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Effects of Sulfamethoxazole and Trimethoprim on Human Neutrophil and Lymphocyte Functions In Vitro: In Vivo Effects of Co-Trimoxazole

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The effects of sulfamethoxazole and trimethoprim individually and in combination on in vitro neutrophil random migration, chemotaxis to autologous endotoxin-activated serum and the synthetic chemotactic tripeptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine, phagocytosis and postphagocytic Nitro Blue Tetrazolium reduction, glycolysis, hexose monophosphate shunt activity, myeloperoxidase-mediated protein iodination, hydrogen peroxide production, and degranulation were assessed. The effects on lymphocyte mitogen-induced transformation were also evaluated. It was found that the test agents individually and in combination at high concentrations (>100 μg/ml) caused the inhibition of neutrophil postphagocytic myeloperoxidase-mediated protein iodination, which was related to the interference with H_2O_2 formation as the enzyme per se was unaffected. Both agents caused the inhibition of lymphocyte transformation at high concentrations (>100 μg/ml). In vivo studies before and after the ingestion of co-trimoxazole by three individuals showed no inhibition of any of the neutrophil functions tested. The inhibition of lymphocyte transformation was observed in one individual after the ingestion of the chemotherapeutic agent. These findings indicate that the concentrations which inhibit neutrophil H_2O_2 production and lymphocyte transformation in vitro are not attainable in vivo.

Combinations of sulfonamides and trimethoprim (TMP) are now widely used in treating various infections. Conventional therapeutic concentrations are without effect on human folate metabolism (4). On the other hand, combinations of the sulfonamides, sulfadiazine, and sulfamethoxazole (SMX) with TMP may have immunosuppressive effects. Ghilchick et al. (6) have reported that TMP per se had an immunosuppressive effect and a prolonged allograft survival in mice. The inhibition of antibody synthesis in animals after the ingestion of co-trimoxazole, the combined SMX-TMP chemotherapeutic agent, has been reported (2, 3). The depressed uptake of tritiated thymidine by mitogen-stimulated lymphocytes after the ingestion of co-trimoxazole by humans has been reported (5). However, Kobayashi et al. (7) failed to detect any subsequent impairment of leukocyte function in seven children with chronic granulomatous disease receiving prophylactic SMX-TMP (Bactrim; Hoffmann-LaRoche, Inc., Nutley, N.J.).

The present study was undertaken to investigate extensively the effects of SMX and TMP individually and in combination (SMX-TMP) on neutrophil motility, phagocytosis, postphagocytic metabolic activity, and lymphocyte mitogen-induced transformation in vitro. In a further series of experiments, neutrophil and lymphocyte functions were evaluated before and after the ingestion of co-trimoxazole by three normal adult volunteers.

MATERIALS AND METHODS

Chemotherapeutic agents. The individual drugs were tested at a final concentration range of 6.25 to 200 μg/ml. The proportions in the SMX-TMP combination were those used in certain commercial preparations: the ratio of SMX to TMP was 5:1. The concentration ranges were 6.25 to 200 μg of SMX per ml and 1.05 to 40 μg of TMP per ml. The drugs were dissolved in the appropriate suspending medium according to the cell function being assessed.

Neutrophil functions: studies of motility. Neutrophils were obtained from heparinized venous blood (5 U of heparin per ml) and were suspended to a final concentration of 10^7/ml in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Sigma Chemical Co., St. Louis, Mo.)-buffered Hanks balanced salt solution (HBSS; GIBCO Laboratories, Grand Island, N.Y.) as previously described (1).

The following two leukoattractants were used: (i) fresh autologous serum activated with 100 μg of bacterial endotoxin (*Escherichia coli* O127:B8; Difco Laboratories, Detroit, Mich.) per ml, which was diluted

eightfold with HBSS before use; and (ii) the synthetic chemotactic tripeptide, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (f-met-leu-phe; Miles Laboratories, Inc., Elkhart, Ind.), which was used at a final concentration of 5×10^{-10} M (previously found to be the optimal leukotactic concentration) in HBSS supplemented with 0.1% bovine serum albumin.

In random migration systems the leukoattractant was replaced with an equal volume of bovine serum albumin supplemented with HBSS (0.8 ml). The cell suspensions were preincubated with the various drug concentrations for 15 min. The drugs remained in the cell compartment throughout the incubation period. The final cell concentration was 5×10^6/ml for studies with endotoxin-activated serum (EAS) and in random migration studies and 3×10^6/ml when f-met-leu-phe was used. The assays of motility were performed in modified Boyden chambers (12) with 5-μm-pore size membrane filters (Millipore Corp., Bedford, Mass.) and a 3-h incubation period. The results are expressed as the number of cells per microscope high-power field as an average of triplicate filters.

Phagocytic studies. Pure neutrophil suspensions prepared as previously described (1) were used for these studies. Phosphate-buffered saline (0.15 M; pH 7.2) was the cell-suspending medium. The phagocytosis of *Candida albicans* was performed as previously described (1) with a polymorphonuclear leukocyte (PMN)/*C. albicans* ratio of 1:3. Drugs were added in 0.1-ml volumes to give a final reaction volume of 1 ml. The results are expressed as the percentage of *C. albicans* ingested per 25 min. The Nitro Blue Tetrazolium reduction was measured by the semiquantitative method of Sher et al. (11) with high-molecular-weight dextran as the stimulant. The results are expressed as the percentage of PMNs containing reduced Nitro Blue Tetrazolium.

(i) HMS activity. The hexose monophosphate shunt (HMS) activity was measured by the method of Wood et al. (13) with modifications (1) by the potassium hydroxide absorption of $^{14}CO_2$ derived from glucose radiolabeled in the C-1 position (D-[1-^{14}C]glucose; New England Nuclear Corp., Boston, Mass.). The reaction mixture contained 2×10^6 PMNs (0.1 ml), autologous serum (0.1 ml), 10^7 *C. albicans* (0.1 ml), drug (0.1 ml), and radiolabeled glucose (0.6 ml) containing 0.06 μCi of [1-^{14}C]glucose. The results are expressed as the corrected mean counts per minute.

(ii) Measurement of H_2O_2 release. In these experiments the effects of the drugs on the release of H_2O_2 into the extracellular medium after the ingestion of opsonized *C. albicans* were determined. The opsonization was performed at 4°C with 1 ml of fresh pooled human serum per 10^8 organisms per 2 h. The *C. albicans* organisms were washed twice and resuspended to a concentration of 5×10^9/ml. Each experimental tube contained 10^7 PMNs (0.2 ml), *C. albicans* (0.1 ml), 10^{-2} M sodium azide (0.1 ml), an appropriate drug concentration (0.1 ml), and phosphate-buffered saline (0.4 ml) containing 10 mM glucose. After a 30-min incubation period, the tubes were centrifuged at $5,000 \times g$ for 10 min, and the supernatant fluid was assayed for H_2O_2 by the method of Root et al. (9) by the reduction of scopoletin fluorescence. Each assay tube contained distilled water (2.5 ml), horseradish peroxidase (50 μl; Sigma) at a stock concentration of 13.8 U/ml, 200 μM scopoletin (20 μl; Sigma), and cell-free supernatant (50 μl). Standard curves were constructed in the range of 0.1 to 10 nmol of H_2O_2. The H_2O_2-dependent reduction in fluorescence was monitored on a Perkin-Elmer model 204 Hitachi fluorescence spectrophotometer at an exciter wavelength of 390 nm and an analyzer wavelength of 460 nm. The results are expressed as nmol per 10^7 PMN per min.

(iii) Quantitative MPO assay. Myeloperoxidase (MPO) was assayed by the increase in fluorescence at 470 nm which accompanied the oxidation of guaiacol. In these experiments 5×10^7 PMNs were incubated with the various drug concentrations for 1 h at 37°C in the presence of autologous serum. The cell suspension was then sonicated in an MSE ultrasonic disintegrator (three 20-s bursts) at an amplitude of 10 μm peak to peak, and the sonicate, after centrifugation at $5,000 \times g$, was assayed for MPO activity by the method of Paul et al. (8) with minor modifications. The assay system contained glycine–NaOH buffer (pH 10) (1 ml), 1.5×10^{-1} M guaiacol (1 ml), 5×10^{-2} M H_2O_2 (1 ml), and sonicate (0.1 ml). The rate of oxidation was measured spectrophotometrically as the increase in optical density at 470 nm. The results are expressed as enzyme units per 10^8 PMN calculated from a standard curve in the range of 0.83 to 275 U of horseradish peroxidase per ml.

(iv) MPO-mediated iodination of ingested protein. The MPO-mediated iodination of ingested protein was determined by the method of Root and Stossel (10) with minor modifications (1). To 0.1 ml of PMN suspension (10^7/ml) were added 0.1 ml of *C. albicans* (10^8/ml), 0.1 ml of fresh autologous serum, 0.1 ml of the various drug concentrations, 0.1 ml of an ^{125}I solution (0.6 μCi/ml) (sodium iodate; New England Nuclear), and 0.5 ml of phosphate-buffered saline. The incubation was for 60 min at 37°C, after which the amount of incorporation of ^{125}I into acid-precipitable protein was determined by solid scintillation counting. The results are expressed as counts per minute.

(v) Lysozyme release. Lysozyme release was used to assess the effects of the chemotherapeutic agents on postphagocytic degranulation. Experimental tubes contained 5×10^6 PMNs (0.5 ml), autologous serum (0.1 ml), an appropriate drug concentration (0.1 ml), and *C. albicans* (5×10^8/ml) (0.1 ml). The tubes were incubated for 60 min at 37°C, after which PMNs and *C. albicans* were removed by centrifugation. The supernatants were assayed for lysozyme by the lysis of a suspension of *Micrococcus lysodeikticus* by a reduction in turbidity at 450 nm. Each assay tube contained phosphate-buffered saline (2 ml), *M. lysodeikticus* (0.1 ml), and phagocytic supernatant (0.5 ml). The starting optical density of the bacterial suspension was 0.15. A standard curve was constructed in the lysozyme concentration range of 1.25 to 400 U/ml. The results are expressed as enzyme units per 5×10^6 PMNs.

Lymphocyte studies. Lymphocyte transformation to the mitogens phytohemagglutinin (PHA) and concanavalin A (ConA) at concentrations of 25 and 50 μg/ml, respectively, was also determined. Mononuclear cells were obtained by density gradient centrifugation and suspended after washing, to a concentration of 4×10^6/ml, in TC 199 (GIBCO). Samples (50 μl) of 2 ×

10^5 cells were added to the wells of 6-nm Linbro tissue culture plates (Flow Laboratories, Inc., Rockville, Md.). The final volume in each well was brought to 0.2 ml by the addition of 110 μl of serum (10%)-supplemented TC 199, 20 μl of the various drugs, and 20 μl of each mitogen. The plates were incubated for 48 h at 37°C in a humidified atmosphere of air and 3% CO_2, after which 0.2 μCi of tritiated thymidine (New England Nuclear) in 20 μl was added to each well, and the plates were incubated for a further 18 h. The plates were harvested with a multiple automated sample harvester, and the extent of incorporation of tritiated thymidine into newly synthesized deoxyribonucleic acid was assessed in a liquid scintillation spectrophotometer.

In vivo studies. Neutrophil chemotaxis to EAS, postphagocytic HMS activity, MPO-mediated protein iodination, and lymphocyte mitogen-induced transformation was assessed before and after the ingestion of co-trimoxazole in three adult volunteers. Each individual ingested four 480-mg tablets (two, twice daily) for 5 days. Repeat testing was performed on day 5.

RESULTS

Calculation and expression of results. Unless otherwise stated, the results are expressed as the mean value with standard error of five separate experiments. Statistical evaluations were performed by the Student *t* test. The significant effects of SMX and TMP were only observed at concentrations of 50 μg/ml. Therefore, only the results obtained with 100 μg of each agent per ml and with 100 μg of SMX per ml plus 20 μg of TMP per ml are shown as a concentration of 100 μg of SMX per ml approaches the maximum safe level in therapeutics.

Neutrophil motility. SMX and TMP, individually and in combination at all concentrations tested, had no effect on neutrophil random migration and chemotaxis to the leukoattractants EAS and f-met-leu-phe. The results for the individual agents at 100 μg per ml and for the combination at 100 μg/ml + 20 μg/ml are shown in Table 1.

Phagocytosis and postphagocytic metabolic activity. SMX and TMP, individually and in combination, were without effect on the phagocytosis of *C. albicans* (data not shown). The Nitro Blue Tetrazolium reduction was depressed by TMP but not by SMX at high concentrations (>100 μg/ml). The results for 100 μg/ml are shown in Table 1. The HMS activity was significantly reduced by high concentrations of TMP but not of SMX (Table 1).

MPO-mediated protein iodination. The MPO-mediated protein iodination was significantly depressed by SMX and TMP individually and in combination at high concentrations. (Table 1 shows 100-μg/ml values.) For determin-

TABLE 1. *Effects of SMX and TMP individually and in combination on human neutrophil and lymphocyte functions in vitro*[a]

Drug (concn)	Function examined (units of measurement)									
	Neutrophils						Lymphocyte transformation (cpm) to:			
	Random migration (cells/HPF[b])	Chemotaxis to: EAS (cells/HPF)	Chemotaxis to: f-met-leu-phe (cells/HPF)	NBT[c] reduction (% positive PMN)	HMS activity (cpm)	MPO-mediated iodination (cpm)	H_2O_2 production (nmol/10^7 PMN)	PHA (25 μg/ml)	PHA (50 μg/ml)	ConA (25 μg/ml)	ConA (50 μg/ml)
Control	36 ± 6.2	251 ± 29	193 ± 21	96 ± 2.1	21,916 ± 1,916	35,059 ± 1,420	5.6 ± 0.52	53,391 ± 7,334	43,995 ± 5,020	20,727 ± 3,035	15,554 ± 1,903
SMX (100 μg/ml)	35 ± 6.1	257 ± 31	208 ± 27	96 ± 1.9	24,765 ± 1,164	21,631 ± 2,901	3.9 ± 0.95	46,984 ± 8,086	32,996 ± 4,001	16,301 ± 3,009	12,243 ± 1,729
TMP (100 μg/ml)	37 ± 7.1	281 ± 31	186 ± 19	83 ± 3.2	16,437 ± 2,302	7,712 ± 990	0.6 ± 0.06	43,780 ± 7,504	36,912 ± 6,392	26,512 ± 4,106	16,665 ± 2,012
SMX-TMP (100 μg/ml) + (20 μg/ml)	37 ± 4.8	263 ± 29	216 ± 21	88 ± 2.6	22,232 ± 1,967	19,633 ± 1,101	4.5 ± 0.62	48,156 ± 8,957	44,456 ± 10,256	23,727 ± 3,057	15,610 ± 2,160

[a] Results are given as the mean and standard error of five separate experiments.
[b] HPF, High-power field.
[c] NBT, Nitro Blue Tetrazolium.

ing if the inhibition of MPO-mediated iodination was due to a direct inhibition of MPO by SMX and TMP, the effects of both agents on the MPO oxidation of guaiacol, the degranulation by lysozyme release, and H_2O_2 production were assessed.

MPO activity. MPO activity was assessed in the presence of added H_2O_2 by the spectrophotometric analysis of the oxidation of guaiacol. No inhibition of MPO activity was detectable in the presence of SMX and TMP individually and in combination at any concentration (data not shown). The control value for guaiacol oxidation was 22.3 ± 4.2 U/108 PMN. The results obtained in the presence of the various drugs were not significantly different from this value.

Lysozyme release. The mean value for postphagocytic lysozyme release was 68 ± 5.4 lysozyme units per 5×10^6 PMNs. The results (data not shown) in the presence of SMX and TMP were not significantly different from those for the control value, thus showing that these agents probably do not interfere with the release of granule-associated MPO.

H_2O_2 production. High concentrations of SMX and TMP individually and in combination caused the inhibition of postphagocytic H_2O_2 production (Table 1). This finding indicates that the SMX-TMP-induced inhibition of postphagocytic MPO-mediated iodination of ingested protein is due to the lack of availability of H_2O_2 for the peroxidase system.

Lymphocyte transformation. SMX and TMP, individually and in combination at high concentrations, caused a dose-dependent inhibition of transformation to both mitogens (Table 1 shows 100-μg/ml values). The most significant inhibition was achieved with the higher mitogen concentrations.

In vivo studies. No inhibition of neutrophil chemotaxis to EAS or postphagocytic HMS activity or MPO-mediated protein iodination was observed after the ingestion of therapeutic doses of co-trimoxazole for a 5-day period (Table 2). Likewise, no inhibition of mitogen-induced transformation was found in two out of three individuals after the ingestion of co-trimoxazole. However, a third individual developed reduced responsiveness to PHA and ConA which was associated with an allergic reaction to SMX (Table 2). The inhibition was approximately 50% of the preco-trimoxazole response.

TABLE 2. *Assessment of neutrophil and lymphocyte functions in three individuals before and after the ingestion of co-trimoxazole*[a]

Drug	Cell function						
	Neutrophils			Lymphocyte transformation (cpm) to:			
	Chemotaxis to autologous EAS (cells/HPF[b])	HMS activity (cpm)	MPO-mediated protein iodination (cpm)	PHA (25 μg/ml)	PHA (50 μg/ml)	ConA (25 μg/ml)	ConA (50 μg/ml)
Pre-co-trimoxazole	261 ± 27	21,798 ± 2,078	33,113 ± 1,334	49,155 ± 5,560	53,095 ± 6,510	31,215 ± 5,009	38,160 ± 6,402
Post-co-trimoxazole	270 ± 22	19,694 ± 1,250	35,278 ± 2,275	40,745 ± 3,560	46,200 ± 4,540	25,890 ± 3,196	30,470 ± 3,234

[a] Results are given as the mean and standard error.
[b] HPF, High-power field.

DISCUSSION

This study has demonstrated that neutrophil phagocytosis, random migration, and chemotaxis are not affected by SMX and TMP individually or in combination. This was found in both

in vitro and in vivo experiments. However, postphagocytic MPO-mediated iodination and H_2O_2 production were impaired at high concentrations of both agents (>100 μg/ml). The precise mechanism of the inhibition is not yet clear. The decreased MPO-mediated iodination is probably secondary to the reduced H_2O_2 production as high concentrations of SMX and TMP had no effect on MPO per se or on degranulation measured by lysozyme release and by electron microscope studies. SMX and TMP may cause the inhibition of HMS activity or alternatively they may compete for H_2O_2. The inhibition of postphagocytic metabolic activity mediated by SMX and TMP is an in vitro phenomenon caused by supraphysiological concentrations of these agents as no alteration of HMS activity or MPO-mediated iodination was observed after the ingestion of co-trimoxazole by three normal individuals. Folic acid at the same concentrations as TMP had similar inhibitory effects on PMN postphagocytic metabolic activity in vitro (unpublished observations).

Lymphocyte mitogen-induced transformation was also decreased at TMP and SMX concentrations of >100 μg/ml. Low concentrations of both agents (6.25 and 12.5 μg/ml) caused a slight, but insignificant, stimulation of mitogen responsiveness. No consistent inhibition of lymphocyte transformation to both mitogens was observed in vivo; one individual showed impaired mitogen responsiveness, but he also developed allergic manifestations and showed a positive immediate response on skin testing with SMX (2 μg/ml). Furthermore, in three children with chronic granulomatous disease receiving prophylactic co-trimoxazole we observed no inhibition of lymphocyte function in vitro.

We have also investigated the effects of sulfadiazine and TMP individually and in combination on the same cellular functions in vitro, with similar findings (results not shown).

Although further research is required on the relationship between the development of sulfonamide allergy and depressed lymphocyte transformation as observed in one individual in this study, no consistent inhibition of cellular immune function was detected after the ingestion of co-trimoxazole. The lack of interference with immune functions is probably an important property of a chemotherapeutic agent or an antibiotic, especially that which is used prophylactically.

LITERATURE CITED

1. **Anderson, R., and A. J. van Rensburg.** 1979. The *in vitro* effects of propranolol and atenolol on neutrophil motility and post-phagocytic metabolic activity. Immunology **37**:15–24.
2. **Arvilommi, H., M. Vuori, and A. Salmi.** 1972. Immunosuppression by co-trimoxazole. Br. Med. J. **3**:761–762.
3. **Arvilommi, H., M. Vuori, and A. Salmi.** 1976. Sulphamethoxazole-trimethoprim: effect on antibody response in man. Chemotherapy (Basel) **22**:37–42.
4. **Bushby, S. R. M., and G. H. Hitchings.** 1968. Trimethoprim, a sulphonamide potentiator. Br. J. Pharmacol. Chemother. **33**:72–90.
5. **Gaylarde, P. M., and I. Sarkany.** 1972. Suppression of thymidine uptake of human lymphocytes by co-trimoxazole. Br. Med. J. **3**:144–146.
6. **Ghilchick, M. W., A. S. Morris, and D. S. Reeves.** 1970. Immunosuppressive powers of the antibacterial agent trimethoprim. Nature (London) **227**:393–394.
7. **Kobayashi, Y., D. Amano, K. Ueda, Y. Kogasaki, and T. Usui.** 1978. Treatment of seven cases of chronic granulomatous disease with sulphamethoxazole-trimethoprim. Eur. J. Pediatr. **127**:247–254.
8. **Paul, B. B., R. J. Selvaraj, and A. J. Sbarra.** 1978. A sensitive assay method for peroxidases from various sources. J. Reticuloendothel. Soc. **25**:407–410.
9. **Root, R. K., J. Metcalf, H. Oshino, and B. Chance.** 1975. H_2O_2 release from human granulocytes during phagocytosis. I. Documentation, quantitation and some regulating factors. J. Clin. Invest. **55**:945–955.
10. **Root, R. K., and T. P. Stossel.** 1974. Myeloperoxidase mediated iodination by granulocytes: intracellular site of operation and some regulating factors. J. Clin. Invest. **53**:1207–1215.
11. **Sher, R., R. Anderson, A. R. Rabson, and H. J. Koornhof.** 1974. Standardization of the nitro blue tetrazolium test and factors affecting its clinical application. S. Afr. Med. J. **58**:209–213.
12. **Wilkinson, P. C.** 1971. Chemotaxis of phagocytic cells towards proteins. The effect of denaturation, p. 59. *In* N. R. Di Luzio and K. Flemming (ed.), The reticuloendothelial system and immune phenomena. Plenum Publishing Corp., New York.
13. **Wood, H. G., J. Katz, and B. R. Landau.** 1963. Estimation of pathways of carbohydrate metabolism. Biochem. Z. **338**:809–817.