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In Vitro Selection of Bacteria Resistant to LY146032, a New Cyclic Lipopeptide

LYNNE D. LIEBOWITZ,* JEAN SAUNDERS, LYNDA J. CHALKLEY, AND HENDRIK J. KOORNHOF

Emergent Pathogen Research Unit, South African Medical Research Council, School of Pathology, University of the Witwatersrand, and South African Institute for Medical Research, Johannesburg, South Africa

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Isolates of *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Enterococcus faecium*, and coagulase-positive and -negative staphylococci were investigated for their abilities, in vitro, to develop resistance to LY146032. Exposure of the organisms to incremental concentrations of LY146032 resulted in MICs 8- to 32-fold higher than those for the original isolates. After three passages on antibiotic-free medium, the high MICs were maintained for the coagulase-negative staphylococci and pneumococci, with a twofold decrease observed for the enterococci and a fourfold decrease observed for *Staphylococcus aureus*. The frequency of spontaneous emergence of resistance was highest with *S. pneumoniae* (1.2×10^{-6} at 16 times the original MIC) and lowest with *S. aureus* (7.0×10^{-10} at 8 times the original MIC). For bacteria surviving time-kill studies MICs were also higher than were those for the original isolates. Exposure to LY146032 in vitro selected for strains with decreased susceptibilities to the antimicrobial agent. However, the emergence of resistance in vivo is unpredictable and can be evaluated only after prolonged clinical use of the drug.

LY146032, a new cyclic lipopeptide antibiotic, has a spectrum of activity similar to that of vancomycin (3, 5, 6, 8–11). Recently, *Staphylococcus haemolyticus* strains demonstrating resistance in vitro to teicoplanin were described (2). Furthermore, on repeated isolation from a patient on adequate vancomycin therapy *S. haemolyticus* demonstrated stepwise increases in vancomycin MICs, the final MIC being of intermediate resistance (14). Similarly, *S. haemolyticus* isolates in vitro have demonstrated stepwise resistance to vancomycin; the resistant strains demonstrated cross-resistance with teicoplanin but were susceptible to LY146032 (14).

In this study, we investigated the propensity of strains of pneumococci, enterococci, and coagulase-positive and -negative staphylococci to develop resistance to LY146032 in vitro.

MATERIALS AND METHODS

Organisms. The bacterial strains used were clinical isolates obtained from patients attending the Johannesburg, Hillbrow, and Baragwaneth hospitals. The isolates were stored in liquid nitrogen until required.

Antimicrobial agent. Standard LY146032 reference powder was obtained from Eli Lilly & Co., Indianapolis, Ind.

Selection of resistant organisms. Organisms resistant to LY146032 were selected for by two methods. Firstly, a gradient plate technique was used to screen for resistant mutants (15). Briefly, plates were poured with two layers of agar. The bottom layer consisted of 20 ml of antibiotic-free nutrient agar (Oxoid Ltd., Basingstoke, England), which was allowed to harden with the plate slanted sufficiently so that the entire bottom was just covered. The plate was returned to the horizontal position, and a further 20 ml of agar containing the appropriate concentration of LY146032 was added. The plates were inoculated with approximately 0.15 ml of suspensions of the organisms, for which the LY146032 MICs were known, at various inoculum sizes and incubated at 37°C overnight. Resistant colonies were picked off and subjected to MIC testing.

Secondly, isolates of each of several bacterial species were exposed to incremental concentrations of LY146032 (16). Each isolate was inoculated onto an agar plate containing approximately half the MIC. The plates were incubated for 24 h, and any colonies arising were serially transferred to plates containing twofold-incremental concentrations of LY146032 until a concentration which prevented further growth was reached. To determine the stability of this resistance, colonies from plates containing the highest concentration of LY146032 which permitted growth were transferred three times sequentially onto antibiotic-free blood agar plates. The MICs of LY146032 against the resultant colonies were determined.

Determination of frequency of spontaneous resistance. The frequency of spontaneous occurrence of resistance of selected isolates to LY146032 was determined. Duplicate pour plates were prepared containing LY146032 at concentrations of 8 and 16 times the MIC for each selected isolate. A suspension (1 ml) of each isolate in the log phase of growth was added to 19 ml of molten antibiotic-containing Mueller-Hinton agar, supplemented with 5% horse blood when streptococci were tested. Plates were allowed to solidify at room temperature and were then incubated at 37°C for 24 h. Frequency of spontaneous resistance was determined by comparing the number of colonies arising at each concentration of LY146032 with the original inoculum size, which was determined by the same method using antibiotic-free Mueller-Hinton agar. These procedures were performed in duplicate, and the mean values were recorded.

Time-kill studies. Time-kill curves were constructed by previously described techniques (12). Cation-supplemented Mueller-Hinton broth was used for the *Staphylococcus aureus* and *Enterococcus faecalis* strains, and Trypticase (BBL Microbiology Systems) soy broth supplemented with 10% horse serum and 50 mg of Ca^{2+} per liter was used for an isolate of *Corynebacterium* group JK. The stationary-phase inoculum was prepared by inoculating 10 ml of broth and incubating for 20 h at 37°C. The cultures were diluted in fresh

* Corresponding author.

medium to give an initial inoculum of ca. 5×10^5 to 1×10^6 CFU/ml. Tests were performed in 25-ml flasks containing 10 ml of culture with the required concentrations of antibiotics. The cultures were incubated at 37°C and 150 rpm on an orbital environmental shaker (New Brunswick Scientific Co., Inc., Edison, N.J.). Samples were taken out at regular intervals and diluted for viable counts by the method of Miles and Misra (13), 20 μl being delivered on each of five spots using 5% horse blood agar for *E. faecalis* and the corynebacterium and nutrient agar for *S. aureus*.

Eighteen colonies of each of the bacterial strains which survived 24 h of exposure to LY146032 were subjected to MIC testing.

MICs were determined by a broth microdilution technique using Mueller-Hinton broth supplemented with 50 mg of Ca^{2+} and 25 mg of Mg^{2+} per liter. Isolates were inoculated into the medium and placed in a water bath at 37°C for 4 h, and the suspensions were adjusted to contain ca. 10^8 CFU/ml. Then, 0.01 ml was inoculated into each well of the microdilution plates with an MIC 2000 inoculator (Dynatech Laboratories, Inc., Alexandria, Va.) (final inoculum size, ca. 10^6 CFU/ml.). The plates were incubated at 37°C overnight for the enterococci and staphylococci and for 48 h for the corynebacteria.

RESULTS

Selection of resistant mutants. A gradient plate technique was used to select for LY146032-resistant strains of *S. aureus* (initial MIC, 0.5 μg/ml) and *Streptococcus pneumoniae* type 3 (MIC, 0.25 μg/ml). Mutants grew in up to a concentration of 4 μg of LY146032 per ml for both the *S. aureus* and *S. pneumoniae* isolates tested. Retesting the resistant subpopulations confirmed the MICs of 4 μg/ml for both organisms. When strains of several bacteria were serially transferred on agar plates containing incremental concentrations of LY146032, colonies for which MICs were between 8 and 32 times the original MICs were obtained (Table 1). After three passages on antibiotic-free medium, a fourfold decrease in the MIC was observed for the *S. aureus* isolate. A twofold decrease was seen for the *E. faecalis* and *Enterococcus faecium* isolates, with the high MICs being maintained for the coagulase-negative staphylococcus and *S. pneumoniae* isolates tested.

The frequency of the spontaneous emergence of resistance is shown in Table 2. The highest frequency of emergence of resistant strains was observed with the pneumococcus (at 16 times the original MIC), followed by the coagulase-negative staphylococcus, enterococci, and, finally, *S. aureus*.

Time-kill studies. Time-kill studies were performed in duplicate with a methicillin-resistant strain of *S. aureus*, an *E. faecalis* strain, and a *Corynebacterium* group JK strain for which LY146032 MICs were 0.06, 0.06, and 0.25 μg/ml, respectively. LY146032 showed little to no bactericidal activity on lag-phase cells, with killing occurring only after the onset of exponential growth, as indicated by the control culture. With all the isolates tested, regrowth due to insufficient antibiotic or the emergence of resistant or tolerant cells was evident by 24 h. Of the bacterial colonies cultured after the 24-h exposure to LY146032, 18 of each species were retested for susceptibility to LY146032. The results are shown in Table 3. The frequency of emergence of isolates for which MICs were higher than those for the wild-type strains was greatest with the corynebacteria, followed by the enterococci, and the lowest frequency again was demonstrated by the staphylococci.

TABLE 1. Stepwise selection of resistance to LY146032

Organism	MIC (μg/ml)		
	Initial	After passage on medium containing LY146032	After three passages on antibiotic-free medium
S. aureus	0.5	16	4.0
Coagulase-negative staphylococcus	0.06	0.5	0.5
S. pneumoniae	0.12	8.0	8.0
E. faecalis	2.0	32	16
E. faecium	2.0	32	16

TABLE 2. Frequency of resistance to LY146032 at concentrations of 8 or 16 times the MIC

Organism	Multiple of MIC	Frequency of resistance to LY146032
S. aureus	8	7.0×10^{-10}
Coagulase-negative staphylococcus	16	3.3×10^{-7}
S. pneumoniae	16	1.2×10^{-6}
E. faecalis	8	2.0×10^{-8}
E. faecium	8	6.6×10^{-8}

DISCUSSION

With the increasing use of broad-spectrum cephalosporins combined with aminoglycoside antibiotics for the treatment of serious infections, superinfections with enterococci are becoming a problem. Also, the incidence of infections caused by group JK corynebacteria and coagulase-negative staphylococci are increasing with the use of prosthetic devices. Worldwide, coagulase-positive and -negative strains of staphylococci are demonstrating methicillin resistance. It has therefore become necessary to look for new antimicrobial agents for the treatment of infections caused by these often highly resistant gram-positive bacteria. LY146032, a new biosynthetic cyclic lipopeptide, demonstrates good in vitro activity against aerobic gram-positive bacteria but is slightly less active against most anaerobic bacteria (3, 5, 9–11). Compared with vancomycin, LY146032 demonstrates greater in vitro activity against methicillin-susceptible and -resistant isolates of staphylococci and equal or greater activity against *E. faecalis* and *Corynebacterium* spp. (3, 5, 9, 10). Furthermore, exposure to antimicrobial agents tends to select for resistant mutants both in vitro and in vivo. Early studies on vancomycin demonstrated the ability of bacteria on exposure to vancomycin in vitro to

TABLE 3. Susceptibility of bacteria after 24 h of exposure to LY146032

Organism	Initial MIC (μg/ml)	Concn of LY146032 (μg/ml)	MICs for 18 colonies surviving 24-h exposure to LY146032[a] (μg/ml)
E. faecalis	0.06	0.5	0.06_2, 1.0_2, 2.0_{14}
S. aureus	0.06	0.25	0.06_{15}, 0.25_1, 0.5_2
Corynebacterium group JK	0.25	1.0	1.0_{18}

[a] The inferior number indicates the number of isolates for which the MIC was as indicated.

develop stepwise increases in vancomycin MICs (7). It was recently reported that a patient undergoing continuous ambulatory peritoneal dialysis developed peritonitis caused by a strain of *S. haemolyticus*. While the patient was on treatment with vancomycin, the organism was repeatedly isolated and demonstrated stepwise resistance to vancomycin (the initial lowest MIC was 2 μg/ml, which after adequate vancomycin therapy increased to 6 μg/ml and then to 8 μg/ml) (14). *S. haemolyticus* isolates exposed to vancomycin in vitro have developed stepwise increases in vancomycin MICs, the highest being 128 μg/ml (14).

We investigated selected bacterial strains to determine whether mutants for which LY146032 MICs were higher than those for the wild-type strains could be isolated. Because stepwise increases in resistance were demonstrated for selected isolates in vitro, this may result in the gradual emergence of organisms for which LY146032 MICs are higher. Stepwise resistance to penicillin has been demonstrated in vitro with pneumococci (8). This phenomenon probably occurred with the emergence of penicillin-resistant strains of pneumococci (1). With LY146032, the highest frequency of spontaneous emergence of resistance was observed with pneumococci and the lowest was observed with *S. aureus*. The frequency of spontaneous resistance to LY146032 noted in *S. aureus* was slightly lower than that recorded for the new quinolone antimicrobial agents (4). However, the development of resistance in vivo is unpredictable and can be determined only after clinical evaluation of an agent for a prolonged period.

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