Effect of Telithromycin (HMR 3647) on Polymorphonuclear Neutrophil Killing of *Staphylococcus aureus* in Comparison with Roxithromycin

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HMR 3647 (telithromycin), a new ketolide, is active on intracellular pathogens. It was previously demonstrated that it inhibits superoxide anion production in a time- and concentration-dependent manner, at concentrations which inhibit 50% of the control response of about 55 µg/ml (5 min) to 30 µg/ml (30 min); these values are similar to those obtained with roxithromycin, a classical erythromycin A derivative. Here we investigated whether these drugs modified the bactericidal activity of human polymorphonuclear neutrophils (PMN) on four strains of *Staphylococcus aureus* with different profiles of susceptibility to macrolides and ketolides. We found that the main factor involved in killing was the antibacterial potency of the drugs, although combinations of antibiotics with PMN were slightly more active than each component used alone against two of the four strains. In addition, high concentrations of the drugs, which impaired the PMN oxidative burst, did not impair PMN bactericidal activity. Likewise, some cytokines which enhance PMN oxidative metabolism did not modify PMN bactericidal activity in the presence or absence of macrolides or ketolides. These data suggest that oxygen-independent mechanisms contribute to the bactericidal activity of PMN on these strains of *S. aureus*. Both live and/or heat-killed bacteria impaired the uptake of telithromycin and roxithromycin (but not that of levofloxacin, a quinolone) in a concentration-dependent manner, owing to a modulation of PMN transductional systems involved in the activation of the macrolide carrier.

Polymorphonuclear neutrophils (PMN) are cornerstones of host defenses against bacterial infection. A major mechanism by which these cells destroy pathogens is the production of reactive oxygen species in the phagocytic vacuole after the activation of a complex enzymatic system, the membrane NADPH oxidase (8). It has been observed that erythromycin A-derived macrolides significantly impair oxidant generation by PMN (1, 2, 3, 19). However, macrolide administration clearly does not result in impaired bacterial eradication in vivo. Synergy between some macrolides and PMN for bacterial killing has even been suggested (4, 5, 20). HMR 3647 (telithromycin) belongs to a new class of semisynthetic erythromycin A derivatives, the ketolides, characterized by a 3-keto group in place of the L-cladinose moiety at position C-3 of the lactone ring (10). HMR 3647 was one of the most active compounds on respiratory pathogens in a series of 11,12-cyclo-disubstituted ketolides synthesized by Roussel-Uclaf (11). In addition, HMR 3647 is active against intracellular microorganisms (e.g., *Chlamydia pneumoniae*, *Legionella pneumophila*, *Rickettsia* spp., and *Toxoplasma gondii*) (6, 9, 25, 26). It accumulates to significantly higher levels than roxithromycin and also inhibits oxidant production (30). Here we examined whether telithromycin modified the bactericidal activity of PMN on four strains of *Staphylococcus aureus* with different patterns of susceptibility to macrolides and ketolides. Roxithromycin was used as a

comparative agent. The effect of bacteria on telithromycin and roxithromycin uptake by PMN was also assessed.

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MATERIALS AND METHODS

Antimicrobial agents. Telithromycin, roxithromycin, levofloxacin, and the radiolabeled drugs [³H]telithromycin (39.4 Ci/mmol) and [³H]roxithromycin (21.9 Ci/mmol) in ethanol-water (7/3, vol/vol) and [³H]levofloxacin (66.2 Ci/mmol) in ethanol-toluene (2/3, vol/vol) were provided by Aventis (Romainville, France). To assess antibacterial activity, unlabeled drugs were solubilized in acetic acid (1 mg in 100 µl) and further diluted in sterile Hanks' buffered salt solution (HBSS; Sigma, Paris, France) to the desired concentrations. To measure drug uptake, tritiated drugs (2.5 µl; about 30 µg/ml) were mixed with 25 µl of unlabeled drugs (1 mg/ml of HBSS) and 222.5 µl of HBSS. Stock solutions were further diluted in HBSS to a final concentration of 2.5 µg/ml.

Chemicals. Phorbol myristate acetate (PMA; a protein kinase C [PKC] activator), formylmethionyl-leucyl-phenylalanine (fMLP; a synthetic tripeptide analogous with bacterial chemotaxins), genistein (a tyrosine kinase inhibitor), and H89 (a protein kinase A [PKA] inhibitor) were obtained from Sigma, Saint Quentin Fallavier, France.

Cytokines. Sterile endotoxin-free tumor necrosis factor alpha (TNF-α [5.88 × 10⁷ U/mg]) and granulocyte-macrophage colony-stimulating factor (GM-CSF [1.82 × 10⁸ U/mg]) were obtained from Genzyme Corporation (Cambridge, Mass.). Purity was greater than 98%. Cytokines were prepared in 0.1% human serum albumin solution (Laboratoire Français de Fonctionnement et de Biotechnologie, Les Ulis, France).

PMN. PMN were obtained from venous blood of healthy volunteers by Ficoll-Paque centrifugation followed by 2% dextran sedimentation and osmotic lysis of residual erythrocytes. The viability and purity of the PMN preparation, as assessed by trypan blue exclusion, were both greater than 96%.

Bacteria. Four strains of *S. aureus* were provided by C. Muller-Seyries (Laboratoire de Bactériologie, Andremont, CHU Xavier Bichat-Claude Bernard,

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TABLE 1. MICs for *S. aureus*

<i>S. aureus</i> strain	MIC (mg/liter) of:		
	Erythromycin	Roxithromycin	Telithromycin
A	>128	>128	>128
B	>128	>128	0.06
C	0.125	1	0.06
D	0.125	0.5	0.03

Paris, France), who also determined antibiotic susceptibility by a macrodilution method with solid Mueller-Hinton agar (Biomérieux, Balme-les-grottes, France). MICs were determined with an inoculum of 10^5 CFU (Table 1). Three strains (A, B, and C) were methicillin resistant. Strain A had a constitutive macrolide-lincosamide-streptogramin B resistance phenotype, and strain B had an inducible macrolide-lincosamide-streptogramin B resistance phenotype.

Bacteria were grown in Trypticase soy broth (Sanofi, Diagnostic Pasteur, Marnes-la Coquette, France). On the day of the experiments, an overnight culture of *S. aureus* was centrifuged and resuspended in Trypticase soy broth at an optical density (490 nm) of 0.01 (approximately 10^6 CFU/ml). The bacterial suspension was further incubated in a shaking water bath to obtain bacteria in the logarithmic growth phase. The bacteria were used after centrifugation and suspension of the pellet in the same volume of HBSS.

Bactericidal activity. Three experimental protocols were used to mimic possible in vivo situations: combination of PMN, normal human serum (NHS), antibiotics, and bacteria (global assay); incubation of PMN with antibiotics before the addition of bacteria (prephagocytosis exposure); and incubation of PMN with bacteria before the addition of antibiotics (postphagocytosis exposure).

(i) **Global assay.** A total of 2.5×10^6 PMN in 1 ml of HBSS containing 10% NHS (from the same blood donor as the PMN) were incubated with the bacterial suspension (2.5×10^6 to 2.5×10^8 CFU in HBSS) and antibiotics or HBSS. Controls were obtained by replacing PMN with HBSS. Samples of 100 μ l were taken immediately after the addition of bacteria (time zero [T0]) and at 30 and 60 min. PMN were lysed by vigorous shaking in 900 μ l of sterile water at 0°C. Serial dilutions were then made in sterile water before plating on Trypticase soy agar (Sanofi, Diagnostic Pasteur). CFU were counted after overnight incubation at 37°C.

In some experiments, PMN were pretreated with cytokines (TNF- α at 100 U or GM-CSF at 0.125 pmol per 2.5×10^6 PMN) for 30 min at 37°C before treatment with bacteria, serum, and antibiotics for a further 30 min. Bactericidal activity was assessed as described above. Controls were composed of PMN alone, cytokines alone, or HBSS incubated for 30 min.

(ii) **Prephagocytosis exposure.** PMN (2.5×10^6) plus 10% NHS and antibiotics or HBSS were incubated for 30 or 60 min at 37°C before treatment with bacteria (T0) for a further 30 min. Controls were HBSS or antibiotics plus 10% NHS incubated for 30 to 60 min at 37°C. CFU were counted as described above.

(iii) **Postphagocytosis exposure.** PMN (2.5×10^6) plus 10% NHS and bacteria were incubated for 30 min at 37°C before addition of the antibiotic solution and incubation for a further 30 min. Samples were taken just after the addition of the antibiotics (T0) and 30 min later. CFU were counted as described above. Controls were composed of bacteria incubated alone in HBSS plus 10% NHS before addition of HBSS or antibiotics.

In the latter two assays, in some experiments with PMN plus bacteria or PMN plus antibiotics, cells were centrifuged at $12,000 \times g$ for 3 min at 22°C through a water-impermeable silicone-paraffin oil barrier (86:14 [vol/vol]), and the pellet was resuspended in an equivalent volume of HBSS plus 10% NHS before addition of antibiotics or bacteria. For prephagocytosis exposure, controls included telithromycin alone at concentrations theoretically associated with the cell pellet, as calculated by assuming cellular concentration/extracellular concentration ratios of about 100 at 30 min and 200 at 60 min and a volume of about 0.6 μ l for a cell pellet containing 2.5×10^6 PMN (30). For an extracellular concentration of 10 μ g/liter, this calculation yields total amounts of 0.6 μ g at 30 min and 1.2 μ g at 60 min; these quantities were diluted in HBSS to reach a final volume of 1 ml, as in the other tests.

Calculations. The following parameters were calculated in the three experimental assays: percentage of bacterial survivors ([CFU in the test sample/CFU in the inoculum] $\times$ 100) and relative bacterial decrease index ([CFU in the presence of PMN plus antibiotics or HBSS/CFU in the presence of antibiotics or HBSS alone] $\times$ 100); this index can also be calculated as the ratio of survival in the presence of PMN to the overall growth for each sample times 100. The former index gives either the percentage of growth (test samples containing

HBSS with or without antibiotics) or the percentage of overall survival (test samples containing PMN with or without antibiotics) compared to data for the inoculum at T0. The latter index reflects the part played by PMN in the bactericidal combination: when the combination (PMN plus antibiotic or HBSS) is as effective as the antibiotic or HBSS alone, then the index is 100; when the combination is less active than the antibiotic alone, then the index is greater than 100; when PMN activity reinforces the effect of the antibiotic (or when the bacterial growth in HBSS is marked, whether or not PMN kill the bacteria), then the index is less than 100.

Drug uptake in the presence of bacteria. A radiometric assay was used to measure macrolide uptake (30). Briefly, 2.5×10^6 PMN in 500 μ l of HBSS plus 10% NHS were incubated at 37°C with the radiolabeled drugs plus live or heat-killed bacteria or the synthetic analog of bacterial chemotaxins, fMLP, at 10^{-7} M (a concentration that does not trigger the oxidative burst) for 5 or 30 min. They were then centrifuged at $12,000 \times g$ for 3 min at 22°C through a water-impermeable silicone-paraffin oil barrier (86:14 [vol/vol]). The pellet was solubilized in Hionic fluor (Packard), and cell-associated radioactivity was quantified by liquid scintillation counting (LS-6000-S; Beckman). Standard dilution curves were used to determine the amounts of cell-associated drug. Results were expressed as nanograms per 2.5×10^6 PMN. The extracellular macrolide concentration used in the assays was 2.5 μ g/ml. The quinolone levofloxacin was assessed in the same experiments.

To analyze the impact of bacterial phagocytosis on drug uptake, PMN were first incubated for 30 or 60 min with bacteria plus 10% NHS before addition of the radiolabeled drugs, and uptake was measured at 5 and 30 min as described above.

In other experiments, PMN were pretreated for 15 min with various pharmacologic agents (PMA at 10 ng/ml, H89 at 50 μ M, and genistein at 50 μ M) that modify macrolide uptake (29, 30, 31); this step was followed by addition of HBSS (control) or *S. aureus* A (2×10^7 to 4×10^7 CFU) and the radiolabeled drugs and incubation for a further 5 min before measurement of drug uptake.

Statistical analysis. Results are expressed as the mean and one standard deviation (SD) of the mean for n experiments conducted with PMN from different volunteers. Each experiment was done in duplicate or triplicate, and the results of each experiment are given as the mean. Intra-assay variability was less than 5%. The bactericidal experiments were performed with PMN from 26 blood bank donors who gave informed consent. PMN from 16 of these donors were also assessed for macrolide uptake. Analysis of variance, regression analysis, and Student's t test for paired data were used to determine statistical significance. All tests were performed with the Statworks program, version 1.2 (Cricket Software).

RESULTS

Global bactericidal assay. *S. aureus* strain A grew moderately during the experimental period (46% at 60 min; $P = 0.039$) (Fig. 1A). Growth was slightly impaired in the presence of PMN, and this effect was significant only at 60 min (88% of the inoculum; the P value was 0.005 for a comparison against the control without PMN). The growth-limiting effect of PMN resulted in a relative bacterial decrease index of less than 100, an effect which was statistically significant (about 56%) only at 60 min (the P value was <0.01 for a comparison against a value of 100) (Table 2). Telithromycin and roxithromycin (1 to 10 μ g/ml) did not modify directly either bacterial growth or the growth inhibition exerted by PMN. The combinations of PMN plus telithromycin at 10 μ g/ml and PMN plus roxithromycin at 1 or 10 μ g/ml seemed to be slightly more rapidly bactericidal than PMN plus HBSS. This conclusion was indicated by the relative bacterial decrease index, which was significantly less than 100 for both combinations (Table 2), and by the percentage of bacterial survivors at 30 min (about 90%; the P value was <0.05 for a comparison against the respective controls without PMN [about 140%]) (Fig. 1A); however, this effect was not significant when compared with PMN alone. A high concentration of telithromycin or roxithromycin (50 μ g/ml), which initially inhibits oxidant generation by PMN by about 50 to 60% (1, 30) and totally abrogates it at 180 min, was also tested.

FIG. 1. Global bactericidal assay. Bacteria were incubated for 30 or 60 min with HBSS (solid lines) or PMN (broken lines) in the presence of HBSS, telithromycin (TEL), or roxithromycin (ROX). Results, expressed as percent survival of the inoculum, are given as the means and one SD for controls and the means alone for antibiotics. (A) *S. aureus* A (3×10^6 to 4×10^7 CFU/ 2.5×10^6 PMN), five to seven experiments. (B) *S. aureus* B (3×10^6 to 1.2×10^8 CFU/ 2.5×10^6 PMN), four to six experiments. (C) *S. aureus* C (2.5×10^6 to 1.2×10^7 CFU/ 2.5×10^6 PMN), three or four experiments. (D) *S. aureus* D (1.5×10^7 to 2.7×10^7 CFU/ 2.5×10^6 PMN), three experiments.

FIG. 1—Continued.

Bacterial growth and PMN bactericidal function were not impaired by this high concentration at up to 180 min (percentages of growth, 153, 150, and 135%, and percentages of bacterial survivors in the presence of PMN, 39, 30, and 35%, respec-

tively, for the control, telithromycin, and roxithromycin [means of two experiments]).
S. aureus strain B was resistant to macrolides and susceptible to telithromycin (Table 1). It did not grow significantly over the

TABLE 2. Relative bacterial decrease indices

Drug concn ($\mu\text{g/ml}$)	Relative bacterial decrease index (no. of experiments) for the following <i>S. aureus</i> strain ^a :			
	A	B	C	D
None				
30 min	74 $\pm$ 26.7 (6)	81 $\pm$ 37.7 (6)	82 $\pm$ 12.1 (3)	67 $\pm$ 9.9 ^b (3)
60 min	56 $\pm$ 21.8 ^b (7)	85 $\pm$ 38.8 (4)	62 $\pm$ 17.6 ^b (4)	43 $\pm$ 4.2 (2)
Telithromycin				
30 min				
0.1	ND	88 $\pm$ 61.8 (4)	70 $\pm$ 10.7 ^b (3)	81 $\pm$ 10.7 (3)
1	85 $\pm$ 26.5 (5)	86 $\pm$ 14.9 (5)	75 $\pm$ 18.5 ^b (3)	89 $\pm$ 24.3 (3)
5	ND	142 $\pm$ 82.7 (2)	62 $\pm$ 14.0 (2)	78 $\pm$ 31.8 (2)
10	72 $\pm$ 22.6 ^b (5)	71 $\pm$ 35.4 (2)	ND	ND
60 min				
0.1	ND	62 $\pm$ 4.4 ^b (3)	53 $\pm$ 17.3 ^b (3)	66 $\pm$ 7.8 (2)
1	65 $\pm$ 10.4 ^b (6)	116 $\pm$ 62.4 (4)	50 $\pm$ 19.6 ^b (3)	94 $\pm$ 8.5 (2)
5	ND	75 $\pm$ 25.4 (2)	67 $\pm$ 24.7 (3)	88 $\pm$ 17.6 (2)
10	58 $\pm$ 17.4 ^b (6)	200 (1)	ND	ND
Roxithromycin				
30 min				
1	67 $\pm$ 19.4 ^b (5)	71 $\pm$ 23.8 ^b (6)	88 $\pm$ 27.5 (3)	96 $\pm$ 24.0 (3)
5	ND	80 $\pm$ 13.5 ^b (2)	67 $\pm$ 11.9 (2)	61 $\pm$ 8.5 (2)
10	68 $\pm$ 17.8 ^b (5)	75 $\pm$ 5.0 ^b (3)	ND	ND
60 min				
1	59 $\pm$ 24.6 ^b (4)	61 $\pm$ 28.4 (4)	62 $\pm$ 10.4 ^b (3)	64 $\pm$ 7.8 (2)
5	ND	75 $\pm$ 16.2 (2)	55 $\pm$ 16.7 ^b (3)	65 $\pm$ 9.2 (2)
10	61 $\pm$ 20.6 ^b (6)	ND	ND	ND

^a The relative bacterial decrease index is defined in Materials and Methods. Results are given as the mean and 1 SD. ND, not done.

^b The *P* value for comparisons with a value of 100% was <0.05 .

experiment period, and PMN did not reduce the inoculum (Fig. 1B and Table 2). Telithromycin at 0.1 and 1 $\mu\text{g/ml}$ was rapidly bactericidal (Fig. 1B), with about 50 and 70% killing, respectively, at 30 min; this effect was not increased at 60 min. Concentrations of 5 and 10 $\mu\text{g/ml}$ were not more effective than 1 $\mu\text{g/ml}$ (35% $\pm$ 35.4% and 49% $\pm$ 23.2% survival at 30 min and 38% $\pm$ 31.1% and 24% survival at 60 min for telithromycin at 5 and 10 $\mu\text{g/ml}$, respectively [one or two experiments]). The presence of PMN did not modify the bactericidal activity of telithromycin, as indicated by a relative bacterial decrease index of about 100 (Table 2), except for the concentration of 0.1 $\mu\text{g/ml}$; the combination of PMN and telithromycin at 0.1 $\mu\text{g/ml}$ was more bactericidal than telithromycin alone at 60 min (37 and 59% survival, respectively; the *P* value was <0.05 for a comparison against telithromycin alone), as shown by a relative bacterial decrease index that was significantly less than 100 (Table 2). Roxithromycin did not impair bacterial growth, but the combination of PMN and roxithromycin (1, 5, and 10 $\mu\text{g/ml}$) was bactericidal at 30 min, with about 75% survival (the *P* value was <0.05 for a comparison against the control [PMN alone]) (Fig. 1B); accordingly, the relative bacterial decrease index was significantly less than 100 (Table 2). This effect was not significant at 60 min.

S. aureus strain C was susceptible to telithromycin and roxithromycin (Table 1). This strain did not grow over the 60-min study period, and PMN were bactericidal only at 60 min (about 60% survival; *P* < 0.01) (Fig. 1C); this effect resulted in a significant relative bacterial decrease index of about 62 (Table 2). At 30 min, the bactericidal activity of telithromycin was not

significant (85, 67, and 68% survival in the presence of 0.1, 1, and 5 $\mu\text{g/ml}$, respectively). However, the combination of telithromycin and PMN was bactericidal, with 60, 48, and 39% survival, respectively, at 0.1, 1, and 5 $\mu\text{g/ml}$ (the *P* value was <0.05 for a comparison against the control [PMN or telithromycin alone]). Direct bactericidal activity of telithromycin was observed at 60 min (69% $\pm$ 11.1%, 44% $\pm$ 4.9%, and 37% $\pm$ 6.0% survival in the presence of 0.1, 1, and 5 $\mu\text{g/ml}$, respectively; the *P* value was <0.01 for three experiments). The bactericidal activity of PMN was not modified by telithromycin at 60 min (Table 2). Roxithromycin (0.1 to 5 $\mu\text{g/ml}$) was not bactericidal for this strain at 30 min, and only the 5- $\mu\text{g/ml}$ concentration was bactericidal at 60 min (77% $\pm$ 1.5% survival; *P* < 0.001) (Fig. 1C). The presence of roxithromycin did not modify the bactericidal activity of PMN.

Macrolide-susceptible *S. aureus* strain D did not grow during the 60-min study period and was significantly killed by PMN at 30 and 60 min (about 60 and 40% survival, respectively) (Fig. 1D). Telithromycin exhibited a rapid bactericidal effect (42 and 44% survival at 30 min and 26 and 30% survival at 60 min, respectively, for 1 and 5 $\mu\text{g/ml}$). At 60 min, the 0.1- $\mu\text{g/ml}$ concentration was also bactericidal (68% survival). The bactericidal activity of the combination of telithromycin plus PMN was mainly due to the drug (68, 37, and 31% survival at 30 min and 44, 24, and 26% survival at 60 min, respectively, at 0.1, 1, and 5 $\mu\text{g/ml}$). Accordingly, the relative bacterial decrease index was about 100 (Table 2). Roxithromycin was not bactericidal over the 60-min study period and did not modify the bactericidal activity of PMN (Fig. 1D).

Owing to the direct bactericidal effect of telithromycin, it was not possible to demonstrate an inhibitory effect of the 50- $\mu\text{g/ml}$ concentration on PMN killing of *S. aureus* strains B, C, and D.

Effect of cytokines. PMN pretreated with TNF- α or GM-CSF exhibit an enhanced oxidative response to stimulation (29). Here, we pretreated cells for 30 min with optimal concentrations of cytokines (TNF- α at 100 U/ml and GM-CSF at 125 pM) before addition of *S. aureus* A or C and antibiotics at 1 or 10 $\mu\text{g/ml}$ and incubation for 30 min. Bacterial growth was not modified by the cytokines, and neither was the bactericidal activity of PMN. Neither the direct bactericidal activity of telithromycin and roxithromycin nor that of PMN in the presence or absence of antibiotics was altered by the two cytokines (data not shown).

Prephagocytosis exposure. PMN were pretreated for 30 or 60 min at 37°C with antibiotics before addition of bacteria and incubation for 30 min. Controls were composed of HBSS or antibiotics plus 10% NHS incubated at 37°C. Against the macrolide- and ketolide-resistant strain A, PMN bactericidal activity was not influenced by HBSS or antibiotic pretreatment (about 70% survival at 30 min). With regard to macrolide-resistant, ketolide-susceptible strain B, the bactericidal activity of telithromycin (1 to 10 $\mu\text{g/ml}$) after incubation in the presence of NHS for 30 min (Table 3) was unchanged compared to that obtained without incubation (about 30% survival) (see above). Telithromycin-pretreated PMN had bactericidal activity similar to that of the drug alone (22 to 24% survival at 30 min). When telithromycin was incubated with NHS for 60 min, it also retained its bactericidal activity, and the bactericidal activity of telithromycin-treated PMN was similar to that of the

TABLE 3. Prephagocytosis exposure

Treatment ($\mu\text{g/ml}$) ^a	Result (%) after pretreatment with antibiotic for the following min ^b :			
	30		60	
	Growth	Survival	Growth	Survival
Strain A				
Control (HBSS)	79 $\pm$ 18.4	73 $\pm$ 5.0	78	75
TEL (1)	76 $\pm$ 12.7	74 $\pm$ 7.1	88	86
TEL (5)	79 $\pm$ 18.4	81 $\pm$ 5.0	91	83
TEL (10)	80 $\pm$ 14.9	65 $\pm$ 6.4	96	97
ROX (10)	93 $\pm$ 9.9	74 $\pm$ 5.0	92	95
Strain B				
Control (HBSS)	107 $\pm$ 32.6	98 $\pm$ 26.1	149 $\pm$ 46.0	112 $\pm$ 64.5
TEL (1)	30 $\pm$ 18.3 ^c	24 $\pm$ 14.3 ^c	17	33
TEL (5)	28 $\pm$ 9.0 ^c	24 $\pm$ 6.6 ^c	61 $\pm$ 43.1	43 $\pm$ 25.5
TEL (10)	28 $\pm$ 12.6 ^c	22 $\pm$ 5.6 ^c	62 $\pm$ 41.0	36 $\pm$ 25.5
ROX (10)	79 $\pm$ 31.6	77 $\pm$ 35.4	123 $\pm$ 49.5	104 $\pm$ 17.0
Strain C				
Control (HBSS)	160 $\pm$ 84.9	170 $\pm$ 14.1	176 $\pm$ 39.6	169 $\pm$ 9.9
TEL (1)	113	84	94 $\pm$ 4.2	82 $\pm$ 14.1
TEL (5)	92	70	75 $\pm$ 14.1	89 $\pm$ 0.7
TEL (10)	71 $\pm$ 21.2	65 $\pm$ 12.7	96 $\pm$ 5.7	62 $\pm$ 2.8
ROX (10)	90 $\pm$ 14.1	121 $\pm$ 35.4	142 $\pm$ 26.2	150 $\pm$ 2.8

^a TEL, telithromycin; ROX, roxithromycin.

^b The growth and survival indices are defined in Materials and Methods. Results are given as the mean and 1 SD. Numbers of experiments for 30 and 60 min: strain A, three and one, respectively; strain B, three to six and one to three, respectively; and strain C, one or two and two, respectively.

^c The *P* value for comparisons with a value of 100% was <0.05 .

drug alone. PMN pretreated with roxithromycin were not more active against *S. aureus* B than were HBSS-treated control cells (Table 3). The growth of strain C was not impaired by control PMN treated for 30 or 60 min with NHS. Telithromycin alone (1 to 10 $\mu\text{g/ml}$) tended to be bacteriostatic, and similar activity was observed with telithromycin-treated PMN. The activities of roxithromycin- and roxithromycin-treated PMN did not differ from those of their respective controls.

In two experiments, PMN were pretreated for 30 min with 10% NHS plus telithromycin at 10 $\mu\text{g/ml}$ or HBSS and then isolated by centrifugation through a water-impermeable oil cushion to exclude the extracellular medium. *S. aureus* B was then added and incubated for a further 30 min. Controls included HBSS plus NHS, telithromycin plus NHS, PMN pretreated with HBSS or telithromycin plus NHS but not centrifuged, and a telithromycin concentration (0.6 $\mu\text{g/ml}$) corresponding to the theoretical cellular accumulation of this drug (see Materials and Methods). The results are shown in Fig. 2. Although PMN did not kill *S. aureus* B (Fig. 1 and Table 3), PMN alone slightly reduced bacterial growth (73% $\pm$ 9.2% survival). Telithromycin alone had a strong bactericidal effect, which did not differ from that obtained in the presence of PMN (19 and 21% survival, respectively). Telithromycin (0.6 $\mu\text{g/liter}$) also strongly reduced bacterial survival (30%). When PMN were centrifuged through the oil cushion, they did not retain their bactericidal activity (143% $\pm$ 24.0% survival), although they appeared to be viable by trypan blue exclusion. It is possible that passage through the oil cushion either impairs their phagocytic function or causes membrane changes and cell deactivation. However, when PMN were pretreated with telithromycin, they still reduced bacterial counts (45% $\pm$ 18.4%

survival, a value comparable to that obtained with the theoretical intracellular concentration of telithromycin [0.6 $\mu\text{g/ml}$]). One of the two PMN samples was also assessed in an experiment with 60 min of PMN pretreatment with HBSS or telithromycin at 10 $\mu\text{g/ml}$. Similar controls were used, except for the theoretical concentration of telithromycin, which was changed to 1.2 $\mu\text{g/ml}$ (see Materials and Methods). After centrifugation through the oil cushion, PMN were unable to kill bacteria (175% survival at 30 min), whereas control PMN were still able to do so (41% survival). PMN pretreated with telithromycin had a similar bactericidal effect whether or not they were centrifuged (19 and 28% survival, respectively), and this effect was comparable to that of the theoretical intracellular telithromycin concentration (20% survival).

Postphagocytosis exposure. Only *S. aureus* strains A and B were analyzed in this assay. We first checked that less than 1% of the bacterial inoculum was able to cross the oil cushion during centrifugation, ensuring that bacteria present in the cell pellet would be intracellular or cell associated. After incubation of strain A with PMN for 30 min, only 62% $\pm$ 12.6% of the inoculum survived and only 6% $\pm$ 2.5% was associated with the PMN pellet (three experiments). At variance with the results obtained in the prephagocytosis experiments, isolated PMN still continued to kill cell-associated bacteria, resulting in almost complete elimination after 30 min (3% $\pm$ 1.02% of the initial inoculum). This result suggests that the decreased bactericidal activity observed after prephagocytosis exposure was due to an impairment of phagocytosis after passage through the oil cushion; in contrast, since in the postphagocytosis experiment phagocytosis had already occurred before the centrifugation procedure, the intracellular bactericidal activity of PMN could be maintained. Telithromycin (1 and 10 $\mu\text{g/ml}$) did not significantly modify PMN bactericidal activity, whether or not PMN were centrifuged after incubation with bacteria.

S. aureus strain B did not grow during the first 30 min (102% $\pm$ 3.5%; three experiments), but the inoculum increased by about 178% over the next 30 min ($P = 0.008$). During this period, telithromycin (1 and 10 $\mu\text{g/ml}$) considerably reduced bacterial growth (17 and 14% of the inoculum, respectively). In the presence of PMN, the inoculum was reduced by about 50% ($P < 0.01$) during the first 30 min. The growth-limiting effect of PMN was further demonstrated in the subsequent 30-min period by the relative bacterial decrease index of 20 ± 6.7 ($P < 0.01$). When telithromycin was added to PMN after bacterial phagocytosis, it did not strongly modify PMN bactericidal activity; the global bactericidal effect (PMN plus drug) was smaller than that observed with the drug alone (37% $\pm$ 25.3% and 45% $\pm$ 34.1% survival versus 17% $\pm$ 1.7% and 14% $\pm$ 1.7% survival, respectively, for telithromycin at 1 and 10 $\mu\text{g/ml}$ with PMN versus without PMN). The resulting relative bacterial decrease indices (bacterial survivors in the presence of PMN plus telithromycin/survivors in the presence of telithromycin) were 225 ± 164.4 (1 $\mu\text{g/ml}$) and 305 ± 204.6 (10 $\mu\text{g/ml}$), suggesting that the drug was more active on extracellular than on intracellular bacteria. When PMN were isolated through the oil cushion, 9% $\pm$ 6.4% of the bacterial inoculum was associated with the cell pellet. In the subsequent 30-min incubation period, the killing activity of isolated PMN continued, with less than 2% survivors at the end of the assay; this result was not modified in the presence of telithromycin.

FIG. 2. Prephagocytosis exposure. A total of 2.5×10^6 PMN were incubated with HBSS or telithromycin (TEL) (10 $\mu\text{g/ml}$) for 30 min. The cells were then either centrifuged through an oil cushion (B) or not centrifuged (A) before the addition of *S. aureus* B (6×10^8 to 8×10^8 CFU) and incubation for a further 30 min. For technical details and controls, see Results. Results, expressed as percent inoculum survival, are given as the mean and one SD of two experiments.

Modulation of telithromycin and roxithromycin uptake by bacteria. Macrolide intracellular activity and blockade of PMN oxidative metabolism are linked to the cellular uptake of these drugs. As telithromycin was less active on intracellular bacteria than on extracellular bacteria in the postphagocytosis assay, we investigated whether opsonized or unopsonized *S. aureus* modified telithromycin and roxithromycin uptake. We first evaluated the effect of NHS. Telithromycin uptake was slightly enhanced in the presence of 10% NHS, and this effect was significant at 30 min ($113\% \pm 11.4\%$ of the control value in seven experiments; $P < 0.05$). In contrast, roxithromycin uptake was moderately reduced at 5 and 30 min ($83\% \pm 30.2\%$ in 15 experiments and $72\% \pm 30.4\%$ in 7 experiments; $P > 0.05$). Bacteria resuspended in HBSS (with or without 10% NHS) were then added to PMN, and drug uptake was measured at 5 and 30 min. At 5 min, the uptake of both drugs was impaired by *S. aureus* strains A, B, and D in a bacterial density-dependent manner. In general, the inhibition was greater in the absence of serum and was slightly more marked with roxithromycin.

We used logarithmic regression analysis to determine the numbers of bacteria (CFU) which impaired drug uptake by about 50%. They were as follows for telithromycin (with or without NHS) and roxithromycin (with or without NHS) (CFU/ 2.5×10^6 PMN; $P < 0.05$ [for all analyses]; $r = 0.712$ to

0.967): *S. aureus* A, 3.5×10^8 , 3.5×10^7 , 4×10^7 , and 2.2×10^7 ; *S. aureus* B, 2×10^8 , 4.5×10^7 , 8×10^7 , and 3.6×10^7 ; and *S. aureus* D, 2.9×10^8 , $>2 \times 10^8$, 4.6×10^8 , and 9.5×10^7 . *S. aureus* C at densities of $\leq 2 \times 10^8$ CFU/ 2.5×10^6 PMN inhibited only roxithromycin uptake in the absence of serum. Heat-killed bacteria and fMLP, a synthetic formylated tripeptide analogous to bacterial chemotaxins and a stimulus of PMN, had the same effect as whole bacteria (data not shown). Similar results were obtained with another macrolide, clarithromycin (data not shown); however, the uptake of levofloxacin, a fluoroquinolone which displays passive accumulation (28), was not modified, whether or not the bacteria were opsonized (90 to 116% of the control values). The inhibitory effect of bacteria on drug uptake at 30 min did not differ from that obtained at 5 min.

In further experiments, PMN were first allowed to engulf bacteria for 30 or 60 min before the drugs were added and incubated for a further 5 or 30 min (Fig. 3). The short pretreatment period with opsonized bacteria did not modify the first phase (5 min) of telithromycin accumulation but resulted in a significant decrease (about 50%; $P < 0.05$) at 30 min (Fig. 3A). However, when PMN were incubated for 60 min with bacteria, there was no significant change in telithromycin accumulation (about 80 to 96% of control uptake at 5 and 30

FIG. 3. Inhibition of antibiotic uptake by bacteria. A total of 2.5×10^6 PMN were incubated with HBSS or bacteria for 30 min (A) or 60 min (B) before the addition of telithromycin ($2.5 \mu\text{g/ml}$) and incubation for 5 to 30 min. Results are given as the mean and 1 SD of three experiments (control) and the mean of two or three experiments (bacteria). CFU of bacteria were as follows: *S. aureus* A, 4×10^6 to 6×10^6 ; *S. aureus* B, 3×10^6 to 13×10^6 ; *S. aureus* C, 2.5×10^6 to 20×10^6 ; and *S. aureus* D, 4×10^6 to 35×10^6 .

FIG. 4. Effect of PMN or inhibitors on telithromycin uptake in the presence of *S. aureus* A (2×10^7 to 4×10^7 CFU). PMN were pretreated with pharmacologic agents (PMA, 10 ng/ml; H89, 50 μ M; and genistein, 50 μ M) for 15 min before the addition of bacteria and telithromycin (2.5 μ g/ml) and incubation for 5 min. Results are given as the mean and 1 SD of control percent uptake in three experiments. An asterisk indicates a *P* value of <0.05 for comparisons with the control (100%).

min) (Fig. 3B). Similar data were obtained with roxithromycin (data not shown).

We further studied the possibility of interactions between bacteria and various PMN activators or inhibitors which are known to modulate macrolide or ketolide uptake (29, 30); we studied PMA, the protein kinase C activator which impairs macrolide and ketolide uptake, and H89, a PKA inhibitor which increases the uptake of azithromycin but impairs that of roxithromycin. *S. aureus* A was chosen as the test bacterium. PMA at a concentration (10 ng/ml) which does not activate the PMN oxidative burst decreased by 40% the uptake of telithromycin, and *S. aureus* A (2×10^7 to 4×10^7 CFU/ 2.5×10^6 PMN) decreased it by about 50% (Fig. 4). When both inhibitors were present, the inhibitory effect was about 80%, suggesting an additive effect. H89 (50 μ M) increased telithromycin uptake by about 50%; however, the inhibitory effect of *S. aureus* A predominated when the combination of H89 and bacteria was used, indicating that the stimulatory action of H89 occurs downstream of the target of the bacterial effect. Interestingly, the tyrosine kinase inhibitor genistein did not significantly modify telithromycin uptake but greatly increased the inhibitory effect of *S. aureus*.

DISCUSSION

A major property of macrolides which largely explains their use in infections caused by intracellular pathogens is their ability to enter and concentrate within host cells (15, 16). In

addition, this cellular accumulation of macrolides may modify cell functions. This behavior is particularly well documented for phagocytes and erythromycin A derivatives (1, 2) and may account, at least in part, for the anti-inflammatory activities of these antibiotics (17, 18). The immunosuppressive properties of macrolides have also been implicated as a potential mechanism facilitating bacterial superinfection (23). However, there are no reported cases of macrolide-induced superinfection, even after long-term administration of erythromycin A derivatives (14), and synergy with the bactericidal functions of PMN has been observed in vitro (4, 5, 20). The ketolide telithromycin has been shown to inhibit PMN oxidant-generating capacity (30). Whether or not telithromycin-mediated inhibition of superoxide production also impairs the bactericidal activity of PMN has not been studied. Here, we analyzed two aspects of the interactions among bacteria, PMN, and two erythromycin A derivatives (telithromycin and roxithromycin, the latter as a comparative agent), namely, (i) the bactericidal activity of these drugs on intracellular or extracellular *S. aureus* with different drug susceptibility profiles in the presence of PMN and (ii) the effect of the bacteria on drug uptake.

Neither drug impaired the global bactericidal function of PMN, whatever the experimental conditions, which were chosen to mimic possible in vivo situations (global assay and prephagocytosis and postphagocytosis exposures). In addition, even high concentrations of the drugs, which totally abolished the oxidative metabolism of PMN, did not impair the bactericidal function of these phagocytes. The PMN antimicrobial

system comprises oxidative and nonoxidative mechanisms, such as bactericidal proteins and peptides (13, 24), and the latter may have played a major role here. Indeed, PMN priming with two cytokines which potentiate the PMN oxidative response did not increase the bactericidal activity of PMN. Bactericidal activity better than that achieved with PMN alone was noted with the combination of PMN and roxithromycin against one species resistant to this drug (*S. aureus* strain B), and a similar effect was observed with telithromycin and susceptible *S. aureus* strain C. Similar results have also been obtained by other authors with resistant and susceptible strains (4, 27); however, the clinical significance of these findings is unclear.

The main value of this study is to highlight the direct antibacterial potency of telithromycin in various bactericidal systems. It must be noted first that telithromycin and roxithromycin appeared to be less active than expected from their MICs, and no clear concentration dependence was demonstrated for the susceptible strains. However, staphylococci were growing slowly under our experimental conditions, and this characteristic may have impeded the action of the antibiotics. In the global assay, the activity of telithromycin was not impaired in the presence of PMN and, at therapeutic (or supratherapeutic) concentrations, it did not impair PMN bactericidal function. With prephagocytosis exposure, the antibacterial activity of telithromycin was maintained after its intracellular accumulation. Last, with postphagocytosis exposure, telithromycin still expressed bactericidal activity, although it seemed to be more active on extracellular than on intracellular staphylococci.

This latter observation is intriguing, as it is widely acknowledged that macrolides and ketolides are strongly concentrated by PMN. Therefore, we examined whether bacteria or their products modified drug uptake, a question which has been rarely investigated. We found that live and heat-killed, opsonized and unopsonized *S. aureus* (whatever the strain) impaired telithromycin and roxithromycin uptake in a bacterial density-dependent manner. Similar data were obtained with a strain of *Pseudomonas aeruginosa* (data not shown). This inhibition appeared to be specific to macrolide uptake, whatever the main cellular location (granules for telithromycin and cytoplasm and granules for roxithromycin and clarithromycin), as the uptake of a quinolone, levofloxacin, was not modified. Likewise, fresh and heated bacterial culture supernatants had inhibitory effects on macrolide uptake (data not shown).

The accumulation of all erythromycin A derivatives appears to be mediated by a common carrier system (30, 31). Despite reports suggesting an active macrolide entry process (29, 30, 31), the underlying mechanism is still poorly understood. Various PMN stimuli, such as fMLP, bacteria (this study), and PMA (29), impair macrolide uptake, whereas some inhibitors of PMN transductional pathways either enhance or inhibit macrolide uptake. For instance, H89, a PKA inhibitor, impairs roxithromycin accumulation (M. T. Labro, H. Abdelghaffar, D. Vazifeh, and A. Bryskier, Program Abstr. 7th Int. Congr. Infect. Dis., abstr. 110-018, p. 278, 1996), while it enhances that of azithromycin and telithromycin. In contrast, inhibition of p38 mitogen-activated protein (MAP) kinase seems to increase macrolide uptake (29). Tyrosine phosphorylation is also necessary for optimal roxithromycin uptake (29).

Recent studies suggested that the pathogenesis of intracel-

lular infections may involve interference with host cell signaling (7, 12, 21, 22). In particular, bacterial phagocytosis by PMN stimulated MAP kinase cascades. Extracellular response kinase and p38 kinase activities were increased within 5 min of phagocytosis of plasma-opsonized *S. aureus*, reached a maximum at 20 to 30 min, and remained elevated through 60 min. Ingestion of nonopsonized *S. aureus* reached only 10% that of opsonized bacteria and caused a minimal increase in extracellular response kinase and p38 kinase activities at 60 min. MAP kinase activation was partially inhibited by genistein and PKC inhibitors (21). Here, we observed that *S. aureus* stimulation of PMN impaired macrolide or ketolide uptake; this effect was strongly enhanced by the tyrosine kinase inhibitor genistein (Fig. 4) and was additive with the inhibitory effect of the PKC activator PMA. These data suggest that phagocytosis- and/or bacterium-induced stimulation of PMN modifies a transduction pathway involved in activating the macrolide carrier. A potential target is PKC activation by PMA and opsonized bacteria (21), leading to an increase in the activity of p38 MAP kinase, which would in turn impair the macrolide carrier (29).

In summary, despite many claims that the intracellular accumulation of macrolides results in synergistic interactions with the bactericidal systems of these phagocytes, we demonstrate here that the intracellular or extracellular antibacterial activities of telithromycin and roxithromycin mainly depend on the direct antibacterial effects of these drugs. The model that we used involved a bacterium that is generally considered an extracellular pathogen, although the intracellular persistence of staphylococci in PMN has been documented (32). Our findings cannot be directly extrapolated to other bacterial species, particularly obligate or facultative intracellular pathogens, which reside and/or multiply in macrophages and are preferential targets of macrolides. The clinical relevance of the potential inhibitory effect of bacteria on macrolide uptake remains to be ascertained, but this observation may aid in an understanding of discrepancies between in vitro and in vivo results. However, it must be noted that macrolide uptake inhibition was observed only during a short period after phagocytosis (30 min) (Fig. 3) when, according to the literature, MAP kinase activation is maximum (21); the effect was not significant after a longer (60 min) activation of PMN by bacteria. The inhibition of macrolide uptake by bacteria may thus be less important in vivo.

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