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Formulation of 'idealized' topical antimicrobial mixtures for use with cultured skin grafts

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In order to develop antimicrobial mixtures which provide broad-spectrum antimicrobial activity for use with cultured cell autografts, several individual antimicrobial agents, in concentrations non-toxic for cells in culture, were tested against a variety of bacteria and *Candida* spp. isolated from burn patients. An agar well diffusion topical assay was used. Antimicrobials active against Gram-positive and Gram-negative bacteria and antifungal agents, individually, were uniformly effective against their respective spectra of organisms. Broad-spectrum antibacterials were uniformly effective against Gram-negative bacteria but their activity varied against Gram-positive bacteria. Adding an agent active against Gram-positive bacteria to all broad spectrum antibacterial agents conferred uniform Gram-positive activity to the mixture. One mixture consisting of specific Gram-negative, Gram-positive and broad spectrum antibacterial agents, was uniformly active against all bacteria tested and the addition of antifungal agents extended the activity to cover *Candida* spp. without interfering with the mixture's overall antibacterial activity. Another mixture showed either additive or antagonistic activities against the battery of microorganisms tested. Thus, these methods can be used to identify mixtures of antimicrobials, in concentrations non-toxic for cells in culture, that have very broad spectra of antimicrobial activity. Such mixtures should be evaluated in patients when cultured skin grafts are used.

Introduction

A new, potentially important approach for the closure of wounds of various types is the use of cultured skin grafts (Carver & Leigh, 1991). Most of these grafts contain a biopolymer implant with keratinocytes and, in some cases, fibroblasts. Because these grafts are avascular and only partially keratinized, they are fragile and readily subject to microbial destruction (Gallico *et al.*, 1984; Hansbrough *et al.*, 1989; Hull, Finlay & Miller, 1990), and the use of topical antimicrobials in conjunction with this type of graft is beneficial. Because of the fragile nature of grafts containing cultured cells, topical antimicrobials must be sought that are, in appropriate concentrations, non-toxic for keratinocytes and fibroblasts but still effective as antibacterial/antifungal agents. Recent studies from this institution have described in-vitro methods to test various concentrations of antimicrobials to determine their toxicity for cells in culture, and

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further, to test those concentrations of antimicrobials shown to be non-toxic for the retention of antimicrobial activity demonstrated by in-vitro testing (Holder & Boyce, 1994; Boyce & Holder, 1995). Using both of these methods, a number of antimicrobials, alone and in combination, have been shown to have potential for use on cultured skin autografts.

This report presents results of studies to formulate 'idealized' antimicrobial mixtures for use with cultured skin autografts that have the following characteristics: (i) concentrations of individual antimicrobials to be contained in the mixture should be non-toxic for keratinocytes and fibroblasts in culture and still retain antimicrobial activity; (ii) antimicrobial coverage should be broad spectrum and include both bacteria and fungi; (iii) individual antimicrobial components of the mixture should have synergic, additive or neutral but not antagonistic activity towards each other; (iv) there should be a redundancy of antimicrobial coverage among the antimicrobial components of the mixture to reduce the emergence of resistant strains and superinfections; (v) for the same reason, individual antimicrobial components with different modes of action should be included and (vi) should not contain any antimicrobial that would be used parenterally to treat sepsis in the institution in which the topical mixtures are being used. There should also be no systemic toxicity caused by absorption of the antimicrobial mixture through the wound, though this last characteristic of our 'idealized' topical antimicrobial mixture was not assessed in this report.

We describe here the results of in-vitro topical testing used to formulate and assess the antimicrobial activity of individual agents and mixtures of antimicrobials shown previously to be non-toxic for cells in culture.

Methods

Antimicrobials

A variety of individual and combined antimicrobial agents previously titrated to determine concentrations which were non-toxic for fibroblasts and keratinocytes in culture (Boyce & Holder, 1993) and which retained antimicrobial activity, assessed by an in-vitro topical testing procedure, were selected (Holder & Boyce, 1994; Boyce & Holder, 1995). The antimicrobials and the concentrations determined to be used for this study are listed in Table I.

Table I. Antimicrobials tested

Antimicrobial	Spectrum of organisms covered	Concentration
Mupirocin	Gram-positive bacteria	40 mg/L
Ramoplanin		1 mg/L
Polymyxin B	Gram-negative bacteria	500,000 U/L
Neomycin + polymyxin*	broad spectrum	neomycin 40 mg/L; polymyxin B 200,000 U/L
Ciprofloxacin	bacteria	20 mg/L
Norfloxacin		20 mg/L
Amphotericin B	fungi	5 mg/L
Nystatin		100 mg/L

Table II. Susceptibility of bacteria and yeasts isolated from burn patient to individual antimicrobial solutions in concentrations non-toxic to cells in culture

Microorganisms	N/P	N/P+	POLY	CIP	NOR	RAM	MUP	AMP	NY
S. aureus	6/10[a]	3/10	0/10	7/10	5/10	10/10	10/10	0/10	0/10
P. aeruginosa	10/10	10/10	10/10	10/10	10/10	0/10	0/10	0/10	0/10
Misc. GNR	10/10	10/10	10/10	10/10	10/10	0/10	0/10	0/10	0/10
Candida spp.	0/10	0/10	0/10	0/10	0/10	0/10	0/10	10/10	10/10

N/P, neomycin and polymyxin; N/P+, N/P plus additional polymyxin B; POLY, polymyxin B; CIP, ciprofloxacin; NOR, norfloxacin; RAM, ramoplanin; MUP, mupirocin; AMP, amphotericin B; NY, nystatin.
[a]Number susceptible/number tested.

The sources of the antimicrobials are as follows: mupirocin—donated, Smith Kline Beecham Pharmaceuticals, Philadelphia, PA; ramoplanin—donated, Marion Merrell Dow Research Institute, Cincinnati, OH; polymyxin B—Burroughs Wellcome Company, Research Triangle, NC; GU irrigant:neomycin + polymyxin B,—Schein Pharmaceutical Inc., Floram Park, NJ; ciprofloxacin—Miles, Inc., West Haven, CT; norfloxacin—donated, Merck Institute for Therapeutic Research, West Point, PA; amphotericin B—E. R. Squibb & Sons, Inc., Princeton, NJ; nystatin—Paddock Laboratories, Inc., Minneapolis, MN, USA.

Microorganisms

Ten strains of *Staphylococcus aureus*, ten strains of *Pseudomonas aeruginosa*, miscellaneous Gram-negative bacteria (four *Enterobacter cloacae*, three *Escherichia coli*, two *Klebsiella oxytoca*, one *Serratia marcescens*) and ten *Candida* spp. (seven *Candida albicans*, three *Candida parapsilosis*) all isolated from patients at the Shriners Burns Institute, Cincinnati Unit, were tested.

Topical antimicrobial assay system

A modification (Holder & Boyce, 1994) of the agar well diffusion topical testing assay, originally described by Nathan *et al.* (1978), was used. 0.1 mL of the test antimicrobials, alone and in various combinations, were placed in 6 mm wells cut in the surface of a Mueller-Hinton agar plate (Baxter Healthcare Corp., OH, USA) which had been uniformly inoculated with the test microorganism grown to the density of an 0.5 McFarland standard. After overnight incubation of the plates at 35°C, the diameters of clear zones of inhibition around the test wells were measured. Zones of $\geqslant 1$ mm around the test well (total zone diameter measurement of $\geqslant 8$ mm including the well) were taken to indicate susceptibility of the microorganism to the individual or mixture of test antimicrobial(s) in the well.

Results

Results of individual antimicrobial testing are presented in Table II. As was expected antibiotics having activity only against Gram-positive bacteria (mupirocin; ramoplanin) were inactive against Gram-negative bacteria but were uniformly active against *S. aureus* while the antibiotic with activity only against Gram-negative bacteria (polymyxin B) was uniformly active against Gram-negative test strains but inactive

against *S. aureus*. Antimicrobials with broad-spectrum antibacterial activity (neomycin + 200,000 U/L polymyxin B, neomycin + 700,000 U/L polymyxin B, ciprofloxacin or norfloxacin) were uniformly active against Gram-negative bacteria but varied in their action against *S. aureus*. Antifungal agents (nystatin, amphotericin B) were uniformly active against *Candida* spp.

All *S. aureus* strains resistant to one or more of the broad-spectrum antibiotics (neomycin + 200,000 U/L polymyxin B, neomycin + 700,000 U/L polymyxin B, ciprofloxacin, norfloxacin) were uniformly susceptible to antibiotics with Gram-positive spectra (mupirocin, ramoplanin).

The addition of other broad-spectrum antibacterials (ciprofloxacin, norfloxacin) or antifungal agents (nystatin, amphotericin B), singly or in combination, to a basic antimicrobial mixture with broad spectrum activity (neomycin + 700 U/mL polymyxin + mupirocin) did not affect the antibacterial activity of the basic mixture. Furthermore, these additions gave a redundancy of broad-spectrum antibacterial coverage while extending the spectrum to include *Candida* spp. The addition of ciprofloxacin, norfloxacin, nystatin and amphotericin B, however, did change assay results when mupirocin was replaced by ramoplanin in the basic antimicrobial mixture (Table III). In this case, the addition of ciprofloxacin or norfloxacin extended the spectrum of activity of the mixture to include two and three of the strains of *Candida* spp. respectively. On the other hand, the combination of amphotericin B with either ciprofloxacin or norfloxacin had an antagonistic effect on the overall effectiveness of amphotericin B against *Candida* spp. with only seven of ten or nine of ten strains, respectively, showing susceptibility.

Discussion

Microbial contamination of graft beds is a major factor in the failure of skin grafts in general, and of cultured cell autografts in particular (Clugston *et al.*, 1991). Significant contamination of the graft arises from the placement of grafts on beds that are already colonized by microorganisms (Greenleaf, Cooper & Hansborough, 1991). While exogenous contamination of the graft sites is a possibility, recent evidence suggests that most colonized burn wound autograft sites are 'self-colonized' by microorganisms from various other sites on the patient (Neely *et al.*, 1991). Appropriate antimicrobial coverage for the microorganisms colonizing the graft site leads to increased graft take (Clugston *et al.*, 1991), and topical antimicrobial treatment has been recommended, especially in the cases where cultured cell autografts are used (Lineaweaver *et al.*, 1985). While some antimicrobials are overtly toxic for the keratinocytes and fibroblasts used to prepare cultured cell autografts, certain concentrations of others are safe to use (Lineweaver *et al.*, 1985; Boyce & Holder, 1993). However, microorganisms important in graft failure are quite diverse and include, among others, *P. aeruginosa*, *S. aureus*, and *C. albicans* (Barillo, Nangle & Farrell, 1991; Greenleaf *et al.*, 1991; Neely *et al.*, 1991). Thus, it appeared that mixtures of antimicrobials would be needed to treat grafts composed of cultured cells. With this in mind, using methods we have described previously to determine antimicrobial concentrations non-toxic for cultured cells, (Holder & Boyce, 1994; Boyce & Holder, 1995) we have confirmed that concentrations of individual antimicrobials non-toxic for cells in culture can retain their antimicrobial activities determined by an in-vitro agar well topical antimicrobial assay (Table II). We have shown in this report, furthermore, that deficiencies in the Gram-positive coverage

Table III. Effect of additional antimicrobials added to a basic N/P+; RAM mixture on the susceptibility of various bacteria and yeasts isolated from burn patients

Microorganisms[a]	N/P+; RAM	+CIP	+NOR	+CIP/AMP	+CIP/NY	+NOR/AMP	+NOR/NY
S. aureus	10/10[a]	10/10	10/10	10/10	10/10	10/10	10/10
P. aeruginosa	10/10	10/10	10/10	10/10	10/10	10/10	10/10
Misc. GNR	10/10	10/10	10/10	10/10	10/10	10/10	10/10
Candida spp.	0/10	3/10	2/10	7/10	10/10	9/10	10/10

N/P+; RAM, Neomycin plus 700,000 U/L polymyxin B and ramplanin; CIP, ciprofloxacin; NOR, norfloxacin; CIP/AMP, ciprofloxacin/amphotericin B; CIP/NY, ciprofloxacin/nystatin; NOR/AMP, norfloxacin/amphotericin B; NOR/NY, norfloxacin/nystatin; GNR, Gram-negative bacilli.

[a]Number susceptible/number tested.

of broad-spectrum antibacterial substances can be overcome by their addition to antimicrobials with specific Gram-positive antibacterial spectra and that no antagonistic effects occur when some anti-Gram-positive or anti-Gram-negative antimicrobials are combined with broad-spectrum antimicrobials. In addition, we have demonstrated that antimicrobial mixtures which have additive or antagonistic activities against some groups of microorganisms can be identified (Table III). Using our assays, antimicrobial mixtures which meet most of the criteria for the 'idealized' antimicrobial mixtures defined in the introduction to this report can be derived, and other mixtures can be excluded from consideration. The successful mixtures are non-toxic for cells in culture, they still retain broad-spectrum antimicrobial activity, and the individual components are not antagonistic to one another. Also, the individual components of the mixtures provide both a redundancy of antimicrobial coverage and a variety of modes of antimicrobial action, which should reduce the risk of development of resistance and prevent superinfection.

While all of our data generated *in vitro* suggest that our methods might be used to formulate 'idealized' mixtures of antimicrobial agents for potential use on patients, a correlation between our results obtained *in vitro* and clinical efficacy has not been established. However, we have shown recently that topical treatment of intentionally contaminated grafts in athymic mice for 14 days with one of the antimicrobial mixtures described in the current study (neomycin + polymyxin B 700 U/mL, mupirocin, ciprofloxacin and amphotericin B) led to significantly less bacterial colonisation and significantly increased wound healing compared to control, sham treated grafts (Boyce *et al.*, 1995). This suggests that results of our in-vitro testing is relevant to efficacy *in vivo*. Because of this, we recommend that the procedures for the formulation of 'idealized' mixtures of antimicrobial solutions described in this report be used to prepare additional antimicrobial mixtures. Our mixture and these mixtures should be tested as topical antimicrobial therapy on autografts made from cultured skin cells. The specific antimicrobials used in any one institution can be formulated to the specific needs of that institution based upon the particular microorganisms that they encounter, the antimicrobial agents that are used for parenteral treatment in that patient population, etc. Thus, using our procedures, topical antimicrobial treatment can be customized to suit the needs of any particular institution using cultured skin autografts.

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