The intracellular activity of ofloxacin and roxithromycin against *Staphylococcus aureus* phagocytosed in human neutrophils

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Some antimicrobial drugs, such as the new quinolones and macrolides, reportedly become highly concentrated in the neutrophils. The effects of ofloxacin (OFLX) and roxithromycin (RXM) against *Staphylococcus aureus* phagocytosed by human neutrophils were examined in this experiment. An *S. aureus* strain isolated from an impetigo and ATCC 29213 strain were studied. Neutrophils separated from human peripheral blood were incubated with *S. aureus* cells at 37°C in the presence of human serum. Non-phagocytosed extracellular bacteria were killed using lysostaphin, and the neutrophils with phagocytosed *S. aureus* were obtained. These were then incubated with 0, 0.2, 0.25, 1, 2, 4, 8, 16, or 32 mg/l of OFLX, or either 2 or 16 mg/l of RXM for 6 h. The neutrophils were then sonicated and the viable bacteria were counted by culturing on plate agar. After incubating with low concentrations of OFLX for 6 h, the viable bacterial counts were not significantly different from those of the control group, which had been incubated for 6 h without antimicrobial agents. A slight decrease in the number of viable bacteria was observed with higher concentrations of OFLX. RXM showed a little effect against *S. aureus* in the neutrophils. The results indicate that *S. aureus* in neutrophils can survive in the presence of OFLX and RXM.

Key words: Neutrophils, ofloxacin, roxithromycin, *Staphylococcus aureus*

Introduction

Staphylococcus aureus, the major cause of skin and skin structure infections, is phagocytosed and killed by neutrophils, although research has shown that it can remain viable in the neutrophils for long periods of time[1]. It has long been reported that *S. aureus* cells ingested by neutrophils can evade antimicrobial agents outside the neutrophils leading to prolongation of the infection[2]. However, some antimicrobials can enter human cells in high concentrations and are believed to act not only on free bacteria but also on bacteria within the cells[3~10]. The effects of some antimicrobials against the *S. aureus* within neutrophils have been investigated[4,10~13] but the results have been contradictory. One reason for these conflicting reports may be that since the life span of neutrophils is short, the effects of antimicrobial agents against *S. aureus* cells in neutrophils can only be observed after a relatively short period of time.

In order to examine the effect of antibacterial drugs against *S. aureus* in cells, intracellular viable bacteria are usually counted by culturing on plate agar. However this method has given varying results due to inconsistencies in the incubation medium, neutrophil concentration, incubation period, and the procedure used to eliminate the extracellular bacteria[4,10,12,13].

Radioassay, fluorescent dye[11], and biophotometry[14] were used as alternative methods. However, the first two methods require special equipment, and fluorescent dye staining does not sufficiently differentiate the viability of the bacteria. Further, while colony counting is time consuming, it is also

a simpler and often more reliable method.

Ofloxacin (OFLX) and roxithromycin (RXM) are known to be useful in the treatment of infections of *S. aureus* in the skin and skin structure and have been shown to penetrate well into the neutrophils[5~9]. The colony-counting method was used in this study to examine the effects of OFLX and RXM against two strains of *S. aureus* ingested by human neutrophils.

Materials and Methods

Staphylococcus aureus. A clinical isolate of *S. aureus* from an impetigo (labeled strain 90-432), and an ATCC 29213 strain were used in all conditions. Both strains do not produce TSST-1 and enterotoxin type A-D.

Neutrophils. Venous blood samples were drawn from healthy volunteers using heparinized syringes, and the neutrophils were separated by centrifuging with the Mono-poly resolving medium Ficoll-Hypaque (Flow Laboratories, McLean, VA. USA) at 1,200×g. After washing with Hanks' balanced salt solution (HBSS, Sigma Chemical Company, Ltd., St. Louis, MO, USA), the contaminated erythrocytes were removed by hypotonic lysis in 0.2% NaCl. An equal volume of 1.6% NaCl was added and the washing procedure was repeated. The cell pellet was resuspended in HBSS to a concentration of 1×10^7 cells/ml. The viability of the neutrophils was confirmed by trypan blue staining. Viable neutrophils constituted more than 95% of all cells.

Human sera. Sera were separated from the blood samples of healthy adults and were stored at −80°C.

Antimicrobial agents. OFLX was obtained from Daiichi Pharmaceutical Company, Ltd., Japan. RXM was obtained from Eizai Company, Ltd., Japan. OFLX was dissolved in 0.1 N NaOH, RXM in methanol, and diluted with distilled water to adjust to the concentration of 1 mg/ml. They were then sterilized using a membrane filter (0.20 μm, Advantec Toyo Company, Ltd., Japan), and diluted with physiological saline.

Lysostaphin. Lysostaphin was purchased from Sigma Chemical Company, Ltd., St. Louis, MO, USA. A solution of 1,000 u/ml in physiological saline was prepared and stored at −20°C. The effect of lysostaphin was checked by incubating 1×10^8 cfu/ml *S. aureus* at 37°C with lysostaphin at a final concentration of 2 u/ml. This procedure killed more than 99.95% of the *S. aureus* cells, of both strains, within 30 min.

Preparation of *S. aureus* cell suspension. *S. aureus* was cultured in Tryptic soy broth (Difco Laboratories, Detroit, MI, USA) at 37°C for 24 h. It was then washed 3 times with 0.9% saline and resuspended in 0.9% saline to a concentration of $4-6\times10^8$ cfu/ml.

Antimicrobial susceptibility testing. The MICs were determined according to the microbroth dilution method using Mueller Hinton broth (Difco Laboratories, Detroit, MI, USA) with an inoculum size of 10^4 cfu.

The MBCs were determined using the macrobroth dilution method, as described by Amsterdam[15]. An inoculum of 2.5×10^5 cfu/ml in the early logarithmic stage was incubated in Mueller Hinton broth at 35°C for 24 h. The MBCs were defined as the concentrations that killed 99.9% of the bacteria.

To examine the time course of viable bacteria in the presence of an antimicrobial drug, bacterial inoculums of approximately 2×10^7 were subcultured in 20 ml of HBSS containing 5% serum for 1 h. They were then washed once by centrifugation and were resuspended in the same volume of HBSS. The test antimicrobial drug was then added at each concentration. After further incubation for 2, 4 and 6 h, the viable bacteria were counted. The bacterial counts were performed using the ten-fold dilution method with heart infusion agar plates (Heart Infusion Agar "Nissui", Nissui Pharmaceutical Company, Ltd., Tokyo, Japan) for 24 h.

Phagocytosis assay. In order to opsonize the bacteria, mixtures of 1 ml of the bacterial suspension, 1 ml of HBSS, and 0.5 ml of serum were preincubated with shaking at 37°C for 10 min. Then, 2.5 ml of neutrophil suspension were added and the mixture was incubated for 15 min. Lysostaphin was added at a final concentration of 2 u/ml in order to kill the extracellular bacteria. After 30 min of incubation at 37°C, trypsin (NACALAI TESQUE, INC., Kyoto, Japan) was added at a final concentration of 0.25%. Neutrophils containing

bacteria were obtained by centrifugation at 1,000 × g and were resuspended in 3.2 ml of HBSS. An aliquot of this solution was smeared on a slide glass and stained by Giemsa stain, and the number of phagocytosed bacteria in 100 neutrophils was counted using a light microscope. OFLX (final concentrations of 0.2, 0.25, 1, 2, 4, 8 and 32 mg/l) or RXM (final concentrations of 2 and 16 mg/l) was added to the neutrophil suspensions in HBSS (cell count 8×10^5 cells/ml) and then incubated with shaking in siliconized test tubes at 37°C. After 6 h, the neutrophil suspensions were sonicated in order to destroy the neutrophils. They were then diluted by serial ten-fold dilution, and then cultured on an agar plate (Heart infusion agar). Since we had previously determined that the number of colonies was not affected by drugs on the plate diluted to 100 - and 1,000- fold prior to culturing, antimicrobial agents were not removed prior to culturing on the agar plates. The colonies on the plate were counted to calculate the number of viable bacteria. The colony forming unit (cfu) was defined as the number of colonies which formed on a medium plate, inoculated with an aliquot of sample, and it represented the number of viable bacterial cells in the aliquot.

Statistical analysis. Data are expressed as mean±standard error of the mean (SEM). The data were analyzed by the Student's T-test. A P value of <0.05 was considered significant.

Results

Antimicrobial susceptibility. Table 1 shows the MICs and MBCs of the strains used in the experiment. The 90-432 strain was resistant against RXM. OFLX showed bactericidal activity against both strains. Fig. 1 shows the growth curves of these opsonized *S. aureus* strains in HBSS in the presence of OFLX and RXM. Bacteria did not grow in 6 h in the absence of antibiotics. In the presence of 8 mg/l (16 MIC, 8 MBC) of OFLX or 16 mg/l (<1/8 MIC) of RXM, the number of strain 90-432 cells did not change over 6 h. The number of strain ATCC 29213 decreased in the presence of 32 mg/l (128 MIC, 64 MBC) of OFLX.

The number of phagocytosed *S. aureus* cells. All of the 100 neutrophils counted contained phagocytosed bacteria. The mean was 1,706 (SEM; ±138) cells/100 neutrophils for strain 90-

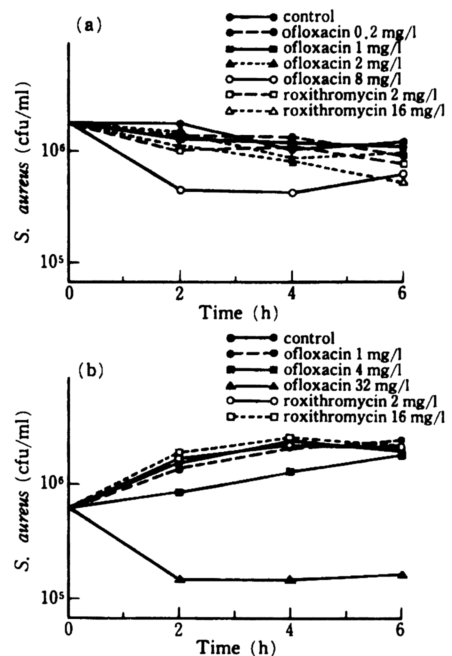

Fig. 1. Influence of ofloxacin and roxithromycin against opsonized *Staphylococcus aureus* in HBSS.
(a) strain 90-432,
(b) strain ATCC 29213 (n=3)

Table 1. MICs and MBCs of ofloxacin and roxithromycin against bacterial strains 90-432 and ATCC 29213

	Ofloxacin		Roxithromycin	
	MIC (mg/l)	MBC (mg/l)	MIC (mg/l)	MBC (mg/l)
Strain 90-432	0.5	1	>128	>128
ATCC 29213	0.25	0.5	4	32

432 (n=6) and 1693 (SEM; ±86.2) cells/100 neutrophils for strain ATCC 29213 (n=7). When 2.5×10^{7} neutrophil cells were incubated with $4-6\times10^{8}$ cfu of bacteria, the mean number of phagocytosed bacteria was approximately 17 cells/neutrophil. The total number of phagocytosed *S. aureus* cells was 4.3×10^{8} cfu. This indicates that the majority of the added *S. aureus* cells had been ingested.

Effects of OFLX and RXM against *S. aureus* in neutrophils. Table 2 shows the numbers of viable *S. aureus* cells. The results varied when the experiments were performed separately. The control group consisted of samples of neutrophils with ingested bacteria following incubation without any antimicrobials for 6 h. Without any antimicrobials, the number of viable *S. aureus* cells in the neutrophils decreased or increased over 6 h. The percentages of the number of viable cells at 6 h against the number at 0 h were 139.7±34.8 (mean±SEM%) (strain 90-432) and 195.7±24.0 (mean±SEM%) (strain ATCC 29213).

Three or four experiments were performed with each concentration of OFLX and RXM. Experiments 1—13 were performed separately. There were no differences in viability of strain 90-432 in the neutrophils in the HBSS containing 0.2 mg/l (0.4 MIC) of OFLX, compared to the sample without any antimicrobials. In the condition with 1, 2 or 8 mg/l (2, 4, 16 MIC) of OFLX, viability decreased as the concentration of the drug increased. The extent of decrease of viability with 1, 2 or 8 mg/l of OFLX was significant in each experiment. In the condition with 2 and 16 mg/l (<1/64, <1/8 MIC) of RXM, the number of viable bacteria was smaller than the number in the control group, but there were no differences in the number of viable bacteria in the experiments performed at the same time between the group with 2 mg/l and group with 16 mg/l of RXM.

In the condition with the ATCC 29213 strain, there were no differences in the number of viable bacteria between the group with 0.25 mg/l (1 MIC) of OFLX and the control group. In the groups with 1, 4 or 32 mg/l (4, 16, 128 MIC) of OFLX, viability decreased slightly as the concentration increased. In the groups with 2 or 16 mg/l (0.5, 4 MIC) of RXM, the number of viable bacteria was slightly smaller than in the control group.

Discussion

The intracellular concentrations of OFLX and RXM have been shown to be much higher than the extracellular concentrations[5~9]. The ratio of intracellular concentration to extracellular concentration is commonly expressed as the C/E ratio. The C/E ratio for OFLX was reported to be 8.15±2.00 for 30 min by high-performance liquid chromatography (HPLC)[7], or 6.7±1.4 for 20 min by fluorometric measurement[9]. The C/E ratio for RXM was reported to be 14±3[5], 19.81±1.49[9], or 32.56±2.08[9] for 30 min by radioassay. It was also reported that one third of the RXM recovered in the cell homogenate was found to be associated with azurophil granules in polymorphonuclear leukocytes, and that the concentration in the granules was much higher than that estimated from the C/E ratio[5]. It seems probable that if the drugs acted on *S. aureus* in the neutrophils in high concentrations, then they should have an effect on the intracellular bacteria with low extracellular concentrations, below the MIC or MBC.

In the present experiment, the number of intracellular viable bacteria was not influenced by the 0.4 MIC of OFLX using the 90-432 strain, or by the MIC of OFLX using the ATCC 29213 strain. OFLX only decreased the number of viable intracellular bacteria in high concentrations. Pascual *et. al.*[12] reported that OFLX at an MIC significantly reduced the number of viable intraphagocytic *S. aureus* ATCC 25923 strain cells. I believe that the effective concentration of a drug will differ for different organisms. Pascual *et. al.* reported a viability of approximately 50-65% after 3 h incubation compared to the number before incubation with OFLX, which is consistent with my results. However, while these results might be significant by calculation they may not be efficacious for clinical use. The intracellular effects of OFLX in neutrophils were found to be less than expected.

Even if intracellular concentrations are high, the effect against intracellular bacteria depends on where a drug is concentrated in the cells and whether or not the drug remains active in the cells.

Table 2. Effect of ofloxacin and roxithromycin on the viability of *Staphylococcus aureus* ingested by neutrophils

Strain 90-432

experiment	(0 h)	control (6 h) (survival rate (%))	ofloxacin (mg/l) (6 h)				roxithromycin (mg/l) (6 h)	
			0.2	1	2	8	2	16
1	3.08±0.20	8.86±0.36 (288)	7.91±0.26	1.40±0.05*	0.840±0.079	0.388±0.030	ND	ND
2	1.93±0.007	1.97±0.06 (102)	1.86±0.12	0.92±0.06*	0.512±0.069	0.316±0.010	ND	ND
3	3.79±0.07	2.02±0.09 (53.3)	2.07±0.12	ND	ND	ND	1.26±0.07*	1.05 ±0.04*
4	4.02±0.34	2.97±0.14 (73.9)	ND	1.74±0.04*	1.18 ±0.07	0.515±0.019	1.61±0.10*	1.31 ±0.10*
5	1.62±0.06	2.43±0.33 (150)	ND	ND	ND	ND	1.06±0.04*	0.772±0.081*
6	2.11±0.71	3.61±0.21 (171)	ND	ND	ND	ND	1.74±0.10*	1.70 ±0.12*
		(139.7±34.8)						

ATCC 29213

experiment	(0 h)	control (6 h) (survival rate (%))	ofloxacin (mg/l) (6 h)				roxithromycin (mg/l) (6 h)	
			0.25	1	4	32	2	16
7	2.56±0.34	3.85±0.13 (150)	3.21±0.20	1.30 ±0.09*	ND	ND	ND	ND
8	2.90±0.38	4.86±0.22 (167)	4.89±0.20	1.41 ±0.05*	ND	ND	ND	ND
9	1.13±0.06	1.70±0.06 (151)	1.72±0.10	0.673±0.022*	0.235±0.095	0.116±0.010	ND	ND
10	1.78±0.16	5.76±0.32 (324)	ND	ND	0.415±0.011*	0.222±0.009	ND	ND
11	1.48±0.14	2.35±0.17 (159)	ND	ND	0.294±0.011*	0.182±0.014	0.888±0.053*	0.830±0.029*
12	1.52±0.06	2.83±0.10 (186)	ND	ND	ND	ND	0.644±0.039*	0.581±0.022*
13	1.07±0.11	2.49±0.71 (233)	ND	ND	ND	ND	0.493±0.074*	0.576±0.056*
		(195.7±24.0)						

($\times 10^{-6}$ cfu/ml)

The numbers indicate the amounts of viable bacteria in each group of neutrophils ($\times 10^{-6}$ cfu/ml) and the ingested *S. aureus* cells at the start of incubation (n=3), and after incubation with ofloxacin or roxithromycin for 6 h (n=5), and, for the control group, after 6 h incubation without any antimicrobials (n=5). All measurements are expressed as the means±SEM for each experiment. Numbers 1−13 indicate different experiments. The survival rate of the bacteria in the control group after 6 h is shown as percentages.

ND: not done * $P<0.05$ versus control

The *S. aureus* cells in the neutrophils stay in the phagolysosomes[10], which are known to have a low pH[5]. For this reason, the effects of drug concentrations in whole cells as measured by radioassay, HPLC, or fluorometric assay will not always represent the actual activity of the drug in the cell. According to W. L. Hand *et. al.*, the ability of an antibiotic to enter the phagocytes is only one of the factors which determine its intracellular antimicrobial activity, since its concentrations in the neutrophils and its intracellular activity are not always consistent with each other[4].

While OFLX is effective in killing free *S. aureus* cells in the growth phase, it is clearly less effective in killing cells in the stationary phase. In the present study, the *S. aureus* cells in the neutrophils were in the stationary phase. Table 2 shows that the change in the number of viable bacteria in the control group incubated without antimicrobial agents for 6 h, was relatively small. Thus, in the stationary phase, OFLX had a greater effect on *S. aureus* cells in the neutrophils than on the free *S. aureus* cells. This indicates that higher concentrations of OFLX may be required within the neutrophils than outside the neutrophils, or that OFLX may activate the neutrophils to kill the bacteria in higher concentrations.

Reportedly, OFLX stimulates the hexose monophosphate shunt of the neutrophils but does not increase the killing ability of the neutrophils[16]. Although I did not examine neutrophil function in the present study, it is likely that the influence of the killing ability of the neutrophils was smaller than that observed *in vivo*, because the killing of *S. aureus* cells in neutrophils requires IgG and complements in the serum[17] and I did not add serum when incubating the neutrophils and phagocytosed *S. aureus* with antimicrobials.

No serum was added because preliminary experiments suggested that its presence stimulated the growth of *S. aureus* outside the neutrophils. Previous research demonstrated that phagocytes have no bactericidal activity in the absence of serum, and thus, they recommended that serum not be used so that the activity of phagocytes could be excluded and so that the activity of the antibiotic alone could be measured[13].

RXM showed little bactericidal activity (Table 1) and did not influence the viability of bacteria in the stationary phase (Fig. 1). Even after the concentration in phagolysosome became high and remained active, the number of viable bacteria did not decrease. My results showed a slight decrease in the number of viable bacteria in both strains tested and I found that the amount of change was not influenced by differences in bacterial susceptibility and drug concentration. This suggests that RXM may increase the killing activity of the neutrophils. RXM was reported to increase the phagocytosis and killing of *S. aureus* by the neutrophils at a concentration of 0.1 mg/l[18]. Anderson *et. al.*[11] used radioassay and fluorescence dye tests to show bacteriostatic activity of RXM and erythromycin (EM) on *S. aureus* cells in human neutrophils because they did not believe that the colony-counting method was sufficiently sensitive. They also noted the synergistic activity of RXM with the O_2-dependent killing mechanism of the neutrophils[11]. The findings of the present study were consistent with those of Anderson *et. al.*

Erythromycin (EM) was reported to be inactive against *S. aureus* cells in neutrophils as measured by counts of the viable bacteria[4]. Others have reported that EM is active in the cytoplasm but not fully active in phagolysosomes, which have a low pH, and that it does not effect the *S. aureus* cells that stay in the phagolysosome[10]. Using biophotometric technology, Vosbeck *et. al.*[14] showed that EM acts on *S. aureus* Wood 46 in neutrophils only in high concentrations, such as 30 MBC (approximately 99 % killing for 30 min), and hypothesized that this was because the drug was inactivated by the low pH in the phagolysosomes. I did not try these high concentrations of RXM because such a condition is not normally found *in vivo*. Since the bacteria in the neutrophils are not proliferating, the influence of low pH may only be one factor contributing to the observed effects.

The method of colony counting has been performed differently at different institutes. Lysostaphin has often been used for discriminating between bacteria in the neutrophils and those outside of the

neutrophils[4,19]. It has been reported that lysostaphin can enter the neutrophils and can remain attached to the neutrophil membrane[20,21]. I used lysostaphin to eliminate any bacterial cells that may have attached to the neutrophil membranes. I hypothesized that the effect of lysostaphin in the cells was negligible in my experiments because the viable bacteria counts were not reduced after incubating the neutrophils with intracellular *S. aureus* cells for 6 h after treatment with lysostaphin.

Several previous studies adopted a 3 h incubation period for the neutrophils with bacteria ingested in HBSS containing an antimicrobial drug[4,10,12,13]. I adopted an incubation period of 6 h because the number of viable bacteria in the cells decreased slightly as incubation time increased, and because, as a rule, it is better to evaluate the effect of a drug after the longest possible incubation period. The half-life of neutrophils *in vivo* is approximately 6—7 h[22]. In my preliminary experiments, the viability of neutrophils with ingested *S. aureus* was approximately 60—80%, after incubation in HBSS for 6 h. It is possible that some of the ingested bacteria may have been released from broken neutrophils, although opsonized bacteria do not proliferate in HBSS and it is also unlikely that an antimicrobial agent could have reduced the number of bacteria outside of the neutrophils, since the concentrations were too low for bactericidal effects.

The maximum drug concentration in human serum is approximately 3 mg/l following oral administration of ofloxacin at a dose of 200 mg[23], and about 10 mg/l following oral administration of RXM at a dose of 150 mg[24]. The crucial factor is whether the drugs have an effect on intracellular bacteria *in vivo*. These results indicate that OFLX might be slightly active against *S. aureus* in the neutrophils only when there is sufficient sensitivity against the drug. The direct effect of RXM against *S. aureus* in the neutrophils was far less than expected. The relationship between the *in vitro* intracellular effect and the clinical effect of RXM is not clear. Many factors work *in vivo* to prevent infection, including the neutrophils, serum, and antimicrobial agents. The present study indicates that the role of the intracellular activities of these two drugs is less than would be expected from the MIC. Further experiments should test the effects of other drugs on different strains of bacteria in order to better understand these effects.

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ヒト好中球に貪食された黄色ブドウ球菌に対するオフロキサシンとロキシスロマイシンの細胞内活性の検討

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抗菌薬の中にはニューキノロン系やマクロライド系等のように，血清中よりも好中球内に高濃度に移行すると報告されているものがある。好中球内濃度が比較的高くなるオフロキサシン(OFLX) とロキシスロマイシン (RXM) について，*in vitro* でヒトの好中球に貪食させた黄色ブドウ球菌に対する効果を調べた。黄色ブドウ球菌は膿痂疹より分離した1株とATCC 29213株とを使用した。血清存在下にヒト好中球に黄色ブドウ球菌を37℃で貪食させ，外の菌はリゾスタフィンで除きOFLX 0.2, 0.25, 1, 2, 4, 8, 16, 32 mg/l またはRXM 2,16 mg/l を6時間作用させた。好中球を超音波で破壊し,平板寒天培地にうえてコロニーを数え生菌数を測定した。抗菌薬を作用させない場合と比較してOFLX 1 MIC程度の低濃度では両菌株とも生菌数に差はなかった。濃度が高くなると生菌数は有意に低下したが，その程度は比較的少なかった。RXMでは生菌数の低下がごく軽度見られた。好中球内の黄色ブドウ球菌は両薬剤の影響を逃れ，生き残ることが考えられた。

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