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Activities of Poloxamer CRL8131 against *Mycobacterium tuberculosis* In Vitro and In Vivo

CHINNASWAMY JAGANNATH,[1] H. S. ALLAUDEEN,[1] AND ROBERT L. HUNTER[2]*

CytRx Corporation, Norcross, Georgia 30092,[1] and Department of Pathology and Laboratory Medicine, Emory University, Atlanta, Georgia 30322[2]

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A poloxamer surfactant, CRL8131, was evaluated for activity against *Mycobacterium tuberculosis* (Erdman) by itself and in combination with antibiotics in broth culture, in a macrophage cell line assay, and in testing with mice. In the broth culture, CRL8131 suppressed the growth of *M. tuberculosis* and produced synergistic effects in combination with isoniazid, rifampin, and streptomycin. It also displayed synergy with isoniazid and rifampin against two drug-resistant isolates. In the macrophage cell line assay, CRL8131 produced a synergistic effect on intracellular killing of *M. tuberculosis* by isoniazid, rifampin, streptomycin, pyrazinamide, thiacetazone, D-cycloserine, ethionamide, amikacin, clindamycin, and *p*-aminosalicylic acid. It demonstrated no synergy or antagonism with ethambutol, gentamicin, kanamycin, ciprofloxacin, or nalidixic acid. Finally, with C57BL/6 mice infected with *M. tuberculosis*, a combination of CRL8131 and either thiacetazone or pyrazinamide produced 100% survival at 40 days whereas the antibiotics produced only 33% survival and CRL8131 produced 0% survival when used as single agents. This improved survival rate was associated with a significant reduction in the number of organisms in the lungs and spleens of infected mice.

The feasibility of using large hydrophobic nonionic surfactants for treating mice infected with *Mycobacterium tuberculosis* was demonstrated over 40 years ago. Cornforth et al. reported that a single injection of Triton WR-1339 significantly prolonged the survival of mice infected intravenously with a large dose of *M. tuberculosis* organisms (6, 8). The injection of a surfactant was effective if given either before or up to 5 days after infection. The surfactant suppressed growth of *M. tuberculosis* in macrophage cultures and in intact mice but was inactive in broth culture. It was found to localize in macrophages after systemic injection (20). This information led to the hypothesis that Triton WR-1339 and similar agents suppressed tuberculosis infection by modulating host-parasite interactions rather than by direct action on the organism. It was reported further that Triton WR-1339 produced a synergistic suppression of *M. tuberculosis* growth in mice when used in combination with rifampin and pyrazinamide (19). In spite of these promising findings, the development of surfactants as drugs was not pursued because of the discovery of more effective drugs and declining interest in the disease. However, the increasing incidence of disease in immunocompromised people and the emergence of multidrug-resistant strains of *M. tuberculosis* have renewed interest in novel antimycobacterial agents.

We have investigated the biologic activities of poloxamers for over 10 years. Poloxamers are simple synthetic surfactants that consist of a central chain of hydrophobic polyoxypropylene (POP) flanked by two chains of hydrophilic polyoxyethylene (POE) (22). By varying the lengths of these chains, a homologous series of compounds which span nearly the entire spectrum of functional properties of nonionic surfactants has been produced. Poloxamers are among the least toxic of known surfactants. They can be produced in pure form and have been used in diverse biological applications, including pharmaceuticals (4, 14, 21, 25, 26, 28).

We previously reported the effects of poloxamer surfactants on the growth of *Mycobacterium avium-Mycobacterium intracellulare* (MAI) complex organisms in broth culture (16, 17). Poloxamers that were similar in size and hydrophobicity to Triton WR-1339 were found to have bacteriostatic effects on fresh clinical isolates with transparent colony morphology but not on older isolates with opaque colony morphology. Furthermore, the poloxamers were synergistic with rifampin against all of the fresh isolates. Following these investigations, new methods for synthesis and purification of poloxamers were developed. These methods facilitated production of agents that are better characterized, more homogeneous, and purer than any previously available. Thirty-two new poloxamers of different structures were evaluated in a radiometric assay for inhibitory effect on *M. tuberculosis* Erdman. A pattern of activities similar to that observed previously with MAI was observed (16, 17). CRL8131 was found to be the most effective.

Here, we report the results of studies on the effects of CRL8131 on *M. tuberculosis* as a single agent and in combination with 15 antibiotics. The antimycobacterial activity of CRL8131 was determined by radiorespirometry, inhibition of intracellular growth of *M. tuberculosis* in human macrophage cell lines, and acute intravenous infection of mice. The effects of CRL8131 with antibiotics in selected drug-resistant organisms were also investigated. The results demonstrate that poloxamer CRL8131 can produce synergistic enhancement of the effectiveness of several antibiotics against *M. tuberculosis.*

MATERIALS AND METHODS

Poloxamer CRL8131. The chemistry and synthesis of poloxamers of defined proportions of POP and POE have been described earlier (4, 22). CRL8131 is a mixture of molecular species, with a mean molecular mass of POP chains of 3,500 Da and POE chains of 200 Da each. Industrial poloxamers contain significant amounts of inactive and toxic low-molecular-weight impurities. CRL8131 was synthesized under near GMP conditions. It has fewer impurities and less heterodispersion than previous preparations. It was maintained in a stock of a suspension (30 mg/ml [wt/vol]) in a vehicle of 2% Tween 80–1% ethanol in water (TE vehicle). For use, CRL8131 was diluted from this 30-mg/ml stock, in either

* Corresponding author. Mailing address: Department of Pathology and Laboratory Medicine, Woodruff Memorial Building, Room 762, Emory University School of Medicine, Atlanta, GA 30322. Phone: (404) 727-4636. Fax: (404) 727-2991.

saline (for radiometry experiments) or growth medium (for macrophage assays). Typically, 100 μl of saline containing 10 μg of CRL8131, injected into a BACTEC vial, contained 0.002 μl of Tween 80. Macrophage assays for testing CRL8131 against *M. tuberculosis* growth received 5 μg of CRL8131 in 50 μl of growth medium as the maximum dose and accordingly contained around 0.001 μl of Tween 80. Combination studies of macrophages used 0.1 μg of CRL8131 per ml, and the level of Tween 80 was even lower. The final concentrations of Tween 80 alone were less than 0.05% in radiometry and ≤0.01 to 0.001% in the macrophage assay. These Tween 80 doses do not cause any effect on their own, as confirmed by the addition of appropriate vehicle controls in both radiometry and macrophage assays.

***M. tuberculosis* strains.** Reference strains of *M. tuberculosis* (ATCC 35801; Erdman strain) and streptomycin-resistant H37Ra (ATCC 35836) were grown in 7H9 broth (Difco Laboratories, Detroit, Mich.) and harvested in log phase. Bacilli washed in saline were briefly sonicated to disperse clumps and matched in turbidity to McFarland suspension 1 prior to storage in aliquots at −70°C. Thawed aliquots were diluted in saline and were plated on 7H11 agar (Difco) to determine CFU counts of stock suspensions. Two clinical isolates of *M. tuberculosis* (CDC2218, resistant to isoniazid [INH; CIBA-GEIGY]; and CDC2227, resistant to rifampin) were kindly provided by Jack Crawford of the Centers for Disease Control and Prevention (Atlanta, Ga.). Both were cultured and stock suspensions were stored as described above.

Radiometry. Radiometry was carried out with the 7H12 broth (Becton Dickinson, Sparks, Md.) containing ^{14}C-labeled palmitic acid. (BACTEC TB 460 instrument; Johnston Laboratories, Becton Dickinson, Sparks, Md.). The inhibitory effect of poloxamers on *M. tuberculosis* was determined in terms of MICs and confirmation of activity by plating broth aliquots on 7H11 agar for CFU counts. BACTEC 12B medium vials allow mycobacteria to grow and release ^{14}C-labeled CO_2, and the TB 460 BACTEC reader measures released radiolabeled CO_2. Drugs were added in a final volume of 0.1 ml to the 4.0 ml of media in order to minimize dilution of the medium. Similarly, mycobacterial suspensions were added in 0.1-ml volumes. All drug-containing vials were inoculated with 0.1 ml of McFarland standard 1 matched suspensions. Control vials for each experiment received 0.1 ml of a further 1/100 dilution of this suspension.

Drug-containing vials and controls were incubated at 37°C for 8 to 12 days and were read daily in the TB 460 reader for the growth index (GI). The MIC was determined as that drug concentration at which the daily increase in GI (delta GI; measured for 2 to 3 consecutive days) was equal to or less than that of the 1/100-dilution control vials. Delta GI was calculated only when the control readings were 30 or above. Isoniazid or rifampin was generally used as a positive control. Since control vials have a 1/100 dilution of the *M. tuberculosis* inoculum present in drug-containing vials, this method of calculation defines MIC as that dose which inhibits 99% of *M. tuberculosis* growth. Combination studies were also performed with the two multidrug-resistant strains identified above, defining synergy of drug action according to established methods (23, 24).

Assays of intracellular growth of *M. tuberculosis*. In order to better understand the susceptibility of *M. tuberculosis* within macrophages and to correlate drug effects and/or macrophage bactericidal functions, we developed susceptibility methods using human monocyte-like cell line U937 (CRL-1593) obtained from the American Type Culture Collection. The cells were maintained by in vitro passage in RPMI 1640 medium with 10% fetal bovine serum and gentamicin at 50 μg/ml (growth medium). Cells were expanded in antibiotic-free growth medium, washed, and suspended in antibiotic-free RPMI 1640 medium containing mycoplasm-free 1% fetal bovine serum (assay medium). For infection studies, 10^8 U937 cells were mixed with a sonicated suspension of *M. tuberculosis* containing 10^6 CFU (in 0.1 ml) in 5 ml of assay medium. Phagocytosis was allowed to occur during gentle mixing at 37°C, and cells were washed with assay medium six times. The cells were then diluted to 10^6 cells per ml and plated out at 1 ml per well of a 24-well Costar plate. After an appropriate addition of drugs in triplicate for each concentration, plates were incubated at 37°C in 5% CO_2. Fresh medium (1.0 ml per well) was added on day 3. Aliquots of macrophages aspirated from wells on day 0 were used for determination of baseline CFU counts. Infected macrophages in drug-free wells of incubated plates served as controls for growth of *M. tuberculosis* over 7 days. On day 7, macrophages from individual wells were collected and pelleted. Lysates were prepared by the addition of 0.5 ml of sterile 0.25% sodium dodecyl sulfate (incubated for 15 min at room temperature) and were then neutralized by the addition of 0.5 ml of sterile 15% bovine serum albumin in saline. Lysates were diluted in sterile saline, 10-fold dilutions were plated on 7H11 agar, and CFU counts were determined after 4 to 6 weeks of incubation of the plates at 37°C. Mean values of replicate CFU counts for each drug concentration were plotted against time to determine drug effects. Dose levels inhibiting 99% of the day 0 inoculum were defined as MICs. Thus MIC doses yielded day 7 CFU counts equal to or less than the day 0 baseline CFU counts.

Interactions between CRL8131 and other anti-*M. tuberculosis* drugs were calculated by using the fractional inhibitory concentration (FIC) and criteria defined by Berenbaum (3). Various doses of drugs, generally doubling concentrations below and above known in vitro MICs, were combined with a single sub-MIC dose of CRL8131 in both the radiometry and macrophage assays. This dose of CRL8131 (0.1 μg/ml) was 1/60 of its radiometric MIC and 1/10 of its macrophage MIC. The FIC was calculated as follows: FIC = [(MIC of drug combined with CRL8131)/(MIC of drug alone)] + [(MIC of CRL8131 combined with drug)/(MIC of CRL8131 alone)]. Synergy was defined by FIC values of ≤0.5, additive effects were defined by FIC values of ≤1.0, and antagonism was defined by FIC values of ≥2.0 (3, 11). In order to monitor reproducibility, MICs for all 15 drugs and CRL8131 in macrophages were determined in triplicate experiments.

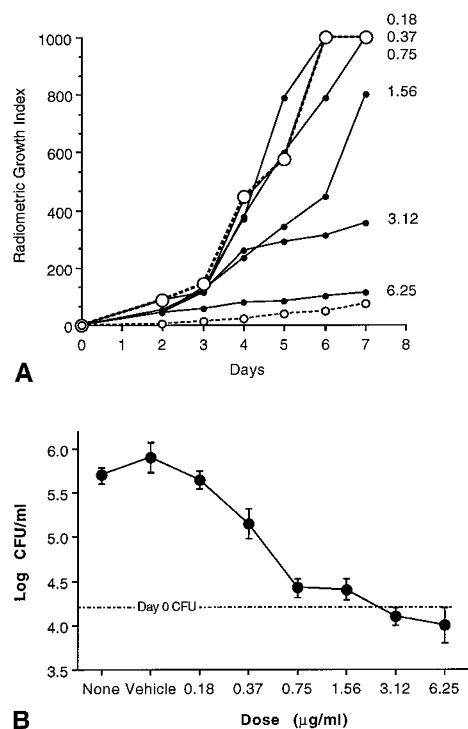

FIG. 1. Effects of CRL8131 on *M. tuberculosis* in broth. (A) BACTEC curves of the growth of *M. tuberculosis* inoculum (large open circles), its 1/100 dilution (small open circles), and the inoculum with multiple doses of CRL8131 (small solid circles) are shown together. The number next to each solid line shows the dose of CRL8131 in micrograms per milliliter. (B) CFU counts of day 10 cultures from this experiment. A dose-dependent decrease in log CFU ± SD of three replicates is observed.

Infection of C57BL/6 mice. The Erdman strain of *M. tuberculosis* was used for in vivo experiments after several passages in C57BL/6 mice to maintain virulence. Mice of either sex were infected intravenously (i.v.) with 10^6 CFU per mouse administered in 0.2 ml of saline and were fed standard pelleted food and water. This dose of *M. tuberculosis* had been standardized to produce 100% mortality of mice by 21 to 28 days after infection. Drugs were administered 24 h after infection and were continued for 5 days/week for 2 or 4 weeks. Groups of mice received either CRL8131 i.v. at 25 mg/kg of body weight diluted in saline for 2 weeks or thiacetazone in saline at 60 mg/kg by gastric gavage for 4 weeks. Pyrazinamide was administered similarly in a dose of 100 mg/kg of body weight. Other groups were treated with both CRL8131 and thiacetazone or remained untreated as controls. Mortality was monitored daily. Surviving mice were sacrificed at 4 weeks after infection. Lungs and spleens of sacrificed and dead mice were collected, and ground homogenates were plated in dilutions onto 7H11 agar for CFU counts. Drug effects were determined by calculating the reduction in mortality and by plotting organ CFU counts for each treatment regimen. Statistical analysis with a two-way analysis of variance was used to test treatment effects.

RESULTS

CRL8131 was evaluated in the radiometric assay for inhibitory effects on *M. tuberculosis* Erdman. It produced a dose-dependent inhibition of growth with an MIC of 6.25 μg/ml (Fig. 1A). CRL8131 did not affect the lag time before the initiation of detectable growth, but it did reduce the slope of the curve. This result suggests that the poloxamer slows the rate of multiplication of the organisms and has a bacteriostatic rather than a bactericidal effect. The results of the radiometric assay were verified by determining the CFU counts per milli-

TABLE 1. Enhancement of drug action by CRL8131 against *M. tuberculosis* strains by radiometry

Strain(s)	Drug tested	MIC (μg/ml)[a]		Dose (μg/ml) tested in synergy		MIC of drug (μg/ml) in combination	FIC[b]
		Drug alone	CRL8131	Drug	CRL8131		
H37Rv, Erdman	Isoniazid	0.12	6.25	0.12–5	0.1	0.02	0.17
	Rifampin	0.12	6.25	0.12–5	0.1	0.008	0.08
	Streptomycin	5	6.25	0.12–5	0.1	0.3	0.07
Isoniazid resistant[c]	Isoniazid	≥4	>10	0.5–8	10	1	0.26
Rifampin resistant[d]	Rifampin	≥8	5	0.5–8	5	2	0.29

[a] MICs were determined by the BACTEC radiometric system.
[b] Refer to Materials and Methods for details on the FIC. Synergy, ≤0.5
[c] CDC2218 strain (resistant to isoniazid and streptomycin).
[d] CDC2227 strain (resistant to isoniazid, streptomycin, and rifampin).

liter of culture at day 10 with replicate platings from BACTEC vials (Fig. 1B).

Studies were undertaken to evaluate the ability of combinations of CRL8131 with isoniazid, rifampin, and streptomycin to inhibit susceptible and drug-resistant *M. tuberculosis* in the radiometric assay system (Table 1). Doubling concentrations of drugs below and above their MICs were combined with a single dose of CRL8131 (0.1 μg/ml) in order to calculate the MIC of the drug in combination with CRL8131. The MIC of isoniazid was reduced sixfold from 0.12 to 0.02 μg/ml when used in combination with CRL8131. This measurement produced an FIC of 0.17 that was evidence of synergy. The effect was even more pronounced with rifampin and streptomycin. The MIC of rifampin was reduced 15-fold from 0.12 to 0.008 μg/ml, and that of streptomycin was reduced 16-fold from 5 to 0.3 μg/ml by CRL8131. These combinations each produced an FIC value of <0.1. These assays each were repeated a minimum of three times, with comparable results.

Additional studies were performed with an *M. tuberculosis* strain resistant to isoniazid and one resistant to rifampin. Doses of isoniazid and streptomycin that produced no detectable effect on the resistant strain when used as single agents nearly produced MIC suppression when used in combination with poloxamer CRL8131 (Fig. 2). The FIC value for both strains with the antibiotic to which they were resistant was <0.3, indicating synergy with CRL8131. It is noteworthy that the MIC of each antibiotic when used in combination with CRL8131 was reduced to a therapeutically attainable level. Combination of drugs with CRL8131 therefore may reduce the doses of drugs required for treatment.

These results were sufficiently promising that studies were initiated with *M. tuberculosis* in monocyte-like cell lines in order to obtain results that might be more physiologically relevant (9). Preliminary experiments demonstrated that infection of U937 cells with *M. tuberculosis* at $>10^7$ CFU of bacilli per 10^8 macrophages resulted in the death of the cells. On the other hand, infection with too small a quantity of organisms did not yield a reproducible growth curve. Infection on day 0 of about 25% of macrophages with 10^3 CFU per 10^6 macrophages resulted in a progressive growth through day 7, at which time 50 to 70% of the cells became infected, yielding 10^5 to 10^6 CFU per 10^6 macrophages. Microscopic examination of acid-fast-stained infected macrophages on day 0 and serially for 7 days did not show any extracellular bacilli. These conditions were therefore used to evaluate the effects of CRL8131 alone and in combination with antibiotics.

CRL8131 was initially incubated at various doses with uninfected U937 cells. It showed cytotoxic effects of granularity, clumping, and loss of cell viability at doses of >25 μg/ml. No detectable sign of toxicity was observed at doses of below 10 μg/ml. The MIC in macrophages was defined as the dose which yields an equal or smaller number of day 7 CFU than day 0 CFU. The MIC of CRL8131 by this criterion was found to be 1.25 μg/ml (Fig. 3). This result was highly reproducible in many replications performed as controls for subsequent studies. A greatly reduced dose, 0.1 μg, was selected on the basis of preliminary studies for evaluation of drug combinations.

M. tuberculosis-infected U937 cells were incubated with 0.1 μg of CRL8131 per ml alone or combined with one of 15 drugs, each at multiple doses. The MIC was determined in three separate experiments for each drug alone and in combination with CRL8131. These data were used to calculate the MIC, FIC, mean FIC, and standard deviation (SD) of the FIC. Statistically significant synergy was obtained if the FIC plus 2 SD was less than 0.5 (3). It should be noted that, because of the amount of work involved, we used only a single sub-MIC dose of CRL8131 instead of performing multiple checkerboard ti-

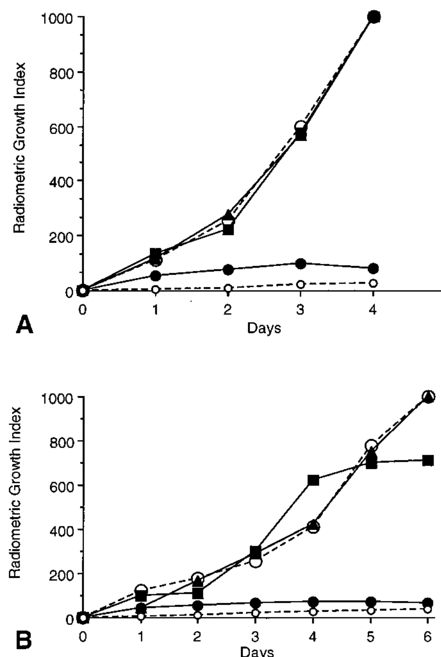

FIG. 2. Effects of CRL8131 with isoniazid and rifampin on resistant strains of *M. tuberculosis*. (A) Radiometric BACTEC curves of the growth of isoniazid-resistant *M. tuberculosis* CDC2218 with isoniazid (1 μg/ml) (triangles), CRL8131 (10 μg/ml) (squares), or both (solid circles) are shown. The medium control (large open circles) and its 1/100 dilution (small open circles) are shown. (B) Effects of CRL8131 (5 μg/ml) with rifampin at 2 μg/ml (triangles) in the same format as panel A, with rifampin-resistant *M. tuberculosis* CDC2227.

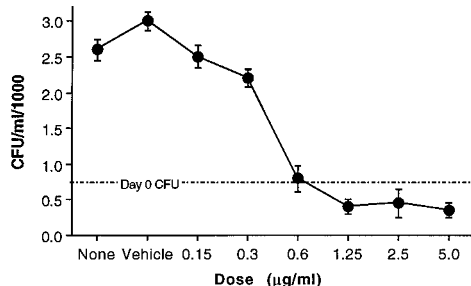

FIG. 3. Effects of CRL8131 on *M. tuberculosis* Erdman growth in macrophages. U937 macrophage-like cells were infected with *M. tuberculosis*, washed, and incubated for 7 days with various doses of CRL8131 as shown. Untreated macrophages and those treated with the vehicle were controls. CFU counts on macrophage lysates prepared on day 7 are shown as the means (± SDs) of triplicate experiments.

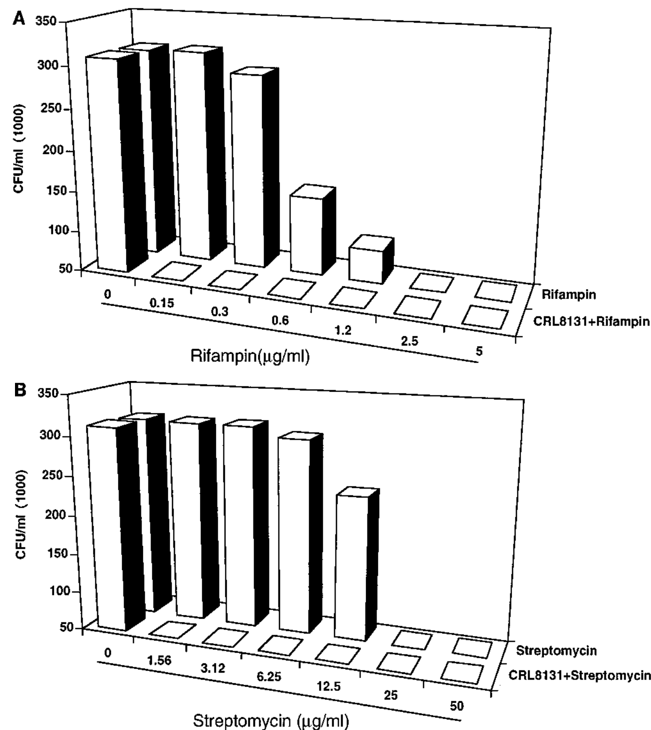

FIG. 4. (A) Effects of CRL8131 and rifampin on *M. tuberculosis* Erdman growth in macrophages. Infected U937 macrophage-like cells were prepared as in the experiment for Fig. 3 and were treated with CRL8131 (0.1 μg/ml) and/or various doses of rifampin as shown. CFU counts on macrophage lysates prepared on day 7 are shown as the means of triplicate experiments. (B) Effects of CRL8131 and streptomycin on drug-resistant *M. tuberculosis* H37Ra in macrophages. U937 macrophage-like cells were infected with streptomycin-resistant *M. tuberculosis* (H37Ra), washed, and incubated for 7 days with CRL8131 (0.1 μg/ml) and/or various doses of streptomycin as shown. CFU counts on macrophage lysates prepared on day 7 are shown as the means of triplicate experiments.

trations. Consequently, the true FIC values may be lower than those obtained. The FIC values should, therefore, be read as less than or equal to the values shown. CRL8131 produced a synergistic effect with 10 of the 15 drugs and significant synergy with 7 of them (Table 2). Representative results of an experiment showing synergy between CRL8131 and rifampin are shown in Fig. 4A. Synergy of this magnitude was not limited to one type of drug but was found with four of five cell wall inhibitors, three of six protein synthesis inhibitors, and one of four drugs with other mechanisms. The drugs that failed to demonstrate synergy all produced FICs of ≤1, indicating additive effects. None produced evidence of antagonism. A streptomycin-resistant strain of *M. tuberculosis* (H37Ra) was tested similarly with streptomycin and CRL8131 (Fig. 4B). The MIC of streptomycin for this organism was reduced 16-fold from 25 to 1.56 μg/ml, producing an FIC of 0.07 and significant synergy.

A final set of experiments was designed to determine if CRL8131 could be effective as an antimicrobial agent in ani-

TABLE 2. Enhancement of drug action by CRL8131 against *M. tuberculosis* (H37Rv and Erdman) in macrophages

Drugs tested	MIC (μg/ml) in macrophages[a]				
	Drug alone	Drug + CRL8131[b]	FIC[c]		
			Mean	SD	Mean + 2 SD
Cell wall inhibitors					
Isoniazid	1.25, 2.5, 1.25	0.15, 0.15, 0.3	0.14	0.09	0.32
Thiacetazone	5, 10, 5	0.6, 0.6, 0.3	0.11	0.06	0.23
D-Cylcoserine	12.5, 25, 25	6.25, 12.5, 12.5	0.5	0	0.5
Ethionamide	10, 5, 5	0.6, 0.6, 0.15	0.07	0.04	0.15
Ethambutol	12.5, 12.5, 6.25	6.25, 12.5, 6.25	0.83	0.28	1.39
Protein synthesis inhibitors					
Rifampin	1.25, 1.25, 2.5	0.15, 0.15, 0.3	0.12	0.06	0.24
Streptomycin	10, 10, 5	0.6, 0.15, 0.3	0.1	0.06	0.22
Amikacin	2.5, 2.5, 5	0.3, 0.15, 0.3	0.06	0	0.06
Clindamycin	25, 25, 12.5	6.25, 3.12, 3.12	0.24	0.21	0.66
Gentamicin	25, 25, 50	25, 25, 50	1	0	1
Kanamycin	12.5, 12.5, 25	12.5, 6.25, 25	1	0.28	1.56
Other metabolic inhibitors					
p-Aminosalicylic acid	6.25, 12.5, 6.25	1.56, 1.56, 3.12	0.28	0.18	0.64
Ciprofloxacin	12.5, 12.5, 25	12.5, 12.5, 25	1	0.28	1.56
Nalidixic acid	12.5, 25, 25	12.5, 25, 25	1	0	1
Pyrazinamide	12.5, 12.5, 50	1.56, 3.12, 3.12	0.12	0.06	0.26

[a] MICs are doses which inhibit 99% of day 0 CFUs. Data from three experiments and corresponding FICs are the means ± SDs of three experiments. The MIC of CRL8131 alone was 1.25.

[b] Various doses of drugs were combined with a sub-MIC dose of CRL8131 (0.1 μg/ml).

[c] For FIC calculations, synergy was ≤0.5, additivity was ≤1.0, and antagonism was ≥2.0.

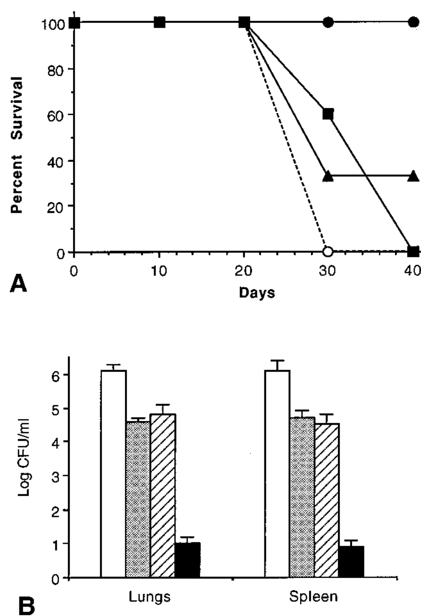

FIG. 5. (A) Survival of mice infected acutely with *M. tuberculosis* Erdman and treated with CRL8131 and/or thiacetazone. Groups of six C57BL/6 mice infected i.v. with *M. tuberculosis* were treated with CRL8131 (25 mg/kg, i.v. for 2 weeks) (squares), thiacetazone (60 mg/kg, orally for 5 days per week for 4 weeks) (triangles), both CRL8131 and thiacetazone (closed circles), or neither (open circles). The percent survival of each group is shown with time after infection. The survival rate was observed daily throughout the course of the experiment. The cumulative survival rates in 10-day increments are shown. (B) Effects of CRL8131 and thiacetazone on CFU counts of *M. tuberculosis* Erdman in organs of acutely infected mice. CFU counts in spleens and lungs were performed on day 30 of the experiment for panel A. The results are shown as mean log CFU/ml ± SD (three mice per group) of untreated controls (open bars) and thiacetazone-treated (shaded bars), CRL8131-treated (striped bars), and thiacetazone-plus-CRL8131-treated (solid bars) mice.

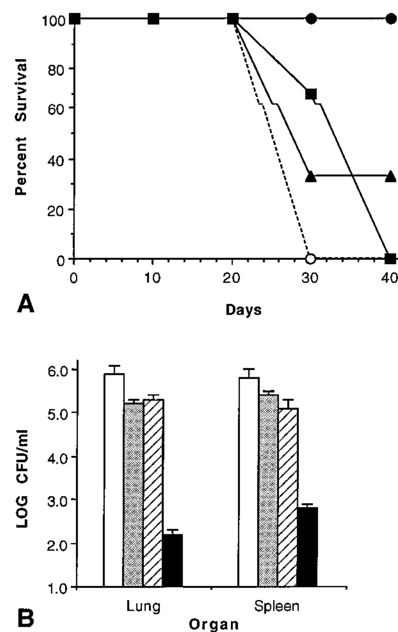

FIG. 6. (A) Survival of mice infected acutely with *M. tuberculosis* Erdman and treated with CRL8131 and/or pyrazinamide. This experiment was similar to that whose results are shown in Fig. 5, except that pyrazinamide at 100 mg/kg was used instead of thiacetazone. (B) Effects of CRL8131 and pyrazinamide on CFU counts of *M. tuberculosis* Erdman in organs of acutely infected mice. CFU counts of spleens and lungs were performed, and the results are shown as in Fig. 5B.

mals with acute tuberculosis. Mice were infected i.v. with a standardized inoculum of *M. tuberculosis* and were then treated with CRL8131, thiacetazone, both drugs, or neither drug. All untreated control mice died of tuberculosis before day 30 (Fig. 5). Those treated with CRL8131 showed 33% mortality by day 30 and 100% mortality by day 40. This observation is consistent with the bacteriostatic effect observed in vitro. Mice treated with thiacetazone alone showed 66% mortality for both 30 and 40 days. All (100%) of the mice that received both CRL8131 and thiacetazone survived for 40 days with no sign of disease. The CFU count of *M. tuberculosis* organisms was determined with the lungs and spleens of three sacrificed mice at 30 days postinfection. Each drug alone reduced the CFU counts by about 1 log. The combination of both drugs reduced the CFU counts by about 4 logs in both the spleens and lungs of the mice (Fig. 5B). The reductions in CFU counts of both lungs and spleens were statistically significant ($P < 0.001$). A similar experiment was carried out to determine the effects of CRL8131 in combination with pyrazinamide. The results demonstrated that the poloxamer enhanced the effect of pyrazinamide just as effectively as that of thiacetazone (Fig. 6).

DISCUSSION

The current treatment of tuberculosis and tuberculosis infection in adults and children recommended by the American Thoracic Society and the Centers for Disease Control and Prevention is a four-drug 6-month regimen consisting of isoniazid, rifampin, and pyrazinamide plus ethambutol or streptomycin (1). CRL8131 produced a statistically significant synergistic effect with four of the five recommended drugs in the macrophage cell line assay. Overall, it produced synergistic effects with 10 of the 15 drugs assayed with *M. tuberculosis* Erdman and with 2 drug-resistant strains. CRL8131 enhanced the effectiveness of drugs in broth culture, in macrophage culture, and in tests with intact mice where its combination with either thiacetazone or pyrazinamide produced a 3-log-greater reduction in the number of CFU in both spleens and lungs than the drug alone and 100% survival with no clinical evidence of disease. CRL8131 produced these synergistic effects with other drugs, even though it produces only a weak bacteriostatic effect by itself. Many aspects of these experiments require discussion.

It has long been known that surfactants like Triton WR-1339 are able to suppress the growth of *M. tuberculosis* in macrophage culture and in mice, even though they have little or no activity in broth culture (7). CRL8131 has physical-chemical properties similar to those of Triton WR-1339 but is chemically distinct and more potent than Triton WR-1339. In considering the mechanisms by which CRL8131 influences mycobacterial infection, several observations seem pertinent. First, the basic structure of CRL8131, POE-POP-POE, is identical to that of poloxamers that have no activity as antimycobacterial agents (2, 7, 16, 17). Changes in the lengths of either the POE or POP chains reduce or abolish activity. Similar structure-function relationships were reported among derivatives of Triton WR-1339 (7, 8). Consequently, it is unlikely that the activity of CRL8131 involves a specific reactive site on CRL8131 that is not present on inactive poloxamers as well.

The surfactants that exhibit antimycobacterial activity have distinctive physical-chemical properties quite unlike those of common detergents. In our studies and in those of Cornforth

et al., these surfactants are large, hydrophobic, nonionic, and poorly soluble in water (7, 8, 16, 17). CRL8131 forms large mycellar aggregates that adhere to hydrophobic surfaces. It has a large, flexible, and weak hydrophobe that is unable to solubilize or denature proteins or membranes. Poloxamer surfactants bind rapidly to hydrophobic surfaces but are typically unable to penetrate them.

The synergy of CRL8131 with antibiotics was more impressive than its action as a single agent. In this study, as in our previous study with MAI, the poloxamer increased the susceptibility of both susceptible and resistant organisms by approximately the same factor. This result, together with the observation that CRL8131 enhanced the activity of a broad spectrum of drugs, argues against there being an effect on the intracellular drug targets. It seems more likely that mechanisms involving reduction of permeability barriers and enhanced drug delivery are operative. This idea is supported by preliminary results of ongoing studies that have demonstrated that poloxamer CRL8131 can increase the uptake of fluorescein-labeled streptomycin by mycobacteria. Other cell wall permeabilizers have been used to enhance movement of drugs into microbes (27). Ethambutol acts as a cell wall permeabilizer and has been used to enhance in vitro susceptibility of *M. avium* (12, 24) and multidrug-resistant strains of *M. tuberculosis* (18). Since drugs that have similar mechanisms of action are expected to display additive rather than synergistic effects, this may explain why ethambutol failed to produced synergistic effects with CRL8131.

At least two other mechanisms of action may contribute to the activity of CRL8131. First, large hydrophobic poloxamers similar to CRL8131 are lipase inhibitors (5, 10). Mycobacteria may be particularly sensitive to lipase inhibitors, since they contain much lipid. We found that a poloxamer induced changes in the lipids extractable from MAI organisms, as analyzed by thin-layer chromatography (2). Second, CRL8131 probably is an immunomodulator since its structure is within the range of poloxamer surfactants that are well-known powerful immunoadjuvants (14, 15). These agents have complex activities that include stimulation of macrophages (13).

Studies with mice were conducted as an initial evaluation of the potential of CRL8131 for treating mycobacterial infections. Thiacetazone was selected because of its chemical similarity to isoniazid and weak bactericidal action on *M. tuberculosis*. When used against acute *M. tuberculosis* infection of C57BL/6 mice, a dose of 30 to 60 mg/kg of body weight did not protect against death. This dose in combination with CRL8131 could therefore be used to demonstrate protection against death. The results demonstrated also that CRL8131 and either thiacetazone or pyrazinamide produced a significantly better therapeutic effect than either agent alone in terms of both survival and numbers of bacilli in the lungs and spleens. Preliminary results of ongoing experiments suggest that similar results will be obtained with isoniazid and rifampin. These data suggest that poloxamers have potential as antimicrobial agents.

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