Assessment of the potential for microbial resistance to topical use of multiple antimicrobial agents

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The goal of this study was to reduce the likelihood of the generation and/or persistence of bacterial resistance to some antimicrobial components contained in a topical antimicrobial mixture (neomycin, polymyxin B, mupirocin and ciprofloxacin) for use with cultured skin grafts, by substitution of alternative antimicrobials, specifically fusidic acid for mupirocin and ofloxacin for ciprofloxacin. The alternative agents failed to serve that purpose. However, with the exception of specific genera of bacteria, *Proteus* sp. and *Providencia stuartii*, 90% or more of all other bacteria tested were susceptible to the action of one or more of the individual antimicrobial agents contained in the original mixture. This was true when bacteria were highly susceptible to the antimicrobials, generally, or when bacteria resistant to specific antimicrobials such as penicillin-class antibiotics and ciprofloxacin, were tested. These results suggest that the redundancy of antimicrobials contained in this mixture reduces the chance that resistant bacteria generated by the use of this mixture or already present on wounds would persist when the mixture is used clinically. **(WOUND REP REG 1999;7:238–243)**

We have previously reported several characteristics for "idealized" topical antimicrobial mixtures for use with cultured skin grafts.[1,2] These characteristics included: that concentrations of individual antimicrobials contained in the mixture should be nontoxic for keratinocytes and fibroblasts in culture and still retain antimicrobial activity; that antimicrobial coverage should be broad-spectrum and include both bacteria and fungi; that individual components should not be antagonistic to each other; that there should be a redundancy of antimicrobial coverage among the components to reduce the emergence of resistant strains and superinfections; that for the same reasons, individual antimicrobial components with different modes of action should be included; and, finally, that the mixture should contain no antimicrobial used parenterally to treat sepsis in the institution in which the topical mixture is being used. In some of these studies, mixtures that we have formulated for topical use with cultured skin cells contained ciprofloxacin (CF) an antibiotic used parenterally to treat sepsis. Because CF is not used in this institution for parenteral treatment, our antimicrobial mixture did not violate one of our characteristics for "idealized" antimicrobial mixtures for use with cultured skin grafts. Other institutions do, however, use CF parenterally, and because resistance to CF has been shown to develop rapidly,[3–6] we were concerned that topical use of our mixture might select for CF resistance. This would obviate the use of CF to treat septic patients. Additionally, mixtures that were proposed contained the antistaphylococcal antimicrobial agent, mupirocin (MUP) formulated for topical use against

CF	Ciprofloxacin
FA	Fusidic acid
GNB	Gram-negative bacteria
GPB	Gram-positive bacteria
MRSA	Methicillin-resistant *S. aureus*
MSSA	Methicillin-susceptible *S. aureus*
MUP	Mupirocin
NEO	Neomycin
OF	Ofloxacin
PB	Polymyxin B

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Staphylococcus aureus.[7,8] Recent publications have indicated that MUP resistance has developed in these bacteria and that this resistance is associated with methicillin resistance, as well.[9,10] This resistance would remove MUP from its useful role to treat staphylococcal wound infections and to eradicate *S. aureus* from nasal carriers and perhaps replace MUP sensitive strains with the more formidable methicillin resistant strains.

Recently, another fluoroquinolone antibiotic, ofloxacin (OF), was tested in vitro and shown be active against both Gram-negative and Gram-positive bacteria equal to or exceeding the activity of CF.[11] Studies have shown that resistance mechanisms of OF may not be the same as those for other fluoroquinolones,[12,13] and OF also appears to have a lower mutational rate to resistance among staphylococcal isolates compared to other antibiotics in this class.[14] Furthermore, other studies showed that fluoroquinolone resistance can be reduced by combining the fluoroquinolone antibiotic with other antibiotics.[15,16] Therefore, we tested clinical isolates of CF-resistant bacteria for their susceptibility to OF, with the idea that, if the test results warranted, we could substitute OF for the CF contained in our topical mixture. In the case of MUP a variety of clinical isolates of Gram-positive bacteria plus specific MUP resistant Gram-positive bacteria were tested against an alternative Gram-positive spectrum antibiotic, fusidic acid (FA).[17]

In addition, we tested the clinical isolates of both CF and MUP resistant bacteria for their susceptibility to the other antibacterial components of our topical mixture, alone and in combination, to determine if the redundancy of antibacterial spectrum coverage associated with our topical mixture as constituted or modified might preclude the emergence of CF and MUP resistant organisms, or their persistence, should our mixture be used clinically.

Bacteria resistant to other specific antibiotics were also tested.

MATERIALS AND METHODS

Thirty bacterial isolates from our patients (10 each *Pseudomonas aeruginosa* and *S. aureus* plus three *Escherichia coli,* four *Enterobacter cloacae,* two *Klebsiella oxytoca*, and one *Serratia marcescens*) were tested for their susceptibility and/or resistance to OF and FA. In addition, 48 CF-resistant bacterial isolates (10 *S. aureus,* 5 coagulase negative staphylococci, 12 *P. aeruginosa*, 5 *E. coli*, 4 *K. pneumoniae,* 3 each of *Proteus mirabilis* and *Providencia stuartii*, and 2 each of *Enterobacter* sp., *Acinetobacter* sp., and *S. marcescens*) were supplied by the University of Iowa, Department of Pathology, Medical Microbiology Division, through the courtesy of Ronald W. Jones, M.D. These strains were determined to be CF-resistant using a variety of standard clinical microbiology procedures. These bacteria were retested for CF resistance and tested for susceptibility and/or resistance to OF and the individual and combined antibacterial antibiotics plus amphotericin B in the concentrations used in an "optimized" topical antimicrobial mixture for use with cultured skin autografts[6] (see below). A variety of Gram-positive clinical isolates for MUP and FA testing as well as organisms resistant to other specific antibiotics were obtained from our own patients or were supplied by the Clinical Microbiology Laboratory, University Hospital of the University of Cincinnati College of Medicine, Joe Staneck, Ph.D., Director. All susceptibility and/or resistance testing was done using the agar well diffusion topical testing assay described below.

Four MUP resistant *S. aureus* and 10 MUP resistant coagulase negative staphylococci were supplied by Dr. S. F. Bradley, Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, MI.

Antibiotics

Concentrations of the following antibiotics were found to be nontoxic for fibroblasts and keratinocytes, grown in cell culture, by methods described previously.[18,19] All individual antimicrobials were used at the highest concentration which was nontoxic to these cells. Further the various antimicrobial combinations used in this study were shown to be, collectively, nontoxic for these cells in culture, also. They include neomycin (NEO), 40 μg/ml: polymyxin B (PB), 700 U/ml: MUP, 20 μg/ml, CF, 20 μg/ml. A mixture of these antibiotics will be referred to as our topical antimicrobial mixture. OF was found to be nontoxic for cells in culture at a concentration of 20 μg/ml and FA at 10 μg/ml.[20] Because amphotericin B (1 μg/ml) was part of our original "idealized" topical antimicrobial mixture[2] and its presence might affect the efficacy of various antibiotics in mixtures, amphotericin B was added to the topical antimicrobial combinations tested in this study. Bacterial isolates were tested for susceptibility and/or resistance to the individual topical antibacterial solutions and combinations which included a base group of antibiotics (NEO and PB) to which were added various combinations of CF, MUP and FA. All antibiotic solutions were prepared by our hospital pharmacy.

Agar Well Diffusion Topical Testing Assay

For topical testing of the bacterial isolates for susceptibility and/or resistance to the individual and combined antibacterial solutions described above, an agar well diffusion topical testing assay was used. This was a modification[21] of the published method originally designed to test the efficacy of topical antimicrobial creams and ointments.[22] The test microorganism, grown up to a density of 0.5 MacFarland standard in brain-heart infusion broth, was poured evenly over the surface of commercially available 150 mm Mueller-Hinton agar plates (BBL; Cockeysville, MD). After the excess inoculum was decanted, the plate surface was dried, and 6-mm wells cut into the surface. The wells were filled with 100 μl of antimicrobial solutions. All plates were incubated (35° C) right-side-up overnight.

After incubation, the diameters of any clear zones around the antimicrobial-containing wells were measured using calipers. Because the antimicrobial agents would be used prospectively as wet soak dressings directly over the cultured skin grafts, and therefore would be in direct contact with the bacteria colonizing the surface of the graft or graft bed, it was decided that a zone of clearing around the agar well of ≥2 mm in radius (i.e., a total zone diameter measurement of ≥10 mm) would be taken as susceptibility of the test bacterial strain to the antimicrobial.

RESULTS

Except for two *S. aureus* strains, 28 other CF-susceptible bacterial isolates from burn patients were susceptible to OF. FA was effective against all 10 CF-susceptible *S. aureus* burn isolates but showed no activity against Gram-negative bacteria (GNB) (data not shown).

Of 42 CF-resistant bacteria tested, only eight were susceptible to OF: 3 methicillin-resistant *S. aureus* (MRSA); 3 methicillin-susceptible *S. aureus* (MSSA); one each *P. aeruginosa* and *Acinetobacter* sp. Thus, OF gave no distinct advantage as a substitute for CF in this mixture (data not shown).

Susceptibility/Resistance of Gram-Positive Bacteria to MUP and FA

Forty Gram-positive bacteria (GPB), selected from the sources described in Materials and Methods, were used for testing against MUP and FA. They included strains that were susceptible and resistant to CF and/or other antibiotics. Both MUP and FA had approximately the same activities against MSSA, MRSA and coagulase negative staphylococci with 32/40 and 29/40 strains tested shown to be susceptible, respectively (Table 1). Both had poor activity against *Enterococcus* sp. with only 4/10 strains susceptible to MUP, and none susceptible to FA.

Susceptibility/Resistance of CF-Resistant Bacteria

Forty-eight CF-resistant bacterial isolates obtained from the University of Iowa, plus 15 additional isolates from this lab, or received from the University of Cincinnati Hospital Clinical Microbiology Laboratory, were tested against the individual nonquinolone antimicrobials that we have proposed as part of this topical antimicrobials mixture; NEO, PB, MUP and FA. These same isolates were tested in a combination mixture containing all of these individual antimicrobials plus CF or OF (Table 2).

While NEO alone showed little activity against GPB or *P. aeruginosa* resistant to CF, 13/23 other GNB were susceptible to its action. While PB, alone, showed poor activity against GPB, many GNB (24/35) were susceptible to its activity. As was expected, except for enterococci, MUP or FA, each alone, showed good activity against GPB. All mixtures containing a base combination of NEO, PB, MUP, and FA plus either CF or OF showed activity against 95% or more of all CF-resistant bacteria tested, except for enterococci. Results did not vary whether CF or OF was the quinolone component added to the basic mixture of NEO, PB, MUP and FA. The addition of amphotericin B did not appear to have any adverse effect on the antibacterial activity of any of the combinations (data not shown).

Susceptibility/Resistance of Bacteria Resistant to Specific Antibiotics

A variety of 15 Gram-negative or Gram-positive CF-resistant bacteria obtained from the University of Iowa plus 59 additional Gram-negative or Gram-positive bacteria resistant to other specific antibiotics or classes of antibiotics, obtained from this laboratory

Table 1. In vitro activity of mupirocin and fusidic acid against Gram-positive bacteria from a variety of sources

		Antibacterial agent	
Organism	***n***	**Mupirocin**	**Fusidic acid**
S. aureus	11	11	11
Methicillin-resistant *S. aureus*	14	13	13
Coagulase negative staphylococcus	5	4	5
Enterococcus sp.	10	4	0
TOTAL	40	32	29

Table 2. Susceptibility of CF-resistant bacteria to NEO, PB, MUP, or FA compared to a mixture of these antimicrobials plus CF or OF.

Organism	*n*	Individual antimicrobials				Greatest activity (# susceptible / # tested) of individual antimicrobials	Antimicrobial mixtures[a]
		NEO	PB	MUP	FA		
Gram-positive							
MRSA	8	0*	0	7	7	7/8	8/8
MSSA	2	0	2	2	2	2/2	2/2
Coagulase negative staphylococci	5	1	2	5	5	5/5	4/5
Enterococcus sp.	13	0	0	4	0	4/13	4/13
Gram-negative							
E. coli	5	4	4	NT[†]	NT	4/5	5/5
Klebsiella sp	4	3	3	NT	NT	3/4	4/4
P. aeruginosa	12	0	12	NT	NT	12/12	12/12
Proteus sp.	3	0	0	NT	NT	0/3	3/3
Enterobacter sp.	2	2	1	NT	NT	2/2	2/2
Acinetobacter sp.	2	1	2	NT	NT	2/2	2/2
S. marcescens	2	2	0	NT	NT	2/2	2/2
P. stuartii	3	0	0	NT	NT	0/3	3/3
C. freundii	2	1	2	NT	NT	2/2	2/2
TOTAL	63	14	28	18	14	45/63	53/63

[a]Mixtures contained NEO, PB, MUP, FA, and either CF or OF. Results were identical with addition of CF or OF.

*Susceptible organisms

[†]NT – not tested

or from the University of Cincinnati Hospital Clinical laboratory, were used to test against a mixture of antimicrobials (NEO, PB, MUP and CF) with or without the addition of FA (Table 3). The addition of FA to this topical antimicrobial mixture increased by four the total number of test bacteria susceptible to the mixture. As expected, these were among the MUP-resistant GPB. The addition of FA did not affect the susceptibility of any bacteria resistant to antibiotics other than MUP. Most (66/74) bacteria, resistant to specific antibiotics, were susceptible to this topical antimicrobial mixture. This included 12/15 quinolone-resistant GNB/GPB. The addition of FA to improve the efficacy of this topical antimicrobial mixture increased the overall effectiveness of the mixture to 70/74 (~95%) of bacteria tested. The four additional microorganisms covered were MUP resistant *S. aureus*. The addition of amphotericin B in any of these mixtures had no effect on the antibacterial activity of the mixtures (data not shown).

Table 3. Susceptibility of bacteria with resistance to specific antibiotics to the action of a topical antimicrobial mixture[a] with or without the addition of fusidic acid

Organism	*n*	Resistant to	– FA	+ FA
S. aureus	4	Mupirocin	1*	4
Coagulase negative staphylococci	10	Mupirocin	9	10
S. aureus	9	Penicillin	9	9
S. aureus	6	Methicillin	6	6
Gram-negative / Gram-positive[†]	15	Cephalosporins	15	15
Gram-negative[‡]	15	Aminoglycosides	14	14
Gram-negative / Gram-positive[§]	15	Quinolones	12	12
TOTAL	74		66	70

[a]Mixtures contained NEO, PB, MUP, FA, and CF.
*Number of susceptible organisms
[†]6 *E. cloacae*, 2 *E. coli*, 2 *S. epidermidis*, one each *S. aureus, A. anitratus, C. freundii, M. morganii, p. mirabilis*
[‡]5 *P. aeruginosa*, 2 *X. maltophilia*, 2 *P. mirabilis*, one each *A. anitratus*, *Enterobacer* sp., *F. meningosepticus, P. stuartii, S. marcescens*
[§]2 each *P. aeruginosa*, *E. coli*, one each *C. freundii, E. faecalis, E. aerogens*, *K. pneumoniae, M. morganii, P. stuartii, S. hemolyticus, X. maltophilia, P. mirabilis, S. marcescens,* MRSA

DISCUSSION

In searching for alternative drugs to replace some components of a topical antimicrobial mixture, in order to reduce the possibility of selecting for resistant organisms, we found that substituting OF for CF did not substantially improve the overall antibacterial coverage of CF-susceptible or CF-resistant bacteria; therefore, OF is not a substitute for CF. Similarly, FA activity against GPB was comparable to, but no better than, MUP against MUP-susceptible GPB (Table 1). Therefore, FA was excluded as a substitute for MUP for these bacteria.

When CF-resistant bacteria were tested against other individual components contained in this topical antimicrobial mixture, it was found that most bacteria were susceptible to the action of one or more of the other components (Table 2). The exception to this were the enterococci where only 4/13 or 0/13 CF-resistant isolates were susceptible to another actual (MUP) or potential (FA) component of the topical antimicrobial mixture, respectively. In addition, three CF-resistant *Proteus* sp. and three *Providencia stuartii* strains tested were resistant to the action of other antimicrobial components of this mixture. However, when base mixtures containing NEO and PB were tested with added combinations of CF or OF and MUP and FA, with the exception of the enterococci, 95+ % of all CF-resistant bacteria were susceptible to the activity of these combined mixtures (Table 2). This finding was true whether amphotericin B, added to extend coverage to fungi, was present or absent (data not shown). This result suggests that some additive antimicrobial activities may have resulted from mixtures of drugs compared to individual antimicrobial components.

These results support our postulate that a redundancy of antimicrobial coverage with antibiotic drugs having different mechanisms of action is a plausible means to control a CF-resistant bacterium, whether it was selected in situ through use of this topical antimicrobial mixture or acquired from the environment. Support of the postulate that redundancy of antimicrobial coverage using antimicrobials with varying modes of action is effective in preventing the generation and/or persistence of resistant strains of bacteria is obtained in results presented in Table 3. The fact that the bacteria most encountered in autografted wounds, including cultured skin autografts, are *S. aureus, S. epidermidis* and *P. aeruginosa*,[23–25] and that these were susceptible to other components in our topical mixture when CF-resistant strains of these genera and species were tested, reduces the concern over the apparent lack of efficacy of the other components against the CF-resistant bacteria cited above. *Candida albicans* is the most common fungal organism associated with autografts,[23–25] and the inclusion of amphotericin B in our mixture allows for antifungal coverage of these organisms as well.[1,2]

In Table 3, of 74 microbial isolates, including GNB and GPB, each individually resistant to one of six specific individual antibiotics representing 5 antibiotic classes, 66 were susceptible to the reported mixture consisting of NEO, POLY, CF, MUP and amphotericin B. If FA was added to this mixture the number of susceptible strains increased to 70. The four additional strains shown to be susceptible when FA was added were MUP-resistant *S. aureus*. Therefore, while our data suggest (Table 1) that FA would not afford increased anti-GPB activity against MUP susceptible bacteria, its addition to a MUP-containing mixture would increase the activity of the mixture to include MUP resistant GPB. Thus, with this redundancy of GPB coverage, the concern about MUP resistance developing with topical use of this mixture, or the persistence of MUP-resistant bacteria contaminating the grafts, would be reduced as well.

We conclude from these results that antimicrobial mixtures for topical use with cultured skin autografts which are formulated to meet the criteria described here for "idealized" topical antimicrobial mixtures, provide broad spectrum antimicrobial activity. Further, by their redundancy of antimicrobial components, these mixtures are formulated to prevent the emergence or persistence of antibiotic-resistant strains and super-infections. Therefore, these concerns should not preclude the use of topical antimicrobial mixtures, such as those described in this article and previously,[1,2] from being used clinically. Further, the spectrum of activity and the redundancy of antimicrobial coverage of our topical antimicrobial mixture can be improved by the addition of FA. Using our testing procedures, mixtures can be formulated to meet the specific needs of any institution.

We must caution the reader that some of the antimicrobials used in our mixtures are not approved for topical use and this off-label use may preclude clinical application in some institutions. Further, fusidic acid is not approved for use in the United States.

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