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Penetration of Brodimoprim into Human Neutrophils and Intracellular Activity

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The entry of an antibiotic into phagocytes is a prerequisite for its intracellular bioactivity against susceptible facultative or obligatory intracellular microorganisms. Brodimoprim is a dimethoxybenzylpyrimidine that has recently entered into clinical use, and its uptake into and elimination from human polymorphonuclear neutrophils (PMNs), together with its effects on normal phagocytic and antimicrobial mechanisms, have been investigated. Brodimoprim uptake by PMNs was determined by a velocity-gradient centrifugation technique under various experimental conditions and was expressed as the ratio of the intracellular to the extracellular drug concentration (C/E) in comparison with the C/E of trimethoprim, which was used as a control drug. After incubation with 7.5 μg of brodimoprim per ml, PMNs accumulated brodimoprim (C/E, 74.43 ± 12.35 at 30 min) more avidly than trimethoprim (C/E, 20.97 ± 6.61 at 30 min). The cellular uptake of brodimoprim was not affected by temperature, 2,4-dinitrophenol, or potassium fluoride and was increased with an increase in the pH of the medium. It was reduced in formaldehyde-killed PMNs. The efflux of brodimoprim was very rapid (46% after 5 min). The liposolubility of brodimoprim was about three times that of trimethoprim, as was the uptake. Therefore, a possible passive transmembrane diffusion mechanism might be proposed. Brodimoprim did not decrease either phagocytosis or phagocyte-mediated bactericidal activity, nor did it affect oxidative burst activity, as investigated by luminol-amplified chemiluminescence. On the basis of the pharmacokinetic data for brodimoprim, the concentration of 7.5 μg/ml was chosen as the highest concentration attainable in serum by oral therapy, and at this concentration of brodimoprim, the amount of drug that penetrated into PMNs was able to maintain its antimicrobial activity without interfering with the functions of the PMNs.

During infection, polymorphonuclear neutrophils (PMNs) migrate to the site of infection to exert their defensive activities, which include phagocytosis, degranulation, and the generation of the phagocyte-derived reactive oxygen species during the respiratory burst. Antimicrobial agents are administered to counteract the bacterial pathogens directly, and when antibiotics enter into the human body they distribute into different tissues, including the site of the infection, but they also enter the PMNs to various extents. Theoretically, this situation could produce synergy. If the infecting microorganism is a facultative or obligatory intracellular organism, the entry of antibiotic into phagocytes is a prerequisite for its effect and may offer a significant advantage in treatment (28, 40). In addition, phagocytes charged with antibiotic can carry it and release it at the site of infection, to which they migrate, thus producing higher local concentrations of active drug (8, 15).

During a therapeutic cycle, antibiotics may enter and leave PMNs cyclically, but they are xenobiotic substances for the human body and their potential influence on phagocytic cells has become increasingly recognized, because the interactions may influence the outcome of therapy against bacterial infections (21, 22). The penetration of some antimicrobial agents is limited, while some others are taken up well (18, 29, 49). For the latter compounds, the activities against intraphagocytic pathogens and their possible adverse influence on the phagocyte antibacterial function must be evaluated (20, 21, 55).

The mechanism, the kinetics, and the biochemical and structural determinants of antibiotic uptake by phagocytes have been characterized mainly for macrolides, clindamycin, and some other antibiotics.

Trimethoprim (TMP) belongs to the class of dimethoxybenzylpyrimidines, and the determinants of its uptake by phagocytes have been only partially investigated. Besides TMP, the class of dimethoxybenzylpyrimidines includes tetroxoprim, epiroprim, and brodimoprim (BMP). BMP [2,4-diamino-5-(4-bromo-3,5-dimethoxybenzyl)pyrimidine] is an antibacterial agent that has recently entered into clinical use. BMP has the classic mechanism of action, inhibition of dihydrofolic reductase, but its improved pharmacokinetic behavior makes once-a-day treatment possible (6, 57, 59).

The uptake of BMP by PMNs is still largely uncharacterized. The present study was designed to quantify some kinetic parameters of its cellular uptake by human PMNs and of its elimination by PMNs and the effects of intraphagocytic concentrations on normal phagocytic and antimicrobial mechanisms. TMP was included in the study as a control drug.

(This study was presented in part at the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, Calif., 17 to 20 September 1995 [5a].)

MATERIALS AND METHODS

Collection of human PMNs. Peripheral venous blood was drawn from healthy adult donors into heparinized syringes (5 U/ml). Blood (5 ml) was stratified on 3 ml of Polymorphoprep (13.8% sodium metrizoate plus 8% dextran [wt/vol]; Nycomed Pharma, Oslo, Norway), and PMNs were separated by the density gradient centrifugation technique (4, 13).

When necessary, residual erythrocytes in the granulocyte preparation were lysed with 0.15 mol of NH_4Cl solution (pH 7.4) per liter. PMNs were collected and washed in RPMI 1640 (HyClone Europe Ltd., Cramlington, United Kingdom) containing glutamine, after passage through a needle (150-μm internal

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diameter) to disrupt cell aggregates, and were then tested for viability by trypan blue exclusion. The final cell suspension was then adjusted to the cell numbers needed for each test by counting in a Bürker chamber (interference-contrast microscopy).

Determination of entry of antibiotic into human PMNs. The antibiotics used in the study were BMP and TMP. Standardized cell suspensions containing 10^7 cells per ml in Hanks balanced salt solution (HBSS; Irvine Scientific, Santa Ana, Calif.) were incubated with agitation at 37°C for different times, as described later, with BMP or TMP at final concentrations of 7.5 μg/ml. The maximum concentration of drug in serum (C_{max}) after administration of a single dose of 400 mg of BMP was 3.25 ± 0.95 μg/ml; after the administration of a single dose of 600 mg of BMP, the C_{max} was 6.22 ± 0.89 μg/ml; and after the administration of repeated doses of 200 mg (steady state), the C_{max} was 5.9 ± 1.3 μg/ml (56, 59). Taking into account the range of possible individual variations, 7.5 μg/ml was adopted (on the basis of previous values) as the highest concentration attainable in serum by oral administration.

The procedure for determining the uptake of BMP and TMP was that described by Koga (32), who used high-performance liquid chromatography (HPLC) to assay antimicrobial drugs.

At predetermined intervals, the PMNs containing antibiotic were separated from the extracellular antibiotic by the velocity-gradient technique (18, 29, 32, 49, 59). Silicone oil, 0.5 ml of a 6:5 mixture of DC550 (Fluka Chemie, Buchs, Switzerland) and DC556 (Dow Corning, Brussels, Belgium), was placed in a microcentrifuge tube, and 0.5 ml of the PMN suspension was layered on top. This was then centrifuged at 12,000 × *g* for 3 min at 4°C in a high-speed refrigerated microcentrifuge (Biofuge A; Heraeus, Hanau, Germany). After a pellet had formed at the bottom of the tube, the preparation was frozen in liquid nitrogen and the layers were separated by slicing with a razor.

The extracellular solution layer was thawed at room temperature and assayed for antibiotics.

The PMN layer was suspended in 300 μl of distilled water and was then disrupted by cycles of freezing, thawing, and mixing with a vortex mixer. The lysate was passed through a membrane filter (pore size, 0.22 μm; Millipore Corporation, Bedford, Mass.) to remove the cell debris, and the antibiotic concentration in this solution was determined.

The intracellular concentrations of BMP and TMP were calculated after establishing the total water in the PMN pellet (^3H_2O uptake), and the extracellular water content was calculated by the methods of Hand and King-Thompson (21) and Klempner and Styrt (30). From the values obtained by this procedure, cell-associated drug concentrations were calculated and expressed as ratios of intracellular to extracellular antibiotic concentrations (C/E ratios) (21). Because small changes in cell volume have a marked effect on the calculated C/E ratio, the cell volume was determined for each individual experiment (30).

HPLC apparatus and chromatographic conditions. The chromatographic system consisted of a PM-30 pump (Waters, Milford, Mass.), a 7125 sample injector (Rheodyne, Cotati, Calif.), and a stainless steel column (300 by 3.9 mm [inner diameter]) (16) packed with Nucleosil C_{18} (reversed-phase; 7-μm particle size; Macherey Nagel, Düren, Germany). The analytical mobile phase (47) consisted of a 60:40 (vol/vol) buffer (0.12 M Na_2HPO_4, 1.0 mM disodium EDTA)–methanol mixture, which was filtered through a 0.22-μm-pore-size vacuum filter apparatus (Millipore Corporation, Bedford, Mass.). To each liter, 20 ml of tetrahydrofuran was added, and the final solution was adjusted to pH 7.0 with 85% H_3PO_4 and was degassed for 15 min before use. The mobile phase was delivered at a flow rate of 1.0 ml/min, and the column was kept at a temperature of 45°C with an LC 22 temperature controller (Bioanalytical Systems, Inc., West Lafayette, Ind.). The UV detection system used was an Uvicord S II (LKB, Bromma, Sweden) spectrophotometric detector with a cell volume of 8 μl and a path length of 2.5 mm, operating at a wavelength of 281 nm with a sensitivity of 0.05 absorbance units, full scale (3, 14, 54). The compounds were quantified from the absorbance obtained, and the peak height was recorded on an OmniScribe recorder (Houston Instrument; Bausch & Lomb, Austin, Tex.). The concentrations of TMP and BMP in each sample were determined by calculating the ratios of the height of each peak to the heights of the calibration curves for the two standards. When a standard solution of the two compounds was injected into the chromatographic system, the elution times were as follows: for TMP, 5.8 min; for BMP, 14 min. The limits of detection (at a signal-to-noise ratio of 2) of this chromatographic system are about 15 ng for TMP and 25 ng for BMP. The linearity of the system was tested from 15 to 400 ng. The equations of the regression lines over the range of 15 to 400 ng for standard solutions of TMP and BMP were as follows: for TMP, $y = 0.0872x - 0.1387$ ($r = 0.999$); for BMP, $y = 0.0264x + 0.0341$ ($r = 0.998$). Every sample was analyzed in duplicate, and the values were averaged.

The coefficients of variation (CV) for the assays were as follows: for the low concentration, the within-day CVs were 3.6% for BMP and 3.4% for TMP; for the low concentration, the between-day CVs were 9.6% for BMP and 8.6% for TMP; for the high concentration, the within-day CVs were 2.3% for BMP and 1.4% for TMP; and for the high concentration, the between-day CVs were 4.0% for BMP and 3.3% for TMP.

Characterization of BMP and TMP uptake. (i) Kinetics of uptake. The kinetics of uptake of the drugs were studied with incubation periods of 15, 30, 60, and 90 min.

(ii) Influence of temperature. The influence of temperature on BMP and TMP entry into PMNs was evaluated by incubation at 4 and 37°C for the incubation periods indicated above.

(iii) Influence of extracellular pH. BMP and TMP uptakes were determined after adjusting the pH of the medium (HBSS) (with 0.5 M NaOH or 0.5 M HCl) to 6, 7.3, and 8. After each adjustment, cell viability was determined by trypan blue exclusion and was always greater than 92%.

(iv) Influence of metabolic inhibitors. The metabolic inhibitors used in the present study were 10^{-4} M 2,4-dinitrophenol (Sigma Chemical Co., St. Louis, Mo.), an uncoupler of oxidative phosphorylation (47), and 10^{-3} M potassium fluoride (Sigma Chemical Co.), which inhibits anaerobic glycolysis (18).

(v) Influence of cell viability. BMP and TMP uptakes by nonviable PMNs were measured after exposure of the cells to 10% formaldehyde (E. Merck AG, Darmstadt, Germany) for 30 min. The cells were then extensively washed and resuspended in fresh medium before the addition of antibiotics.

(vi) Kinetics of efflux of PMN-associated BMP and TMP. PMNs were incubated for 30 min at 37°C with 7.5 μg of BMP or TMP per ml, collected by centrifugation, and immediately resuspended in drug-free medium at 37°C. PMN-associated BMP or TMP concentrations were measured at various times (5, 10, 30, and 60 min) after removal of the extracellular drugs.

Influence of BMP and TMP on normal PMN functions. (i) Phagocytosis. The phagocytic capacity of the PMNs was determined by the method of Lehrer and Cline (35), with the slight modifications introduced by Patterson-Delfield and Lehrer (46) and Metcalf et al. (44). Briefly, 1×10^6 PMNs per ml preincubated with BMP, TMP, or control medium (HBSS) were mixed with 5×10^7 cells of heat-killed *Candida albicans* per ml in HBSS and serum in polystyrene tubes. The tubes were rotated end over end at 10 rpm for 30 min at 37°C.

The PMNs were separated from nonphagocytized *Candida* cells by centrifugation. The final pellets were resuspended on microscope slides in a small volume of HBSS, and trypan blue was added to determine the rate of phagocytosis.

The number of PMNs containing *C. albicans* cells in 100 PMNs is the percent phagocytosis, while the average number of *C. albicans* cells in each phagocytizing cell is the phagocytic index. Tests were done in duplicate. For each drug and control, six separate experiments were performed.

(ii) Assessment of the bactericidal capacities of PMNs. Intracellular killing of the microorganisms was determined by the method of Leijh et al. (36–38). Isolates of *Staphylococcus aureus* which were either susceptible or highly resistant to the tested antibiotics were used (2, 24, 33).

Briefly, microorganisms were incubated for about 18 h in Trypticase soy broth (Difco, Detroit, Mich.). Bacteria in the logarithmic phase of growth were harvested after 10 min of centrifugation at 1,500 × *g* and were washed twice with phosphate-buffered saline. A suspension of 2.5×10^7 bacteria per ml was prepared in gelatin-HBSS.

Preopsonization of the bacteria was performed by incubation of bacterial suspensions (2.5×10^7/ml) with human serum for 25 min at 37°C under slow rotation (4 rpm). The suspensions were washed twice with HBSS and were resuspended to a concentration of 2.5×10^6 bacteria per ml in HBSS.

Suspensions of 2.5×10^6 bacteria per ml and suspensions of 2.5×10^6 PMNs previously incubated with BMP, TMP, or control medium (HBSS) were mixed in a 1:1 ratio and were rotated end over end (6 rpm) at 37°C. The incubation time of Leijh et al. (7 min [37]) was used; phagocytosis was stopped by shaking the tubes in crushed ice. Noningested bacteria were removed by differential centrifugation (110 × *g* for 4 min) and two washes. No decrease in the number or the viability of the PMNs was observed after this procedure.

The PMNs containing the ingested bacteria were suspended in HBSS and were reincubated at 37°C under rotation for 20 min. The intracellular killing was stopped by shaking the tubes in crushed ice. After removal of the supernatant, distilled water containing 0.01% bovine albumin was added, and the cells were lysed by alternating freezing and thawing. This procedure does not affect the viability of bacteria (37). Smears of the suspensions were made and analyzed with a Nomarski interference-contrast microscope to check that all PMNs had been lysed. The number of viable intracellular bacteria was then determined by a microbiological plate method.

(iii) Measurement of the oxidative burst response by LACL. Luminol-amplified chemiluminescence (LACL) was performed by the procedure of Robinson et al. (51) for pathogenic organisms, with slight modifications. In short, 0.1 ml of a suspension of PMNs (5×10^6 cells per ml) preincubated with BMP, TMP, or control medium (HBSS) and 0.30 ml of HBSS (with Ca^{2+} and Mg^{2+}) plus 0.05 ml of 2.5×10^{-4} M luminol (Sigma Chemical Co.) were introduced into a 3-ml flat-bottom polystyrene vial. This vial was placed in the lightproof chamber of the Luminometer 1250 instrument (Bio Orbit, Turku, Finland), and the carousel was rotated to bring the sample in line with the photomultiplier tube to record the background activity.

A suspension of *Staphylococcus aureus* cells (10^8 cells per ml), preopsonized as described above, was added in a final volume of 0.05 ml, and the resulting light output (in millivolts) was recorded continuously on a chart recorder, simultaneously with a digital printout set, for 1- to 10-s recording intervals. All constituents of the mixture were kept at 37°C during the reaction by passing water from a thermostatically controlled circulation through a polished hollow metal sample holder. No mixing was done during recording.

TABLE 1. Effects of cell viability, environmental temperature, pH, and metabolic inhibitors on intracellular penetration of BMP and TMP in human PMNs

Condition	C/E ratio[a]	
	BMP	TMP
Viable cells, 37°C	74.43 ± 12.35	20.97 ± 6.61
Viable cells, 4°C	67.24 ± 19.81	18.80 ± 7.04
pH 6	52.28 ± 7.98[b]	12.67 ± 2.91[b]
pH 7.3	74.43 ± 12.35	20.97 ± 6.63
pH 8	80.57 ± 7.52	26.31 ± 7.37
KF	57.89 ± 12.39	23.36 ± 6.79
2,4-Dinitrophenol	52.14 ± 16.61	14.67 ± 5.16
Dead cells, 37°C	47.12 ± 18.07[b]	11.54 ± 4.65[b]

[a] Data are means ± SDs at 30 min. The number of the experiments was six for every test. After 5 min, effluxes were 46.12 and 49.7% for BMP and TMP, respectively.
[b] $P \leq 0.05$.

The gain control was set to give a reading of 10 mV for a built-in standard. A background subtraction control zeroed the instrument prior to the addition of the opsonized cells.

For each LACL response, the initial shape, peak (in millivolts), time to peak, and slope of declining response were recorded and the area under the curve (AUC) was calculated.

Since oxidant radical production is a phenomenon with its own time course, the simple peak, which freezes the measure at a single time, does not completely characterize the phenomenon in time, so the data are also expressed as a curve, which is a better representation of this important phenomenon.

Determination of the liposolubilities of BMP and TMP. The liposolubilities of BMP and TMP were investigated by determination of their distributions between *n*-octanol and an aqueous buffer (distribution coefficients) (62). The drug samples were dissolved in phosphate buffer, and an aliquot of the buffer was shaken with an equal volume of *n*-octanol for 1 h. The mixture was then left to separate for 30 min and centrifuged for 1 min, and the layers were separated (62). The concentration of drug present in the aqueous layers was measured by HPLC and was compared with that in the original aqueous solution before partition.

Data analysis. The results of the different tests are expressed as means ± standard deviations (SDs). The statistical significance of the differences was calculated by the Student *t* test and, when necessary, by analysis of variance; when the differences were statistically significant, this was followed by a multiple-pair comparison by the Dunnett test.

RESULTS

Characteristics of the cell population and viability. Microscopic examination of the cell suspension revealed that the proportion of the cells that were PMNs was always ≥92%. Viability determined by exclusion of trypan blue dye was always ≥95% and was not influenced by incubation with the concentrations of BMP and TMP used.

Determination of intracellular volume. The mean intracellular volume calculated from the different experiments was 2.59 ± 0.51 μl for 10^7 PMNs. This corresponds to an average volume of 0.26 pl per cell and is of the same order of magnitude as those obtained by various investigators, who have reported values ranging from 0.22 to 0.34 pl (10, 15, 26, 28, 30, 32).

For further confirmation of the cell volume, samples of PMNs were also studied by scanning electron microscopy to determine the mean diameter. The value was 7.66 ± 0.52 μm.

Uptake of BMP and TMP by PMNs. BMP was rapidly taken up by normal PMNs and reached its highest intracellular concentration after 30 min (Table 1). The C/E ratio was 74.43 ± 12.35 at that time. After 30 min there was a plateau, and this was sustained for up to 90 min, with a small decrease at that time to a C/E ratio of 69.59 ± 11.59 (Fig. 1). The curve for TMP shown in Fig. 1 had the same pattern as that for BMP, but the levels were about one-third those of BMP.

Uptake and temperature. The uptake of the PMNs as a function of ambient temperature is illustrated by the curves in Fig. 2. For both BMP and TMP, lowering the temperature to 4°C was followed by a decrease in the C/E ratios from the values obtained at 37°C. This behavior was more evident for BMP, but the differences were not significant (Table 1).

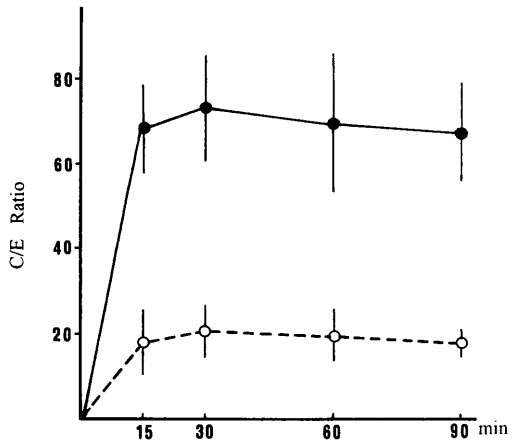

FIG. 1. Kinetics of BMP (●) and TMP (○) uptake by human PMNs. Data are expressed as the C/E ratios and are means ± SDs ($n = 6$).

Influence of extracellular pH. Figure 3 presents the patterns of BMP and TMP uptake according to the different environmental pHs. The extent of intraphagocytic accumulation was proportional to the pH for both BMP and TMP (Table 1). For lipophilic weak bases with a pK_a of ≃7.3, such as BMP and TMP, the passage through biological membranes is correlated with the extent of the protonated form, which depends on the pH. Antibiotic incorporation increased linearly with increasing alkalinity of the medium (BMP, $y = 14.5x - 33.9$ [$r = 0.85$]; TMP, $y = 6.7x - 28.0$ [$r = 0.72$]).

Influence of metabolic inhibitors. In the presence of potassium fluoride and 2,4-dinitrophenol, the entry of TMP and BMP into PMNs was slightly but not significantly reduced from the control value (Table 1; Fig. 4).

Influence of cell viability. Optimal uptake occurred only in live phagocytes. In formaldehyde-killed PMNs, the accumulation of both BMP and TMP was significantly reduced (Table 1; Fig. 5).

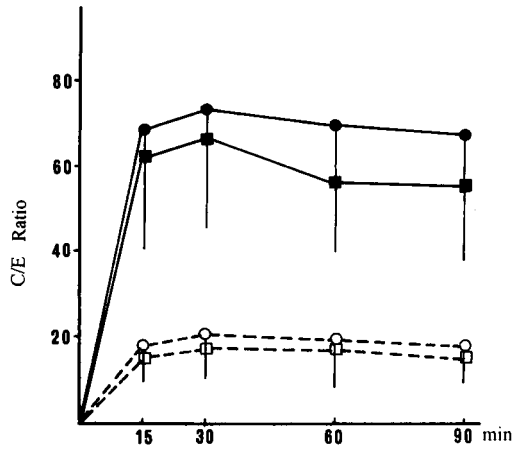

FIG. 2. Influence of environmental temperature on BMP and TMP uptake by human PMNs. ●, BMP at 37°C; ○, TMP at 37°C; ■, BMP at 4°C; □, TMP at 4°C. Values are means ± SDs ($n = 6$).

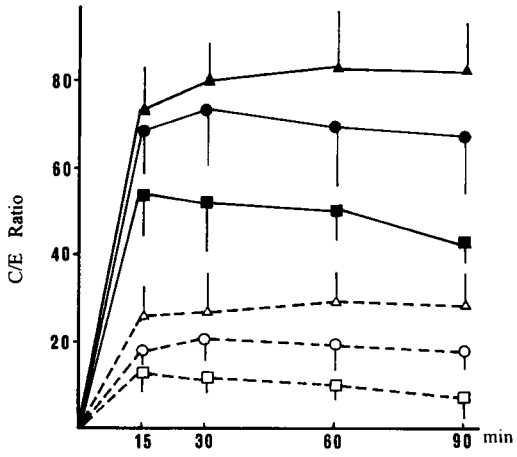

FIG. 3. Influence of environmental pH on BMP and TMP uptake by human PMNs. ●, BMP at pH 7.3; ■, BMP at pH 6.0; ▲, BMP at pH 8.0; ○, TMP at pH 7.3; □, TMP at pH 6.0; △, TMP at pH 8.0. Values are means ± SDs (n = 6).

Antibiotic release. The release of both drugs from PMNs to extracellular medium was very rapid and significant (Fig. 6). To indicate the difference in the ratios of the drugs' uptake, the values for both BMP and TMP were normalized by setting the intracellular amount of BMP after 30 min of incubation equal to 100. The intracellular antibiotic concentrations had fallen to about 46% of the prewashed levels in 5 min for BMP and 49% for TMP. These findings indicate that these compounds are not tightly bound or trapped within intracellular compartments (Table 1; Fig. 6).

Phagocytosis. Table 2 summarizes the data for phagocytosis after incubation with both BMP and TMP. There were no significant differences in the values for *C. albicans* ingestion from the control values.

Killing. The intraphagocytic concentrations of BMP and TMP did not interfere with the bactericidal capacities of the PMNs (Table 2).

LACL measurement. The oxidative burst investigated by chemiluminescence (LACL) was impaired by TMP at 7.5 μg/ml. The figure for LACL indicates a significant reduction in oxidant radical production. At the same concentration, BMP reduced the value for LACL from the control value, but not significantly (Table 2). Figure 7 presents the LACL curves for BMP and TMP in comparison with that for the control.

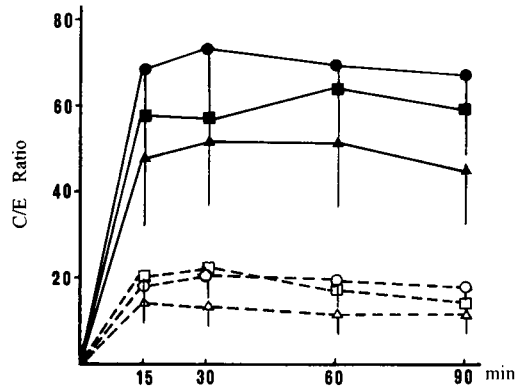

FIG. 4. Effects of metabolic inhibitors on entry of BMP and TMP into human PMNs. ●, BMP; ■, BMP plus KF; ▲, BMP plus 2,4-dinitrophenol; ○, TMP; □, TMP plus KF; △, TMP plus 2,4-dinitrophenol.

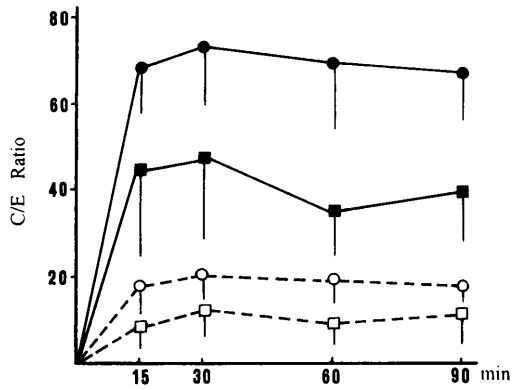

FIG. 5. Influence of cell viability on BMP and TMP uptake by human PMNs. ●, BMP, viable cells; ○, TMP, viable cells; ■, BMP, killed cells; □, TMP, killed cells. Values are means ± SDs (n = 6).

DISCUSSION

TMP, investigated as a control drug, was readily taken up by PMNs at 37°C. The C/E ratio of TMP was maximal within 30 min, reaching a value of 20.97 ± 6.61, and then the values plateaued at a final C/E ratio of 19.44 ± 3.83 at 90 min. These data are comparable to those of Seger et al. (52), who found a C/E ratio of 14.1 ± 2.2 (they used 10 μg of TMP per ml). Hand et al. (23) and Hand and King-Thompson (22) also found that TMP is markedly concentrated by PMNs, with C/E ratios of 9.02 ± 0.77 at 37°C and 12.79 ± 1.66 at 25°C (they used 7.28 μg of TMP per ml).

We have found in the literature only a few other data on C/E ratios for TMP in PMNs. Koga (32) reported a value of 6.10 ± 0.63, Jacobs and Wilson (27) reported a value of 4, Gründer and Seger (17) reported a value of 4.1 ± 0.6 (they used 5 μg of TMP per ml), and Höger et al. (25) reported a value of 3.15 (they used 10 μg of TMP per ml); however, the last three groups of investigators did not indicate the intracellular volumes of their PMNs, and this makes it difficult to compare their results with ours. There is also the value of Climax et al. (7) of 0.5 μg of TMP per ml per 10^7 cells, but this is difficult to analyze and to transform into a C/E ratio because they provided no information about the exact starting concentration or the intracellular volumes of the PMNs.

A review of the literature indicates that there is no apparent preferential method for determining drug uptake but that several methods can be used (12, 29, 32, 49).

One possible major pitfall in the measurement of drug uptake by leukocytes, which might explain some of the differences in the values reported in the literature, is the measurement of

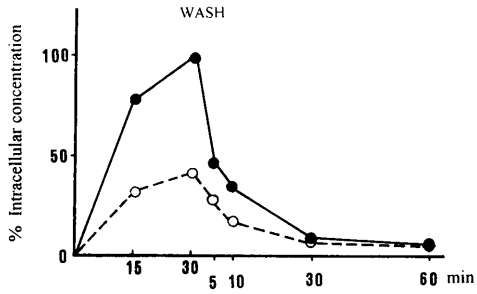

FIG. 6. Kinetics of release of BMP (●) and TMP (○) from PMNs after removal of the drugs from the incubation medium. Values are means ± SDs (n = 6).

TABLE 2. Phagocytosis, killing, and chemiluminescence (LACL) of human PMNs before and after incubation with BMP and TMP[a]

Drug and incubation time	% Phagocytosis	Phagocytic index	% Killing		LACL (mV)
			Resistant *S. aureus*	Susceptible *S. aureus*	
BMP					
Before	74.86 ± 6.01	3.66 ± 0.50	51.35 ± 16.20	56.21 ± 22.73	24.98 ± 6.52
After	77.00 ± 3.85	3.98 ± 0.46	54.10 ± 13.82	62.08 ± 18.96	20.19 ± 4.65
TMP					
Before	76.67 ± 9.79	3.39 ± 0.48	55.12 ± 14.55	60.20 ± 22.72	24.98 ± 6.52
After	74.83 ± 11.21	3.25 ± 0.47	51.83 ± 15.80	69.26 ± 19.64	14.95 ± 2.66[b]

[a] Data are means ± SDs.
[b] $P \leq 0.05$.

the intracellular volume of the PMNs, because small changes in cell volume have a marked effect on the calculated C/E ratio (30, 32, 39, 50). The mean intracellular volume calculated from all our determinations was 0.26 ± 0.08 pl per cell. For further confirmation, we also measured the mean diameter of the PMNs by scanning electron microscopy, and the value was 7.66 ± 0.52 μm. Assuming that the cells have a theoretical spherical shape, the mathematical calculation gives a result of 0.23 pl, which is very close to that found by measurement of aqueous cell volume. These findings agree with those of Koga (32), who assumed a PMN diameter of 8 μm and considered to be reasonable a fixed estimate of the PMN volume of about 0.27 pl per cell.

We found that BMP also entered PMNs rapidly, with a C/E ratio of 69.67 ± 9.33 at 15 min, reaching a plateau at 30 min; the C/E ratio was only slightly lower at 90 min (69.59 ± 11.84). The BMP curve has the same pattern as the one for TMP, but about three times more BMP than TMP enters the cell. This was also confirmed by the calculation of the AUC. The penetration of a drug into a cell is a phenomenon with its own time course, so the AUC expresses this parameter better than a single value, such as the C/E ratio. The AUC for TMP was 16.8 ± 4.3, while the AUC for BMP was 59.7 ± 8.5.

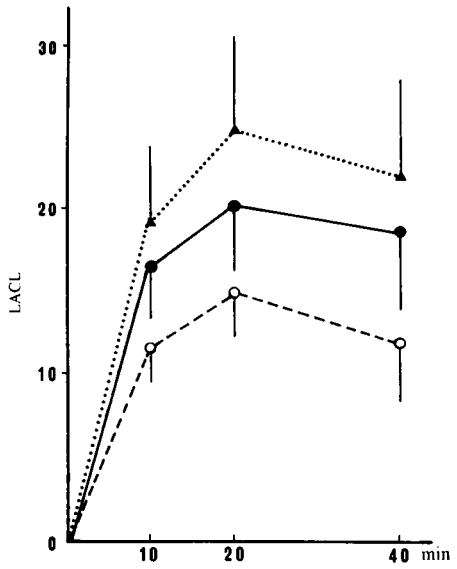

FIG. 7. Effect on LACL response of incubating human PMNs with BMP (●) and TMP (○) in comparison with that for incubation with the control (▲). Values are means ± SDs ($n = 8$).

It has been shown for structurally related groups of compounds that the degree of uptake by mammalian cells is a reflection of the lipid solubilities of the compounds. Lipid-soluble antibiotics penetrate biological membranes well, in contrast to water-soluble antibiotics, which penetrate biological membranes poorly (29, 33, 38, 40, 47). The partition coefficients in *n*-octanol–buffer for BMP and TMP were investigated by Then et al. (57), and the values were 1.82 and 0.64, respectively ($pK_a = 7.15$ for both), indicating that the liposolubility of BMP is about three times that of TMP. Our values for liposolubility were 4.8 for TMP and 26.5 for BMP. Our findings are thus consistent with the concept of lipid solubility-related uptake mentioned above. This concept is further supported for BMP by the data of Seger et al. (52), who reported that tetroxoprim (TXP), another molecule structurally related to TMP, but with a lower lipid solubility ($P_{\text{octanol-buffer}}$ for TXP = 1.8; $P_{\text{octanol-buffer}}$ for TMP = 4.4), enters PMNs at lower levels ($C/E_{TXP} = 5.0 \pm 1.0$; $C/E_{TMP} = 14.1 \pm 2.2$). The observed C/E ratios for PMNs follow the order BMP > TMP > TXP, which exactly mimics the order of the liposolubility of TMP analogs. These findings thus indicate that the uptake of TMP and its analogs (BMP and TXP) is probably by passive transmembrane diffusion (25).

Figure 3 indicates that the entry of BMP and TMP was influenced by environmental pH. At pH 6, the uptake was lower, and at pH 8, it was higher. Lipid-soluble drugs with pK_a values of ≃7.3, such as BMP and TMP at plasma pH (≃7.3), exist as 50% in the ionized form and 50% in the nonionized form, and only the nonprotonated portions of these lipid-soluble molecules diffuse across the lipid cell membrane of PMNs (17, 52, 61). At pH 6 these drugs are mainly ionized (protonated), with reduced diffusion across the membranes, while at pH 8 drugs with pK_a values of ≃7.3 are mainly nonionized, and their diffusions across the membranes are greater than those at an environmental pH of 7.3.

At 4°C, a reduction in uptake was observed, but it was not significantly different from that at 37°C for either BMP or TMP, while formaldehyde pretreatment reduced uptake by 36.7% for BMP and 44.9% for TMP. This last finding can be explained by the observations of Koga (32) and El Benna et al. (11) that formaldehyde alters the fluidity or the structure of the PMN membrane, thus reducing the penetration of drugs.

Metabolic inhibitors did not significantly impair TMP uptake by PMNs, and our data agree with the findings of Hand et al. (23). BMP uptake was also not significantly modified by potassium fluoride or 2,4-dinitrophenol.

After uptake by PMNs, release of BMP and TMP into a medium free of antibiotics was rapid. Intracellular antibiotic concentrations had become about 46% of the prewashed levels

for BMP and 49% for TMP in only 5 min, indicating that these molecules are not tightly bound to any cellular components of PMNs.

As for the effects of intraphagocytic concentrations of BMP and TMP, a general statement is that a documented intracellular accumulation of antibiotic does not prove that it will be effective. To be useful clinically, it must retain its antimicrobial activity and not have negative effects on phagocytosis, killing, or the oxidative burst.

At a concentration of 7.5 μg/ml, neither BMP nor TMP affected phagocytosis of *C. albicans* by PMNs, and this is in agreement with our previous findings (5) with *C. albicans* as the test organism and with the findings of Vosbeck et al. (58) for *S. aureus* and of Melby and Midtvedt (42, 43) for *Escherichia coli*. The amount of BMP and TMP that enters the PMNs did not produce negative effects on the ability of PMNs to exert their bactericidal function.

The metabolic events following phagocytosis result in the generation of oxidative species ($O_2^{\cdot -}$, $^{\cdot}OH$, H_2O_2, and $^1O_2$) (1) that can be investigated by LACL. The intracellular TMP concentrations reached during incubation with TMP at 7.5 μg/ml significantly reduced the oxidative burst investigated by LACL. This is in agreement with the data of Welch et al. (60), Dunker and Ullmann (9), Siegel and Remington (53), and Braga et al. (5). This interference by TMP has been attributed to a direct interaction with the mechanism of the respiratory burst, since the effects are not seen in a cell-free system (9, 31). It has been reported that TMP inhibits the phospholipase D–phosphatidic acid phosphohydrolase pathway in activated PMNs, leading to decreased diradylglycerol production and, consequently, to a reduction of superoxide generation (19, 48).

BMP up to 7.5 μg/ml did not interfere with oxidant radical production (LACL).

We found that although TMP influences the oxidative burst (LACL diminution), it nevertheless does not influence neutrophil functions (phagocytosis, killing); this finding is in agreement with the findings of Welch et al. (60) and Höger et al. (25), but it still opens up discussion about the different factors that influence the complex interactions of the intracellular environment of PMNs with the biological activity of the drug and with the susceptibility of the microbial host. The apparent discrepancy between retention of killing activity and reduction of the oxidative burst might be explained by the intrinsic antibacterial properties of the given antibiotic, as reported by Hand and King-Thompson (21), which are probably crucial in determining the intraphagocytic activity of the drug. In any case, the ability of phagocytes to kill cannot be attributed entirely to the oxidative system. Other mechanisms are also involved, such as release of lytic enzymes.

PMNs charged with antibiotics which are rapidly released may represent a useful biological delivery system. When they have migrated to the site of infection, they can further increase the concentration of antibiotic locally; an example of this phenomenon is that of azithromycin (15). On that basis, for a 70-kg man with a total circulating PMN pool of 4×10^{10} cells, a PMN volume of 260×10^{-15} liter, a C/E ratio of 74, and an average concentration in serum of 7.5 μg of BMP per ml during the first 20 min after the administration of a dose of 200 mg of BMP at steady state, about 5.80 mg of BMP, that is, approximately 3% of the dose, will be in the circulating PMNs, thus forming the delivery pool.

To summarize, replacement of the OCH_3 group in position 4 of the benzyl ring in the TMP molecule with a bromine atom produced a new molecule called BMP. Not only does BMP bind better to dihydrofolate reductase and have a better pharmacokinetic profile (56, 59), but also its greater lipophilicity increases the penetration of BMP into PMNs without significant effects on other PMN functions.

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