other peroxide generating systems or by added H_2O_2 (17, 36).

Early attempts to treat the persistent infections in CGD failed (33) until the advent of antibiotic therapy. Although nafcillin and rifampicin are effective (13, 26, 33), to date most physicians use a sulfonamide or preferentially sulfamethoxazole/trimethoprim (SMX/TMP) not only for acute infections, but also for long term prophylaxis (1, 7, 18, 24, 29, 35, 42) with one reported failure (26). Since then the frequency and severity of bacterial infections in CGD have been substantially reduced.

Apart from previous investigations by Johnston *et al.* (18), who showed improved *in vitro* bacterial killing by CGD-PMN after the addition of sulfisoxazole, the use of SMX/TMP in CGD is purely empiric. Since the low frequency of the disease ($1:10^6$) precludes

clinical studies, it was decided to elucidate the mechanisms involved by *in vitro* experiments. Four possible modes of action were evaluated. (1) Inhibition of bacterial catalase by SMX/TMP or one of its metabolites allowing hydrogen peroxide of microbial origin to diffuse out of the bacteria and to restore the deficient bactericidal complex in CGD-PMN. (2) Improvement of oxygen metabolism in CGD-PMN or direct generation of hydrogen peroxide by SMX/TMP or one of its metabolites. (3) Synergistic action between nonoxygen-dependent PMN killing mechanisms (normal in CGD) and SMX/TMP. (4) A purely antimicrobial effect of SMX/TMP on ingested bacteria dependent on intracellular concentration of the antibiotic as the result of a favourable partition coefficient. Bacteria, resistant to serum levels of the antibiotic, would then be inhibited or killed after phagocytosis.

MATERIALS AND METHODS

ISOLATION OF PMN

PMN were obtained from 10 healthy volunteers and three CGD patients. Informed consent of the donors was obtained before the start of this investigation.

PMN were separated from heparinized blood by dextran and ficoll-hypaque sedimentation (6), and resuspended in modified Krebs-Ringer phosphate buffer (KRP): 16 mM sodium phosphate 120 mM NaCl, 5 mM KCl, 0.9 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 4 g liter⁻¹ human serum albumin. The pH (pH 7.4 at 37°C) was adjusted with 1 M NaOH and 1 M HCl. Cell counts were performed in a Coulter counter. The normal PMN remained intact and functional for at least 4 h after isolation, as determined routinely by latex particle-induced metabolic burst and by bacterial killing tests. Viability of the CGD-PMN was established by stimulation of the metabolic burst with phenazine methosulfate.

OXYGEN CONSUMPTION

Oxygen metabolism before and after latex particle stimulation was recorded on an oxygraph (Gilson, model 5/6), according to Segal and Coade (40). The reaction mixture contained in a final volume of 1.6 ml KRP (reaction temperature 37°C) 5×10^6 ml⁻¹ PMN. Latex particles 2.5×10^8 ml⁻¹ (Difco, dialysed against KRP) served to initiate the metabolic burst. Oxygen consumption during the following 4-8 min was used to calculate the stimulated consumption as nmol O_2 per h per 5×10^6 PMN. The oxygraph was calibrated with air-saturated water, in which 210 μM oxygen are dissolved at 37°C and 101.3 kPa (10).

GENERATION OF H_2O_2

Generation of hydrogen peroxide by SMX/TMP or one of its metabolites in human serum was investigated in an oxygraph after the addition of ascorbate. Serum was obtained from normal volunteers before and 3 h after oral administration of SMX/TMP 18 mg kg⁻¹ body weight and was heated at 62°C for one h to

inactivate endogenous catalase. Similar experimental conditions have been described by Deamer *et al.* (9) for the production of hydrogen peroxide by the alloxan-dialuric acid cycle.

CATALASE ACTIVITY

Catalase activity was assayed in an oxygraph as described by Del Rio *et al.* (10). Bacteria were preincubated for 3 h in the test media (10^5 cells ml^{-1}) and were then transferred to an oxygraph. When a stable base line was established, hydrogen peroxide (9 mM end-concentration) was added and oxygen liberation was then recorded during the next 10 min.

GENERATION OF O_2^-

Superoxide (O_2^-) production was stimulated by addition of NaF (20 mM end-concentration), and measured by superoxide-dismutase-inhibitable reduction of ferricytochrome C using the technique described by Babior *et al.* (3).

PREPARATION OF GRANULE-RICH FRACTION (GRF)

Purified PMN from 400 ml of blood of a healthy volunteer were homogenized according to Dewald *et al.* (11) in 0.1 M Sörensen buffer, pH 7.4 at 4°C and 0.34 M sucrose. The postnuclear supernatant ($1000 \times g$, 5 min) was centrifuged for 20 min at $15,000 \times g$. The sediment contained, besides other cell organelles, abundant cytoplasmic granules and was designated as the GRF (4). The membranes of the GRF were lysed with 0.02% (v/v) triton X 100 and the lysate stored in small quantities in liquid nitrogen. Under these conditions microbicidal activity is maintained for at least 4 wk.

MICROORGANISMS

Staphylococcus aureus strains (KS 1, SMX/TMP resistant and KS 2, SMX/TMP susceptible, both isolated from patients) were maintained on Columbia sheep blood agar (Gibco) and subcultured monthly. For routine experiments, the bacteria were grown in two passages in Isosensitest broth (Oxoid) in a shaking water bath. Determination of colony forming units (CFU) was done as described by Meynell and Meynell (30).

SYNERGISTIC ACTION OF SMX/TMP WITH GRF

Staphylococci (about 10^5 ml^{-1}) were incubated in broth at pH 7.4 or 6.0. Antimicrobials and/or GRF were added at minimal inhibitory concentrations (MIC): 30 μg protein ml^{-1} GRF for both strains, 0.2 μg ml^{-1} flucloxacillin for both strains, 50 μg ml^{-1} SMX/TMP (5:1, by weight) for strain KS1 and 1.5 μg ml^{-1} SMX/TMP (5:1, by weight) for strain KS2.

CELL PENETRATION OF ANTIBIOTICS

According to the method of Mandell (27) PMN/buffer partition coefficients were determined. Buffer was used instead of serum to avoid PMN aggregation. The cells were incubated for 1 h in KRP buffer (pH 7.4, 37°C) before centrifugation in a miniature centrifuge (Microfuge B; Beckman Instruments). During this time they were exposed to radioactive-labelled antibiotics at concentrations obtainable *in vivo*: SMX 24 μg ml^{-1} , TMP 5 μg ml^{-1} , Penicillin G12 μg ml^{-1} , and tetracycline 1 μg ml^{-1} . Total and extracellular water spaces in the PMN sediment were assayed with $^3\text{H}_2\text{O}$ and [^{14}C]-carboxyvinulin, respectively (38), and the intracellular water space calculated as their difference. Intracellular antibiotic concentration was calculated from extracellular antibiotic concentration (assumed to be identical with the concentration in the supernatant) and intra- and extracellular water spaces in the sediment. Partition coefficients were then expressed as concentration of the antibiotic in the water space of the PMN divided by the concentration in the KRP phase. Radioactive-labelled compounds were obtained from New England Nuclear ($^3\text{H}_2\text{O}$, [^{14}C]-carboxyvinulin and tetracycline [^3H -(N)], Amersham Radiochemical Centre

([^{14}C]-benzylpenicillin = potassium 6-phenyl-1-[^{14}C] acetamidopenicillanate and [^{35}S]-sulfamethoxazole = 3-[^{35}S]sulfanilamido-5-methylisoxazole) and Wellcome Research Laboratories ([^{14}C]-trimethoprim = 2,4-diamino-5-(3,4,5-trimethoxybenzyl)-2-[^{14}C]pyrimidine).

RESULTS

INHIBITION OF BACTERIAL CATALASE

The results are summarized in Table 1. Bacterial catalase activity remained unchanged when two strains of *S. aureus* were incubated for 3 h in (1) buffer (2) buffer with SMX/TMP, (3) human serum, heat inactivated, and (4) human serum obtained 3 h after oral SMX/TMP administration (18 mg kg^{-1}) and heat inactivated. Serum concentrations of antimicrobials were in the range of 40–60 μg ml^{-1} for SMX and 2–3 μg ml^{-1} for TMP. Serum bacteriostatic tests before and after heat inactivation showed no loss of antibiotic activity. In contrast to SMX/TMP, azide and cyanide at 0.1 mM completely inhibited the bacterial catalase under the same assay conditions.

IMPROVEMENT OF GRANULOCYTE OXYGEN METABOLISM

There was no improvement of oxygen uptake by resting and stimulated PMN (four normal and two CGD donors) (Table 2) after the *in vitro* addition of SMX/TMP in KRP (120 μg ml^{-1}) or of human serum after SMX/TMP administration (see above). Normal or CGD-PMN (three donors each) isolated 3 h after oral administration of SMX/TMP in various doses did not show an increase in stimulated superoxide production (Table 3). When ascorbate was added to heat inactivated serum drawn before and 3 h after oral administration of SMX/TMP (Fig. 1b and 1c) there was no generation of hydrogen peroxide. This contrasted with hydrogen peroxide generation by the ascorbate driven oxidation of dialuric acid to alloxan (Fig. 1a).

SYNERGISTIC ACTION OF SMX/TMP WITH GRF

As shown in Fig. 2, growth of staphylococci without GRF or antibiotics was exponential for strain KS1, which is clinically resistant to SMX/TMP (MIC for SMX/TMP, flucloxacillin and GRF see "Materials and Methods"), or nearly exponential for strain KS2, which is clinically susceptible to SMX/TMP (see also "Materials and Methods"), during the first 6 h of incubation. The growth was unaffected by Triton X 100 (which was used in the same concentrations as those resulting from the addition of triton-lysed GRF to the broth). Addition of 1.0 MIC of either GRF (isolated from normal PMN of a single volunteer), flucloxacillin or SMX/TMP alone inhibited the growth of both strains. While

Table 1. Catalase activity of *S. aureus*: influence of SMX/TMP

Medium	Catalase activity	
	(nmoles H_2O_2 10^{-5} cells min^{-1})	
	<i>S. aureus</i> KS1	<i>S. aureus</i> KS2
KRP buffer	11.5 $\pm$ 0.6 ¹	19.4 $\pm$ 0.2 ¹
100 μg ml^{-1} SMX and 20 μg ml^{-1} TMP ² in KRP buffer	11.6 $\pm$ 0.9	19.1 $\pm$ 0.8
Heat inactivated ³ human serum before SMX/TMP administration	11.6 $\pm$ 0.8	19.8 $\pm$ 0.6
Heat inactivated ³ human serum 3 h after SMX/TMP administration	11.0 $\pm$ 0.8	19.8 $\pm$ 0.8
Cyanide or azide 0.1 mM	0	0

¹ Mean $\pm$ S.D. ($n = 4$).

² Corresponds to 0.4 mM SMX and 0.07 mM TMP.

³ 62°C, 1h.

Table 2. *Oxygen consumption of normal and CGD-PMN: influence of SMX/TMP*

Test Medium	Oxygen Consumption		Normal PMN Stimulation	CGD-PMN Resting	CGD-PMN Stimulation
	(nmoles O ₂ 5 × 10 ⁻⁶ cells h ⁻¹)	Normal PMN Resting			
KRP buffer	112 ± 107 ¹ (n = 4)		813 ± 347 ¹ (n = 4)	0 ² (n = 2)	0 ² (n = 2)
100 µg ml ⁻¹ SMX and 20 µg ml ⁻¹ TMP in KRP	0 (n = 4)		702 ± 115 (n = 4)	0 (n = 2)	0 (n = 2)
50% KRP and 50% serum before SMX/TMP administration	66 ± 74 (n = 4)		409 ± 129 (n = 4)	ND ³	ND ³
50% KRP and 50% serum 3 h after SMX/TMP administration	40 ± 44 (n = 4)		404 ± 148 (n = 4)	0 (n = 2)	0 (n = 2)

¹ Mean ± S.D.² Values, mean.³ ND = not done.Table 3. *Stimulated superoxide production of normal and CGD PMN: influence of SMX/TMP*

PMN	Dose of SMX/TMP		Stimulated O ₂ ⁻ production		Serum level of SMX/TMP
	(mg kg ⁻¹)		(nmoles O ₂ ⁻ 2.5 × 10 ⁻⁶ cells min ⁻¹)		
			Before SMX/TMP	3 h after SMX/TMP	(µg ml ⁻¹)
a ² . CGD	18		3.7 ¹	0.8	30.2
Control	18		19.8	26.4	
b. CGD	28		0.5	2.0	75.7
Control	28		36	35.2	
c. CGD	48		1.6	1.1	82.5
Control	48		26.6	33.7	

¹ Values, mean.² Three different CGD patients.

Fig. 1. Oxygraph tracings showing lack of generation of hydrogen peroxide in human serum before (b), and after (c) SMX/TMP administration. Production of hydrogen peroxide by the alloxandialuric cycle (a) served as a control. Addition of 0.5 M alloxan in buffer or of human serum (1), addition of 0.5 mM ascorbate (2), and addition of 80 µg ml⁻¹ bovine catalase (3).

the combination of flucloxacillin and GRF resulted in killing, the combination of SMX/TMP and GRF produced no change. Results from experiments at pH 6.0 are not shown, as they did not differ from the values at pH 7.4.

CELL PENETRATION OF ANTIBIOTICS

The results (obtained with normal PMN) are summarized in Table 4. Except for penicillin G all antibiotics (SMX, TMP,

Fig. 2. Lack of synergistic action of SMX/TMP with GRF (a, c) as opposed to synergism of flucloxacillin with GRF (b, d). GRF was obtained from normal PMN (Material and Methods). Growth without inhibitors (· · · · ·), with 1.0 MIC GRF (—+—), with 1.0 MIC SMX/TMP (—○—) with 1.0 MIC flucloxacillin (—△—), and with both, 1.0 MIC GRF and either SMX/TMP (—●—) or flucloxacillin (—▲—). Strain KS1 has a MIC of 50 µg ml⁻¹ SMX/TMP (5:1, by weight), strain KS2 has MIC of 1.5 µg ml⁻¹ for the same drug. Both strains have identical MIC for flucloxacillin (0.2 µg ml⁻¹) and GRF (30 µg ml⁻¹ protein). Results are given as the mean ± S.D. (n = 3).

Table 4. *Partition coefficients (P) of various antibiotics*

Antibiotic	^P PMN/KRP
Penicillin G	0.30 ± 0.06 ¹
SMX	1.7 ± 0.2
TMP	4.1 ± 0.6
Tetracycline	7.1 ± 0.7

¹ mean ± S.D., n = 12 (four samples from three normal donors).

tetracycline) showed a partition coefficient greater than 1. Only the partition of penicillin G was in favour of the extracellular space. The water volume of the PMN as assayed with this method was 257 ± 23 fl (11 healthy donors), with an osmolality of 289 mosmoles of the buffer used.

DISCUSSION

The administration of sulfonamides alone or of SMX/TMP for prophylaxis and treatment of infections in CGD is now a generally accepted procedure. The efficacy of SMX/TMP can be partly explained by its broad spectrum of bactericidal activity (39), and by selective intestinal decontamination. Many anaerobic bacteria are not affected, so that changes in the intestinal flora are minimal and are mostly restricted to the elimination of *S. aureus* and of Enterobacteriaceae (22, 23, 32) to which CGD patients are unduly susceptible. However, the striking clinical success of SMX/TMP has given rise to speculations that these drugs might act in a way other than the classical antibiotic one; that is, other than by inhibition of tetrahydrofolate biosynthesis. The following alternative modes of action have been considered.

INHIBITION OF BACTERIAL CATALASE

Inhibition of bacterial catalases by sulfonamides would permit the release of peroxide generated by bacteria, thus rendering them susceptible to killing even by CGD-PMN. Johnston *et al.* (18) however, found no inhibition of bacterial or bovine catalases by sulfisoxazole. Our results (Table 1) confirm and extend these observations to SMX/TMP. SMX at 0.4 mM and TMP at 0.07 mM (corresponding to $120 \mu\text{g ml}^{-1}$ SMX/TMP (ratio 5:1 by weight) and human serum drawn 3 hr after administration of SMX/TMP showed no significant effect on catalase activity.

IMPROVEMENT OF GRANULOCYTE OXYGEN METABOLISM

Pyesmany and Cameron (35) reported an SMX/TMP mediated increase of the oxidative metabolism in CGD-PMN. However, this observation was not confirmed by others (18, 42). Anderson *et al.* (2) found no improvement of normal neutrophil respiratory functions by SMX/TMP. Our results also indicate that normal and CGD-PMN do not produce more hydrogen peroxide when SMX/TMP or its metabolites are present, as shown by unaltered oxygen consumption (Table 2), and unaltered superoxide anion production (Table 3) of resting and stimulated cells. Sulfonamides inhibit peroxidases (25). Such action could allow tiny amounts of oxygen radicals to survive inside CGD-PMN and to reconstitute the oxygen-dependent killing mechanisms. If enough perhydroxy radical (HO_2) was generated, nonenzymatic oxidation of NADPH could occur and create a self-perpetuating chain reaction (41). This possible mode of action of SMX/TMP seems also not to take place as shown by unaltered superoxide anion production (Table 3). Also SMX/TMP or its metabolites present in human serum after SMX/TMP administration *per se* do not directly reduce oxygen to hydrogen peroxide (Fig. 1c).

SYNERGISTIC ACTION OF SMX/TMP WITH GRF

The effectiveness of SMX/TMP could be due to a synergistic action with the intact nonoxygen-dependent killing mechanisms of CGD-PMN (21). Such a synergism was reported for nafcillin by Warren and Gray (43) and by Friedman and Warren (14). These authors suggested that nafcillin, even at low concentrations, may profoundly affect the integrity of the bacterial cell wall, leading to increased susceptibility to cell wall lytic enzymes of the phagocyte. Root *et al.* (37) showed that subinhibitory (sub-MIC) concentrations of penicillin G increased susceptibility of *S. aureus* to killing by nonoxygen-dependent bactericidal mechanisms. Using electron microscopy, Klainer and Perkins (20) demonstrated changes on the surface of *S. aureus* organisms when these were pretreated with subbactericidal concentrations of penicillins or SMX/TMP. Evidence for inhibition of cell wall synthesis by blockade of folate metabolism was also provided by Daschner (8)

and by Dulaney and Marx (12). We investigated a possible synergism between the nonoxygen-dependent killing mechanisms of PMN and subbactericidal antibiotic concentrations using two strains of *S. aureus* (SMX/TMP resistant, and SMX/TMP susceptible). The nonoxygen-dependent killing mechanisms are located in the granules, which burst into the vacuole formed after phagocytosis (44). Incubation of staphylococci with 1.0 MIC GRF from normal granulocytes and 1.0 MIC flucloxacillin was bactericidal while the combination of 1.0 MIC GRF with 1.0 MIC SMX/TMP had no killing effect (Fig. 2). From these data it seems likely that sublethal concentrations of SMX/TMP equally fail to improve killing of bacteria by the nonoxygen-dependent mechanisms of CGD-PMN. However, as the above experiments were performed with disrupted cells the normal vacuole environment might not have been completely mimicked. It is planned therefore to extend our studies of synergism to intact normal and CGD-PMN which have taken up SMX/TMP-pretreated bacteria into vacuoles.

CELL PENETRATION AND ACCUMULATION OF SMX/TMP

SMX/TMP is very effective in the treatment of infections caused by intracellular microorganisms such as *Pneumocystis carinii* and *Toxoplasma gondii*. This suggests that its unique beneficial effect in CGD patients might be related to penetration and accumulation of the drug inside the leukocyte. This was tested experimentally by determining PMN/buffer partition coefficients. We found the partition coefficients of SMX/TMP and Tetracycline to be greater than 1 (Table 4), which seems to indicate not only cell membrane penetration, but also concentration. Berneis and Boguth (5) obtained a red blood cell/medium partition coefficient of 1.3 for SMX and 4.2 for TMP, which agrees with our observations. On the other hand, we found cell-associated concentrations of penicillin G of less than a third of the extracellular levels. Mandell (27) also showed that penicillin G did not penetrate the PMN leukocyte. This could explain the penicillin resistance of phagocytosed bacteria reported by Holmes *et al.* (15) in 1966.

Partition along pH gradients may be the important mechanism for directing antibiotics like TMP into the cell. As intracellular pH in granulocytes is about 7.2 (31) and intravacuolic pH of normal and CGD-PMN is even lower between 4.5 and 5.0 (16) weak acids, such as penicillin, would be excluded from the more acidic cell interior, whereas weak bases such as trimethoprim and tetracyclines would be accumulated. This explanation, however, does not hold for sulfamethoxazole, which is slightly acidic.

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45. The authors thank Dr. Baggiolini for his help in the preparation of the GRF extract, Dr. Böhm and Dr. Crevoisier, Hoffmann-La Roche, for the determination of MICs and SMX levels, Prof. Hitzig for his encouragement of this study, Mrs. Gubler and Miss Schmid and Miss Vitte for expert technical assistance.
46. The Swiss National Science Foundation (grant number 3.895-0.79) and the Theodor and Ida Herzog-Egli Stiftung is gratefully acknowledged.
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49. Received for publication December 29, 1980.
50. Accepted for publication May 18, 1981.