

# Inhibition by Sulfonamides of the Candidacidal Activity of Human Neutrophils

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**ABSTRACT** Sulfonamides reduced substantially the ability of normal human neutrophils to kill strains of *Candida albicans* and *Candida tropicalis*, and impaired to a lesser extent their activity against *Staphylococcus aureus* 502A and *Serratia marcescens*. Sulfonamides also inhibited (a) iodination of *Candida* cells by normal neutrophils; (b) candidacidal activity in cell-free systems containing purified human myeloperoxidase, hydrogen peroxide, and potassium iodide; and (c) accumulation of molecular iodine in analogously constructed cell-free systems. In contrast to these effects on reactions catalyzed by myeloperoxidase, sulfonamides exerted relatively little effect on the levels of microbicidal activity manifested by human neutrophils that lacked myeloperoxidase. Sulfonamides appear to influence the function of human neutrophils predominantly by interfering with myeloperoxidase-mediated pathways. Certain basic and clinical implications of these data are discussed.

## INTRODUCTION

In recent studies, leukocytes from some patients with kidney transplants manifested an impaired ability to kill *Candida albicans* when tested in autologous serum, despite normal candidacidal activity if tested in normal serum.<sup>1</sup> During serial studies of the leukocytes and serum of one such patient, this pattern of in vitro candidacidal activity appeared after the initiation of therapy with a sulfonamide. Experiments were then performed to determine whether sulfonamides influence leukocyte microbicidal function.

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<sup>1</sup>Lehrer, R. I., and S. Kountz. Unpublished observations.

## METHODS

**Patients.** Myeloperoxidase-deficient leukocytes were obtained from two patients. C. J. B. is a 51 yr old man with hereditary myeloperoxidase (MPO) deficiency; his case has been described in detail (1). The second patient (H. H.) was a 61 yr old man with mosaic MPO<sup>2</sup> deficiency associated with refractory megaloblastic anemia.<sup>3</sup> At the time of these experiments, more than 99% of his neutrophils were completely deficient in peroxidase.

**Leukocyte preparation.** Blood was obtained from normal adult volunteers and from the two MPO-deficient subjects. 5 USP U/ml of sodium heparin<sup>4</sup> were added, and the red cells were sedimented by adding 1 volume of 3% dextran<sup>5</sup> in normal saline for each 2 volumes of blood. The leukocyte-rich supernatant was centrifuged at 150 g for 8 min at room temperature. The cells were suspended in Hanks' balanced salt solution (HBSS) containing 15% fetal calf serum and 5 U/ml of heparin, centrifuged again and suspended as above. Immediately before addition to the incubation mixtures, the leukocytes were centrifuged at 150 g for 8 min and suspended at the desired concentration in plain HBSS.

**Microorganisms.** The previously characterized *C. albicans* strain 820 (2) was grown in yeast phase in Sabouraud's 2% dextrose broth<sup>6</sup> at 33°C. Experiments requiring colony-count procedures were performed with 7-day old cultures. Under these conditions, a colony-forming unit (CFU) had a mean of 1.8 yeast cells (range, 1.6-2.2). All other experiments employed 3- to 5-day old cultures. An isolate of *C. tropicalis*<sup>7</sup> was cultured in Sabouraud's broth for 2-5 days at 33°C. The concentration of either *Candida* species was determined by counting suitable dilutions in a hemocytometer. *Staphylococcus aureus* strain

<sup>2</sup>Abbreviations used in this paper: CFU, colony-forming unit; HBSS, Hanks' balanced salt solution; MPO, myeloperoxidase.

<sup>3</sup>Lehrer, R. I., L. S. Goldberg, N. P. Rosenthal, and M. A. Apple. Refractory megaloblastic anemia with myeloperoxidase-deficient neutrophils. *Ann. Intern. Med.* In press.

<sup>4</sup>Riker Laboratories, Inc., Northridge, Calif.

<sup>5</sup>Clinical grade, mol wt 100,000-200,000, Nutritional Biochemicals Corp., Cleveland, Ohio.

<sup>6</sup>Difco Laboratories, Detroit, Mich.

<sup>7</sup>Generously provided by Dr. Seymour Klebanoff.

502A and a chromogenic strain of *Serratia marcescens* were cultured overnight at 37°C in tryptic soy broth (Difco), washed with distilled water containing 0.01% gelatin (gel-water), and adjusted to the desired concentration spectrophotometrically.

**Sulfonamides.** Sulfonamides were obtained from the Pharmaceutical Service of this institution as anhydrous powders; they met USP or National Formulary (sulfathiazole) criteria for purity. Sodium sulfadiazine and sodium sulfacetamide were dissolved in HBSS. Sulfathiazole and sulfisoxazole were dissolved in a small amount of 0.1 N NaOH, and diluted with HBSS to appropriate concentrations. Sulfonamide solutions to be used with intact leukocytes were adjusted to pH 7.4 with 0.1 N HCl and sterilized by passage through a Millipore filter,<sup>8</sup> pore size 0.22 μ. Leukocytes and sulfonamides were incubated together for 30 min before bacteria or fungi were added. These experiments employed final concentrations of sulfonamides ranging from 5 to 100 mg/100 ml.

**Candidacidal assays.** The ability of leukocytes to kill *C. albicans* was measured in three ways: dye exclusion, specific stain, and colony count. In the dye-exclusion assay (2), equal numbers of neutrophils and *Candida* cells were incubated together at 37°C in HBSS containing 25% normal human group AB serum. After 60 min the leukocytes were lysed with sodium desoxycholate and methylene blue was added in a concentration that stained only non-viable *C. albicans*. The specific-stain method was based on analysis of the morphological and tinctorial changes of *Candida* ingested by neutrophils. In this test, the percentage of *Candida* cells within neutrophils that were "ghosts" (i.e. had not germinated and had lost their cytophilic basophilia by Giemsa stain) after 2.5 hr of incubation was taken to indicate the percentage of ingested organisms killed and partially degraded by neutrophils (3). Preliminary experiments demonstrated that sulfonamides did not alter the staining properties of viable and non-viable *Candida* cells examined in the dye-exclusion and specific-stain assays.

In studies with the colony-count method, leukocytes were suspended in a final volume of 1 ml of HBSS with 25 or 95% normal group AB serum; sodium sulfadiazine was added to certain tubes. In two colony-count experiments with *C. albicans*, a low ratio of organisms to leukocytes was used. In these tests, assay tubes contained  $5 \times 10^6$  neutrophils and  $2 \times 10^5$  CFU of *C. albicans*. In all other colony-count studies with *C. albicans* and *C. tropicalis*, assay tubes contained  $5 \times 10^6$  neutrophils and  $2 \times 10^8$  CFU of the desired *Candida* species. Leukocyte-free control tubes contained *Candida* cells, serum, and HBSS, with or without sodium sulfadiazine. All tubes were prepared in duplicate; incubation and sampling techniques are described below.

*Candida* cells were added to all tubes after the other components had incubated together for 30 min at 37°C. Immediately after the addition of *Candida* to a tube, the contents were thoroughly mixed and three 10 μl samples were removed; each was diluted in 2.5 ml of gel-water. 25 μl of each dilution was spread over the surface of a Sabouraud's agar plate that was then incubated at 37°C for at least 24 hr to permit colony development. Assay and control tubes were incubated at 37°C with rotation (30 rpm). After 90 and 180 min of incubation, additional triplicate samples were taken from each tube and handled as above. Control tubes containing HBSS and serum, with

or without sulfadiazine, did not undergo any fall in colony counts during a 180 min incubation period with *C. tropicalis*, or during a 90 min period with *C. albicans*. The mean percentage of leukocytes that contained ingested *Candida* cells and the mean number of *Candida* cells ingested per phagocytic leukocyte was the same whether or not the tubes contained sulfonamide.

**Bactericidal assays.** Bactericidal activity was measured as previously described (4). Duplicate assay mixtures (in a final volume of 1 ml of HBSS containing 10% normal group AB serum) contained  $1 \times 10^7$  neutrophils,  $2 \times 10^6$  to  $4 \times 10^6$  CFU of *Staph. aureus* 502A or *S. marcescens*, and, where indicated, 1 mg of sodium sulfadiazine. Neither organism was inhibited or killed by the concentrations of serum or sulfadiazine employed in these studies. Differential centrifugation (1) was used to estimate the extent of ingestion of *Staph. aureus* 502A in the presence or absence of sulfadiazine. After 15 min of incubation under these conditions, the assay tubes were centrifuged for 6 min at 50 g, and the concentration of bacteria in the supernatant was determined by the usual procedure for colony counting.

**Myeloperoxidase-linked candidacidal activity.** The purified human MPO<sup>9</sup> (5) used in these studies had an activity of 15,300 U/mg when assayed with orthodianisidine; calculations were made with the Worthington formula (6):

$$U/mg = \frac{A_{460}/min}{11.3 \times \text{mg of enzyme/ml of reaction mixture}}$$

Lysates from erythrocyte-free human peripheral blood leukocytes, prepared in 0.01 M acetate buffer as previously described (1), provided an alternate source of peroxidase activity. Crystalline horseradish peroxidase, activity stated as 300 purpurogallin U/mg, was purchased from Sigma Chemical Company, St. Louis, Mo.

Candidacidal activity in a cell-free system was measured by minor modifications of a previously described method (7). All tubes contained sodium citrate-phosphate buffer, pH 6.0, 40 μmoles; *C. albicans*,  $1 \times 10^6$  CFU; and sufficient distilled water to bring the final volume to 0.5 ml. Selected tubes also contained one or more of the following: MPO, 1.25 μg; KI, 0.025, 0.05, or 0.125 μmole; H<sub>2</sub>O<sub>2</sub>, 0.05, 0.125, or 0.25 μmole; and sodium sulfacetamide, 0.5 μmole, or sodium sulfadiazine, 0.18 μmole.<sup>10</sup> Sulfonamide solutions were adjusted to pH 6.0 by addition of 0.1 M citric acid. H<sub>2</sub>O<sub>2</sub> was the last component to be added and the tubes were then incubated at 37°C for 60 min before samples were taken for colony count.

**Peroxidase assays.** Peroxidase activity was measured by the Worthington method with orthodianisidine as substrate (6), and by an iodometric method adapted from the macro-method of Davis (8). The latter assay was performed in tubes containing sodium citrate-phosphate buffer (pH 5.0 or 5.6), 50 μmoles; KI, 1 μmole; H<sub>2</sub>O<sub>2</sub>, 1 μmole; sodium thiosulfate, 10–50 nmoles; enzyme (1 μg of MPO or an approximately equiactive quantity of leukocyte lysate); boiled starch, 0.1%; and distilled water to bring the final volume to 1 ml. Sulfonamide solutions to be tested were adjusted to the appropriate pH with 0.1 M citric acid. The reaction was started by adding H<sub>2</sub>O<sub>2</sub> and the number of seconds that elapsed before the sudden appearance of a

<sup>9</sup> Generously provided by Dr. Julius Schultz.

<sup>10</sup> These concentrations correspond to 20 mg/100 ml of sodium sulfacetamide and 10 mg/100 ml of sodium sulfadiazine.

<sup>8</sup> Millipore Corporation, Bedford, Mass.

TABLE I  
Inhibition of Leukocyte Candidacidal Activity by Sulfonamides

Sulfonamide concentration	Leukocyte candidacidal activity (per cent of control*) in the presence of			
	Sulfadiazine (5 subjects)	Sulfathiazole (4 subjects)	Sulfisoxazole (4 subjects)	Sulfacetimide (3 subjects)
mg/100 ml				
0 (control)	100	100	100	100
5	78.9 ± 6.3	67.6 ± 10.5	87.4 ± 10.9	64.1 ± 3.8
10	56.5 ± 6.3	48.4 ± 14.5	55.6 ± 8.8	65.7 ± 2.3
25	28.0 ± 4.9	13.5 ± 9.5	19.0 ± 6.5	29.8 ± 10.0
50	10.5 ± 4.4	NT†	NT	NT

\* This figure was derived by dividing the percentage of *C. albicans* killed by a subject's leukocytes in the presence of sulfonamide by the percentage killed by these leukocytes in the absence of sulfonamide (control) × 100. Data are expressed as mean ± SEM. Candidacidal activity was measured by the dye-exclusion assay.

† NT, not tested.

blue color (denoting the molecular iodine-starch complex) was recorded. The time, in seconds, to reach the endpoint was inversely proportional to the enzyme concentration and directly proportional to the thiosulfate concentration and indicated the time required to generate iodine in excess of the reducing capacity of the thiosulfate. In a given study, a concentration of thiosulfate was selected that gave an endpoint at approximately 5 sec in the absence of inhibitors.

**Quantitative iodination.** The ability of normal human neutrophils to iodinate *C. albicans* was studied by slight modifications of the method of Pincus and Klebanoff (9). Incubation mixtures contained  $10^7$  peripheral blood leukocytes,  $2.5 \times 10^7$  heat-killed *C. albicans*, 10 nmoles of KI, and 0.2  $\mu$ Ci of carrier free [ $^{131}$ I]NaI<sup>11</sup> in a final volume of 0.5 ml of HBSS containing 10% normal group AB serum. Sodium sulfadiazine or sodium azide was added to selected tubes. In some studies, zymosan<sup>12</sup> replaced heat-killed *C. albicans*; in some, calcium-free Krebs-Ringer phosphate buffer (10) replaced HBSS. All assay components were adjusted to a pH of 7.4 before addition to incubation mixtures. Tubes were incubated for 60 min at 37°C and processed as described (9).

## RESULTS

***Candida albicans.*** Normal human neutrophils, tested by the dye-exclusion assay, kill approximately one-third of ingested *C. albicans*, strain 820, in 1 hr (1, 2). Sulfadiazine, sulfathiazole, sulfisoxazole, and sulfacetimide all diminished the candidacidal activity of normal neutrophils; the extent of inhibition was dependent on the concentration of sulfonamide in the incubation mixture (Table I). This inhibition was not caused by a decrease in ingestion of the organism. Microscopic examination of unstained wet preparations and of fixed and stained slides prepared 15 min after addition of *C. albicans* cells to the incubation mixtures revealed that all of the added

<sup>11</sup> New England Nuclear Corp., Boston, Mass.

<sup>12</sup> Schwarz/Mann, Orangeburg, N. Y.

organisms were ingested at every concentration of sulfonamide tested.

Equal and virtually complete inhibition resulted whether cells were preincubated with sulfadiazine (50 mg/100 ml) for 1, 15, or 30 min before the addition of fungi. This same concentration of sulfadiazine, added to normal leukocytes 15 min after addition of *Candida*, resulted in only about half of the potential inhibitory effect. If the addition of sulfadiazine was delayed until 30 min after the addition of *Candida*, killing was inhibited by only 20%. Leukocytes preincubated with sulfadiazine and washed free of the drug killed *C. albicans* normally. These observations suggest that magnitude of the inhibitory effect was determined primarily by the concentration of sulfonamide present during the early post-phagocytic period.

In addition to these dye-exclusion studies, candidacidal activity was also measured by a standard colony-count technique and by the specific staining assay. Whether the criterion of candidacidal effectiveness was the percentage of *C. albicans* cells stained by  $2 \times 10^{-4}$  methylene blue (dye exclusion), the percentage fall from initial colony-count levels, or the percentage of organisms within neutrophils that were "ghosts" (specific stain), sulfadiazine inhibited the normal candidacidal activity of neutrophils. At a concentration of 100 mg/100 ml, this inhibition was shown to be essentially complete by all test systems (Table II). In the two colony-count studies conducted at

TABLE II  
Comparison of Three Methods of Demonstrating the Ability of Sulfonamides to Inhibit the Killing of *C. albicans* by Normal Neutrophils\*

Assay method	Serum	Neutrophils	Sulfadiazine	
			mg/100 ml	"Nonviable" <i>C. albicans</i> %
Dye exclusion, 60 min	+	+	0	42.3
	+	+	5	38.3
	+	+	10	32.7
	+	+	25	16.8
	+	+	50	9.3
	+	+	100	0.3
Colony-count, 90 min	+	+	0	61.5
	+	+	100	10.8
	+	0	0	10.8
Specific stain, 150 min	+	+	0	44.5
	+	+	100	3.0

\* The experimental conditions and criteria for determining candidacidal activity are described in the text. +, presence of component; 0, its absence. The data are the results of a single experiment on leukocytes from a normal subject.

TABLE III  
Effect of Sulfadiazine and Serum on the Ability of Normal Human Neutrophils to Kill *C. albicans*\*

Incubation medium		Specific stain assay				Colony-count assay	
		Ghosts in neutrophils		Germination in neutrophils		Colony-count reduction	
		150 min	240 min	150 min	240 min	90 min	180 min
I 25% serum	Mean	43.9	43.0	7.6	27.9	42.9	49.1
	SEM	2.8	2.8	2.0	2.3	3.8	4.4
II 95% serum	Mean	17.1	18.6	19.4	58.7	23.1	20.0
	SEM	3.4	3.3	3.1	3.6	4.5	5.7
III 95% serum plus sulfadiazine (25 mg/100 ml)	Mean	8.8	6.8	22.6	74.5	16.8	13.4
	SEM	2.2	1.7	4.2	3.7	3.4	5.9
Null hypothesis		Statistical significance, paired <i>t</i> test					
I = II	<i>P</i>	<0.001	<0.001	<0.001	<0.001	<0.01	<0.001
I = III	<i>P</i>	<0.001	<0.001	<0.005	<0.001	<0.005	<0.005
II = III	<i>P</i>	<0.005	<0.005	NS†	<0.005	NS	NS

\* Data represent six experiments on leukocytes from different normal subjects. The rate and extent of ingestion was comparable in all tubes.

† NS, *P* > 0.05.

very low ratios of *Candida* to leukocytes, normal leukocytes effected a 59.1% (mean) fall in colony count at 90 min in HBSS and a 10.4% fall in HBSS with sodium sulfadiazine (100 mg/100 ml).

Candidacidal activity was measured by both colony count and by specific-stain technique in a series of six experiments undertaken to examine the effect of moderate concentrations of sulfadiazine on neutrophil function in media with high concentrations of serum. Without sulfadiazine, neutrophil candidacidal activity was more than twice as effective in medium containing 25% serum than in otherwise identical medium containing 95% serum (Table III). The ability of neutrophils to kill ingested *C. albicans* in medium containing 95% serum was further curtailed by the presence of 25 mg of sodium sulfadiazine per 100 ml. This was most clearly shown by a greater than 50% decrease in the percentage of *Candida* within sulfadiazine-treated neutrophils that were "ghosts" after 150 and 240 min of incubation. The inhibitory effect of sulfadiazine on neutrophil function was also shown by the increased ability of *C. albicans* to form pseudogerm tubes after 240 min of intracellular residence in sulfadiazine-treated neutrophils. Although the colony-count data also suggest that 25 mg/100 ml of sulfadiazine depress the candidacidal activity of leukocytes in 95% serum, the results with this technique were more variable and the differences did not achieve statistical significance.

*Candida tropicalis*. A strain of *C. tropicalis* killed, albeit slowly, by the MPO-deficient neutrophils of patient

C.J.B. (11) was used in an attempt to elucidate the mechanism of inhibition by sulfonamides. If the effect of sulfonamides resulted solely from interference with the MPO-mediated candidacidal mechanisms of neutrophils, MPO-deficient neutrophils should not be affected by sulfonamides. On the other hand, if sulfonamides inhibited microbicidal components unrelated to MPO, these drugs should also inhibit MPO-deficient neutrophils. These propositions were tested in five experiments wherein

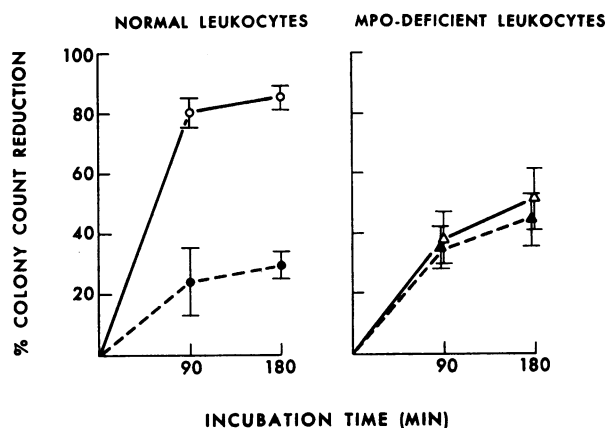


FIGURE 1 Effect of sulfadiazine, 100 mg/100 ml, on the ability of normal and MPO-deficient leukocytes (patient C. J. B.) to kill *C. tropicalis*. ○ and △, in the absence of sulfadiazine; ● and ▲, in the presence of sulfadiazine; vertical bar represents  $\pm 1$  SEM. Four paired experiments were done by using leukocytes from a different normal subject in each.

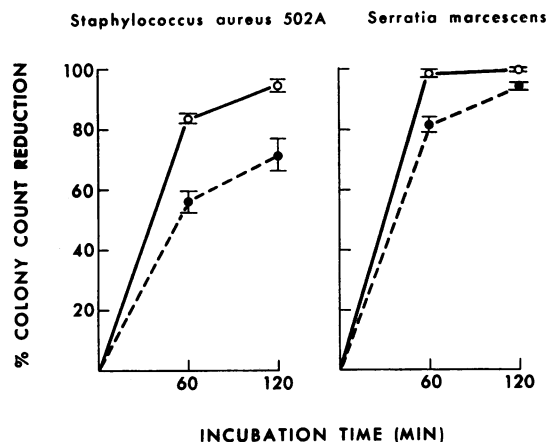


FIGURE 2 Effect of sulfadiazine on bactericidal activity of normal leukocytes.  $\circ$ , in the absence of drug;  $\bullet$ , in the presence of sodium sulfadiazine, 100 mg/100 ml; vertical bar represents SEM of percentage of colony-count reduction within this representative experiment.

leukocytes from different normal subjects were compared with those from the MPO-deficient subjects C. J. B. (four experiments) and H. H. (one experiment) in the presence and absence of sodium sulfadiazine (100 mg/100 ml).

In the absence of sulfonamides, C. J. B.'s MPO-deficient neutrophils killed *C. tropicalis* less effectively than did normal cells (90 min,  $P < 0.025$ ; 180 min,  $P < 0.05$ ) (Fig. 1). Similar results were obtained with leukocytes from H. H. (not shown). Sulfadiazine (100 mg/100 ml) greatly inhibited the candidacidal activity of normal leukocytes (90 min,  $P < 0.025$ ; 180 min,  $P < 0.01$ ); however, it had a considerably smaller effect on the neutrophils of C. J. B. (90 min,  $P > 0.2$ ; 180 min,  $P < 0.05$ ), and did not inhibit those of H. H. These findings are consistent with the hypothesis that sulfonamides exert their inhibitory effect predominantly through interference with MPO-dependent mechanisms. In the presence of sulfadiazine, MPO-deficient (C. J. B.) and normal neutrophils did not differ significantly in their ability to kill *C. tropicalis* (90 min,  $P > 0.20$ ; 180 min,  $P > 0.05$ ), although the number of experiments is too small to exclude the possibility that such a difference exists.

**Bactericidal activity.** In light of previous studies suggesting that MPO promotes the killing of *Staph. aureus* 502A and *S. marcescens* by normal neutrophils (4), it was of interest to examine the effect of sulfonamides on the ability of neutrophils to kill these organisms. At a concentration of 100 mg/100 ml, sodium sulfadiazine consistently decreased the rate at which these organisms were killed by normal neutrophils (Fig. 2). Ingestion, as estimated by differential centrifugation, was unimpaired. In a study comparing the effects of sulfadiazine on the

staphylocidal activity of normal and MPO-deficient neutrophils (C. J. B.), the MPO-deficient cells, unlike normal leukocytes, were only slightly inhibited (Fig. 3). It is noteworthy that, even in the presence of sulfadiazine, normal leukocytes killed staphylococci more effectively than did MPO-deficient cells.

**MPO-linked candidacidal activity.** The above data suggest that the inhibition of microbicidal activity by sulfonamides arises from interference with the neutrophil's MPO-linked intracellular mechanisms. A system composed of MPO,  $H_2O_2$ , and KI has been proposed as a model of the neutrophil microbicidal system (12, 13); this system kills *Candida* species in vitro (7). The possibility that sulfonamides might inhibit this system was examined in a series of experiments with *C. albicans*.

Potassium iodide was tested at concentrations ranging from  $2.5 \times 10^{-4}$  M to  $5 \times 10^{-5}$  M, and  $H_2O_2$  was used at concentrations between  $5 \times 10^{-4}$  M and  $1 \times 10^{-4}$  M. *C. albicans* was not killed when MPO, KI, and  $H_2O_2$  were added singly or in combinations of two to the incubated material, or when the lowest concentrations of both KI and  $H_2O_2$  were combined with MPO. Of the 12 trials (within three large experiments) wherein all three reactants were combined, effective candidacidal activity resulted in 10. When sulfonamides were added to combinations of reactants that induced a greater than 99% fall in the colony count, they inhibited candidacidal activity in only two of five trials. In contrast, when added to combinations of reactants that decreased the number of viable colonies by 40 to 99%, sulfonamides inhibited the candidacidal activity in five of five trials. Three

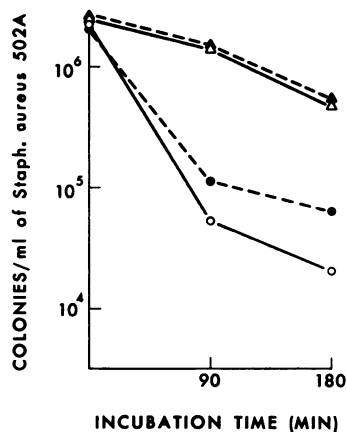


FIGURE 3 Effect of sulfadiazine, 100 mg/100 ml, on the ability of normal and MPO-deficient leukocytes to kill *Staph. aureus* 502A. Colony counts are plotted on a logarithmic scale. Rate of ingestion, as determined by differential centrifugation, was unaltered by sulfadiazine.  $\Delta$  and  $\blacktriangle$ , MPO-deficient leukocytes from patient C. J. B.;  $\circ$  and  $\bullet$ , simultaneously tested normal leukocytes; solid circles and triangles indicate the presence of sulfadiazine.

such trials, from two separate experiments, are shown in Table IV.

**Peroxidase activity.** Even in extremely high concentration (500 mg/100 ml), sulfacetimide did not inhibit the rate of oxidation of orthodianisidine by purified human MPO, leukocyte lysates, or horseradish peroxidase. In contrast, low concentrations of all sulfonamides tested inhibited the rate of accumulation of molecular iodine in a system containing leukocyte lysate (or purified MPO), H<sub>2</sub>O<sub>2</sub>, and KI (Table V).

**Quantitative iodination.** Sulfadiazine inhibited the iodination of heat-killed *C. albicans* after their ingestion by normal leukocytes (Table VI). The magnitude of inhibition was dependent on the concentration of sulfonamide and broadly paralleled the inhibition of candidacidal activity as measured by the dye-exclusion assay (Table I). Almost identical levels of iodination were observed when zymosan (0.5 mg) replaced the heat-killed *C. albicans* in these tests, and an equivalent reduction of iodination attended the inclusion of sulfadiazine (100 mg/100 ml) under these conditions as well. Iodination and its inhibition by sulfonamides were also observed when *C. albicans* or zymosan was ingested by leukocytes in Krebs-Ringer phosphate buffer, but both processes occurred with somewhat reduced efficiency relative to their levels

TABLE IV  
Effect of Sulfonamides on a Myeloperoxidase-Iodide-Hydrogen Peroxide Candidacidal System

MPO	KI	H <sub>2</sub> O <sub>2</sub>	Sulfonamide	<i>C. albicans</i> colonies/ml
Experiment I				
0*	0	0	0	1.9 × 10 <sup>6</sup>
+	0	0	0	1.8 × 10 <sup>6</sup>
0	2.5 × 10 <sup>-4</sup> M	0	0	2.0 × 10 <sup>6</sup>
0	0	5 × 10 <sup>-4</sup> M	0	1.7 × 10 <sup>6</sup>
0	2.5 × 10 <sup>-4</sup> M	5 × 10 <sup>-4</sup> M	0	1.7 × 10 <sup>6</sup>
+	2.5 × 10 <sup>-4</sup> M	0	0	1.9 × 10 <sup>6</sup>
+	0	5 × 10 <sup>-4</sup> M	0	1.7 × 10 <sup>6</sup>
+	1 × 10 <sup>-4</sup> M	5 × 10 <sup>-4</sup> M	0	2.2 × 10 <sup>6</sup>
+	1 × 10 <sup>-4</sup> M	5 × 10 <sup>-4</sup> M	Sulfacetimide‡	1.4 × 10 <sup>6</sup>
+	1 × 10 <sup>-4</sup> M	5 × 10 <sup>-4</sup> M	Sulfadiazine§	1.4 × 10 <sup>6</sup>
Experiment II				
0	0	0	0	2.6 × 10 <sup>6</sup>
+	0	0	0	2.8 × 10 <sup>6</sup>
0	1 × 10 <sup>-4</sup> M	0	0	2.6 × 10 <sup>6</sup>
0	0	2.5 × 10 <sup>-4</sup> M	0	2.5 × 10 <sup>6</sup>
0	1 × 10 <sup>-4</sup> M	2.5 × 10 <sup>-4</sup> M	0	2.5 × 10 <sup>6</sup>
+	1 × 10 <sup>-4</sup> M	0	0	2.8 × 10 <sup>6</sup>
+	0	2.5 × 10 <sup>-4</sup> M	0	2.3 × 10 <sup>6</sup>
+	5 × 10 <sup>-5</sup> M	2.5 × 10 <sup>-4</sup> M	0	7.7 × 10 <sup>5</sup>
+	5 × 10 <sup>-5</sup> M	2.5 × 10 <sup>-4</sup> M	Sulfacetimide‡	2.3 × 10 <sup>6</sup>
+	5 × 10 <sup>-5</sup> M	2.5 × 10 <sup>-4</sup> M	Sulfadiazine§	1.9 × 10 <sup>6</sup>
+	5 × 10 <sup>-5</sup> M	1 × 10 <sup>-4</sup> M	0	2.6 × 10 <sup>6</sup>
+	5 × 10 <sup>-5</sup> M	1 × 10 <sup>-4</sup> M	Sulfacetimide‡	2.3 × 10 <sup>6</sup>
+	5 × 10 <sup>-5</sup> M	1 × 10 <sup>-4</sup> M	Sulfadiazine§	1.5 × 10 <sup>6</sup>

\* +, the presence of a reactant; 0, its absence.

‡ 20 mg/100 ml (1 × 10<sup>-3</sup> M).

§ 10 mg/100 ml (3.7 × 10<sup>-4</sup> M).

TABLE V  
Inhibition by Sulfonamides of Peroxidase-Catalyzed Iodine Accumulation from H<sub>2</sub>O<sub>2</sub> and KI

Sulfisoxazole		Sulfathiazole		Sodium sulfadiazine		Sodium sulfacetimide	
nmoles*	sec‡	nmoles	sec	nmoles	sec	nmoles	sec
0	5.2	0	5.2	0	5.2	0	5.2
12.4	7.6	13	7.9	30.5	6.3	41	6.5
31	10.5	32.5	14.1	61	9.8	82	9.1
62	19.9	65	21.7	122§	13.8	163§	16.1
124§	37.5	130§	60.8	183	25.2	NT	NT

\* nmoles of the indicated sulfonamides in 1 ml of reaction mixture containing 25 nmoles of sodium thiosulfate, 10 μl of leukocyte lysate and other components as described in the text. pH was 5.0.

‡ Mean time elapsed to development of blue color.

§ Sulfonamide concentration, 3.3 mg/100 ml.

in HBSS. Sodium azide (2 mM) was a more effective inhibitor of iodination than the highest concentration of sulfonamide tested in these studies. Additional studies of these reactions are in progress.

## DISCUSSION

After ingestion by mammalian leukocytes, bacteria and fungi are exposed to conditions within the phagocytic vacuole that can result in the death of many of these microorganisms. One of the microbicidal systems of neutrophils and monocytes involves the granule enzyme, myeloperoxidase, and its oxidant substrate, hydrogen peroxide (1, 12). Klebanoff has recently advanced evidence suggesting that this may be the major microbicidal effector of the human neutrophil (11).

The evidence reported here indicates that sulfonamides

TABLE VI  
Inhibition of Iodination of Heat-Killed *C. albicans* by Sulfonamides

Inhibitor	Concentration	Iodination % of control*
None	—	100‡
Sulfadiazine	5 mg/100 ml	78.9 ± 5.3
Sulfadiazine	10 mg/100 ml	61.0 ± 4.4
Sulfadiazine	25 mg/100 ml	41.1 ± 3.1
Sulfadiazine	50 mg/100 ml	23.8 ± 1.8
Sulfadiazine	100 mg/100 ml	11.2 ± 0.9
Sodium azide	2 mM	2.2 ± 0.3

\* This figure was derived by dividing the mean number of acid-precipitable counts measured in the presence of the indicated inhibitor by the mean number of acid-precipitable counts measured in paired control tubes that lacked added inhibitors (mean ± SEM, n = 4).

‡ Normal leukocytes fixed 4.43 ± 0.38 nmoles of iodide per 1 × 10<sup>7</sup> leukocytes into an acid-precipitable form under the conditions of these experiments (mean ± SEM, n = 5).

can decrease the ability of normal human neutrophils to kill *C. albicans*, *C. tropicalis*, and, to a lesser extent, certain bacteria. Several features of these data suggest that the inhibitory effect arises from a relatively specific interference with the peroxidase-mediated microbicidal pathways of the neutrophil. Sulfonamides were found to inhibit the iodination of ingested *C. albicans* by intact normal leukocytes, a reaction shown by Klebanoff to be catalyzed by MPO (9, 12). The drugs inhibited the candidacidal activity of an in vitro system containing MPO, H<sub>2</sub>O<sub>2</sub>, and iodide, and also interfered with accumulation of iodine in this system. In contrast, sulfonamides exerted little effect on the microbicidal activity of MPO-deficient human neutrophils.

In vitro, MPO requires H<sub>2</sub>O<sub>2</sub> and an additional cofactor, such as iodide or chloride, to exert maximally effective bactericidal (12, 13) and fungicidal (7) activity. When iodide is employed as the cofactor in cell-free systems, or is added to mixtures containing phagocytic leukocytes and appropriately opsonized microorganisms, the microorganisms are iodinated as well as killed (9, 12). The identity of the halide or halide-like cofactor presumed to operate under physiologic conditions in the intact human neutrophil has not been established with certainty (12, 14).

Two general classes of chemical compounds, peroxidase inhibitors and reducing agents, have been found to inhibit the bactericidal activity of MPO-H<sub>2</sub>O<sub>2</sub>-halide systems. Cyanide and azide ions typify the former class of compounds. Cysteine, reduced glutathione, thiosulfate, and certain compounds with antithyroid activity such as thiourea, thiocyanate, and 1-methyl-2-mercaptoimidazole (Tapazole)<sup>18</sup> are included among the latter (12, 13, 15). Most classes of antithyroid substances are either competitive substrates or inhibitors of various peroxidases (16); it is interesting to recall that sulfonamides also have antithyroid properties and are goitrogenic in rats (17). Possibly, the ability of sulfonamides to inhibit the function both of leukocytes and of thyroid cells shares a common biochemical basis: interference with peroxidase-mediated reactions involving halide ions.

Although sulfonamides have been reported to inhibit the activity of certain plant peroxidases (18-20), activity of MPO per se (orthoanisidine assay) was not inhibited by sulfacetimide or sulfadiazine under our experimental conditions. However, other mechanisms could account for the effects of sulfonamides on intracellular iodination and iodine accumulation. For example, sulfonamides could react with intermediate or final products generated by the MPO-H<sub>2</sub>O<sub>2</sub>-iodide system. The apparent inhibition of peroxidase activity by various thiols in iodometric assays is a well-recognized model of such an occurrence (21). Another possibility is that MPO could

catalyze the oxidation of sulfonamides in the presence of H<sub>2</sub>O<sub>2</sub> and iodide. In their studies on the inhibition of horseradish peroxidase by isonicotinic acid hydrazide, Andrejew, Gernez-Rieux, and Tacquet (22) noted appreciable peroxidase-catalyzed oxidation of that compound. Perhaps further elucidation of the precise mode of action of sulfonamides and other compounds (compounds that, while not peroxidase inhibitors, can inhibit the peroxidase-mediated antimicrobial activity of intact leukocytes) will facilitate the identification of the physiologic cofactor(s) for H<sub>2</sub>O<sub>2</sub> in the MPO-catalyzed antimicrobial activity of the human neutrophil.

As therapeutic doses of sulfonamides regularly produce concentrations that would result in partial inhibition of candidacidal activity by normal neutrophils, it is necessary to consider whether this effect ever attains clinical significance. Many years of clinical use have produced no evidence to suggest that conventional doses of sulfonamides administered to otherwise normal subjects significantly increase their risk of developing systemic *Candida* infection.

However, systemic candidiasis typically affects, not otherwise normal patients, but persons with serious underlying disorders: recipients of renal transplants (23) and patients with acute leukemia or other neoplasms (24, 25). Such high-risk patients often receive treatment with many drugs, including those with immunosuppressive, antiinflammatory, cytotoxic, and antibiotic actions. These patients undoubtedly have sustained multiple breaches in their antifungal defense mechanisms and are exposed to additional factors that may increase the risk of opportunistic infection. What is the effect of sulfonamide therapy on resistance to *Candida* infection in this group? This question cannot be answered either from available clinical information or by extrapolation from experience with relatively normal patients. It may be that patients with other impaired host defenses are more dependent on intact function of their phagocytic leukocytes to maintain adequate over-all resistance to *Candida* infection (25, 26). If this were so, sulfonamides (or other drugs with comparable actions on leukocyte function) might have substantially greater effects on the over-all resistance of this group of patients than could be surmised from their effects on otherwise normal patients.

That sulfonamides impair the resistance of certain groups of patients to systemic candidiasis remains to be proved. Until the question is resolved by appropriate clinical studies, sulfonamides should not be prescribed unnecessarily to patients at high risk of developing this infection by virtue of other underlying conditions. If their use is required in such patients, appropriate monitoring can prevent the development of excessive serum concentrations and the attendant inimical effects on leukocyte function.

<sup>18</sup> Eli Lilly and Company, Indianapolis, Ind.

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## REFERENCES

1. Lehrer, R. I., and M. J. Cline. 1969. Leukocyte myeloperoxidase deficiency and disseminated candidiasis: the role of myeloperoxidase in resistance to *Candida* infection. *J. Clin. Invest.* **48**: 1478.
2. Lehrer, R. I., and M. J. Cline. 1969. Interaction of *Candida albicans* with human leukocytes and serum. *J. Bacteriol.* **98**: 996.
3. Lehrer, R. I. 1970. Measurement of candidacidal activity of specific leukocyte types in mixed cell populations. I. Normal, myeloperoxidase-deficient, and chronic granulomatous disease neutrophils. *Infect. Immunity.* **2**: 42.
4. Lehrer, R. I., J. Hanifin, and M. J. Cline. 1969. Defective bactericidal activity in myeloperoxidase-deficient human neutrophils. *Nature (London).* **223**: 78.
5. Schultz, J., and H. W. Shmukler. 1964. Myeloperoxidase of the leucocyte of normal human blood. II. Isolation, spectrophotometry, and amino acid analysis. *Biochemistry.* **3**: 1234.
6. 1967. Peroxidase [horseradish]. donor: H<sub>2</sub>O<sub>2</sub> oxidoreductase. I.U.B. 1.11.1.7. *In* Worthington Manual of Enzymes and Enzymes Reagents. Worthington Biochemical Corporation, Freehold, N. J.
7. Lehrer, R. I. 1969. Antifungal effects of peroxidase systems. *J. Bacteriol.* **99**: 361.
8. Davis, W. B. 1942. Quantitative field test for estimation of peroxidase. *Ind. Eng. Chem., Anal. Ed.* **14**: 952.
9. Pincus, S. H., and S. J. Klebanoff. 1971. Quantitative leukocyte iodination. *N. Engl. J. Med.* **284**: 744.
10. Cohen, P. P. 1957. Suspending media for animal tissues. *In* Manometric Techniques. W. W. Umbreit, R. H. Burris, and J. F. Stauffer, editors. Burgess Publishing Co., Minneapolis, Minn. 3rd edition. 149.
11. Klebanoff, S. J. 1970. Myeloperoxidase: contribution to the microbicidal activity of intact leukocytes. *Science (Washington).* **169**: 1095.
12. Klebanoff, S. J. 1967. Iodination of bacteria: a bactericidal mechanism. *J. Exp. Med.* **126**: 1063.
13. Klebanoff, S. J. 1968. Myeloperoxidase-halide-hydrogen peroxide antibacterial system. *J. Bacteriol.* **95**: 2131.
14. Paul, B. B., A. A. Jacobs, R. R. Strauss, and A. J. Sbarra. 1970. Role of the phagocyte in host-parasite interactions. XXIV. Aldehyde generation by the myeloperoxidase-H<sub>2</sub>O<sub>2</sub>-chloride antimicrobial system: a possible in vivo mechanism of action. *Infect. Immunity.* **2**: 414.
15. Klebanoff, S. J. 1970. Myeloperoxidase-mediated antimicrobial systems and their role in leukocyte function. *In* Biochemistry of the Phagocytic Process: Localization and the Role of Myeloperoxidase and the Mechanism of the Halogenation Reaction. J. Schultz, editor. North-Holland Publishing Co., Amsterdam. 89.
16. Rosenberg, I. N. 1952. The antithyroid activity of some compounds that inhibit peroxidase. *Science (Washington).* **116**: 503.
17. MacKenzie, C. G., and J. B. MacKenzie. 1943. Effect of sulfonamides and thioureas on the thyroid gland and basal metabolism. *Endocrinology.* **32**: 185.
18. Carrara, G., and F. M. Chiancone. 1941. Sul meccanismo d'azione dei solfamidici. Nota III: primi dati sull'azione sulle redossasi. *Chim. Ind. (Milan).* **23**: 435.
19. Lipmann, F. 1941. The oxidation of *p*-aminobenzoic acid catalyzed by peroxidase, and its inhibition by sulfanilamide. *J. Biol. Chem.* **139**: 977.
20. Santarato, R. 1949. Azioni su alcuni enzimi delle sulfonamido-associazioni. *Boll. Ist. Sieroter. Milan.* **28**: 317.
21. Saunders, B. C., A. G. Holmes-Siedle, and B. P. Stark. 1964. Peroxidase: The Properties and Uses of a Versatile Enzyme and of Some Related Catalysts. Butterworth & Co. (Publishers) Ltd., London. 134.
22. Andrejew, A., Ch. Gernez-Rieux, and A. Tacquet. 1959. Inhibition de la peroxydase par l'hydrazide de l'acide isonicotinique (INH) et destruction de l'INH par la peroxydase. *Bull. Soc. Chim. Biol.* **41**: 1047.
23. Rifkind, D., T. L. Marchioro, S. A. Schneck, and R. B. Hill, Jr. 1967. Systemic fungal infections complicating renal transplantation and immunosuppressive therapy: clinical, microbiologic, neurologic and pathologic features. *Amer. J. Med.* **43**: 28.
24. Bodey, G. P. 1966. Fungal infections complicating acute leukemia. *J. Chronic Dis.* **19**: 667.
25. Lehrer, R. I., and M. J. Cline. 1971. Leukocyte candidacidal activity and resistance to systemic candidiasis in patients with cancer. *Cancer.* **27**: 1211.
26. Lehrer, R. I. 1970. Defective candidacidal activity of leukocytes from patients with systemic candidiasis. *Clin. Res.* **18**: 443.