Selection of Topical Antimicrobial Agents for Cultured Skin for Burns by Combined Assessment of Cellular Cytotoxicity and Antimicrobial Activity

Steven T. Boyce, Ph.D., and Ian Alan Holder, Ph.D.

Cincinnati, Ohio

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Cultured epidermal skin has become an adjunctive therapy for treatment of major burn injuries, but its effectiveness is greatly limited due to destruction by microbial contamination. To evaluate candidate drugs for use with cultured skin, a combined cytotoxicity-antimicrobial assay system was developed for determination of toxicity to cultured human keratinocytes and fibroblasts, and to common burn wound organisms (20 bacterial and 4 fungal strains). Candidate agents including Hibiclens ($n = 3$), amikacin, piperacillin, norfloxacin, and nystatin were tested separately and in combination ($n = 6$ each) for inhibition of growth of human cells and lytic activity on microorganisms in the wet disc assay. The data showed that: (1) Hibiclens was uniformly toxic to both cultured human cells and microorganisms; (2) norfloxacin had dose-dependent toxicity to human cells and broad effectiveness against microorganisms; and (3) norfloxacin (25 μg/mL) plus nystatin (100 U/mL) had low toxicity to human cells and high toxicity to both Gram-positive and Gram-negative bacteria (20 of 20) and fungi (4 of 4). Selection of topical antimicrobial drugs by these assays may improve effectiveness of cultured skin for burns and may be extended to the control of other surgical wound infections. (*Plast. Reconstr. Surg.* 92: 493, 1993.)

Recent advances in the grafting of burns and giant congenital nevi include cultured epithelium,[1–3] alone or in combination with biopolymer implants[4–6] or allodermis.[7,8] However, incomplete epidermal barrier and lack of vascular and immune components at grafting make all models[9–11] of cultured skin more subject to destruction by burn organisms than native skin grafts. These biologic deficiencies also make cultured epidermal keratinocytes more subject to cytotoxicity of topical antimicrobial agents. Most parenteral antimicrobial drugs are effective and have low toxicity, but topical use may induce resistant organisms that complicate treatment of sepsis. Therefore, requirements for topical antimicrobial agents for cultured skin include: (1) low toxicity to cultured human skin cells; (2) high activity against common burn wound organisms; and (3) no overlap of activity with parenteral drugs. Assays for drugs that meet these requirements have tested cytotoxicity to cultured human fibroblasts[12] or keratinocytes,[13] antimicrobial activity,[14–16] or combinations of fibroblasts and burn organisms.[17–19] This report describes a combined cytotoxicity-antimicrobial assay system in which candidate agents may be tested for toxicity to cultured human epidermal keratinocytes and fibroblasts, and also for antimicrobial activity to common burn wound organisms. Three antibiotics (amikacin, piperacillin, norfloxacin), an antimycotic (nystatin), and an antiseptic (Hibiclens) were tested, alone and in combination, on cultured cells and four isolates each of burn organisms (*Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter sp., Escherichia coli, Candida sp.*). The data show that combinations of norfloxacin (25 μg/mL) plus nystatin (100 U/mL), or amikacin

From the Shriners Burns Institute and Department of Surgery, University of Cincinnati, Cincinnati, Ohio.

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(150 μg/mL) plus piperacillin (300 μg/mL) plus nystatin (100 U/mL) are nontoxic to cultured human cells and effective against a broad spectrum of burn organisms. The data suggest that the combination of norfloxacin, a quinolone, with nystatin may have potential as a topical antimicrobial on cultured skin for burns.

Materials and Methods

Experimental Design

Figure 1 shows the procedural plan by which candidate antimicrobial agents are qualified as having no/low toxicity to cultured human skin cells, and antimicrobial activity to common burn wound organisms. This strategy for serial testing was developed from an initial protocol of parallel testing to reduce the number of tests required for each compound. After determination of the highest concentration that is not cytotoxic to cultured keratinocytes and fibroblasts, that concentration was tested for antimicrobial activity on representative samples of microorganisms. In this study, both serial and parallel testing of agents on cultured cells and microorganisms were performed, but serial testing as diagrammed in Figure 1 is recommended for efficiency.

Antimicrobial Agents

Table I lists the antimicrobial agents tested, the highest test concentration, and the pH and osmolarity. For human cell assays, each agent was diluted serially to 100-fold of the highest test dose into respective growth media at half-log concentrations. For antimicrobial assays, the highest concentration was tested on 24 clinical isolates of burn organisms (see below).

Human Cell Assay

Human epidermal keratinocytes and dermal fibroblasts were isolated from surgical discard tissue obtained with the approval of the University of Cincinnati Institutional Review Board. Epidermal keratinocytes were grown as described previously[20, 21] in serum-free MCDB 153 medium with 0.2 mM calcium and increased amino acids,[4] and supplemented with 0.5% bovine pituitary extract, 1 ng/mL epidermal growth factor, 5 μg/mL insulin, and 0.5 μg/mL hydrocortisone. Dermal fibroblasts were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 1 ng/mL EGF, 5 μg/mL insulin, and 0.5 μg/mL hydrocortisone. Keratinocytes or fibroblasts were inoculated into petri dishes at 2×10^4 cells/dish into the respective culture media described above that also contained penicillin (100 U/mL)-streptomycin (100 μg/mL)-amphotericin B (PSF) (0.25 μg/mL). Cells were incubated for 4 days at 37°C in 5% CO_2 with saturated humidity. On day 4, media were changed to remove PSF, baseline cell counts were performed, and test compounds were titrated into the cell cultures. Cells were incubated for 4 additional days and then harvested and counted.[13] Controls included media with PSF and elevated osmolarity (keratinocytes, 370 and 400 mOs; fibroblasts, 350 and 400 mOs). Each experimental condition

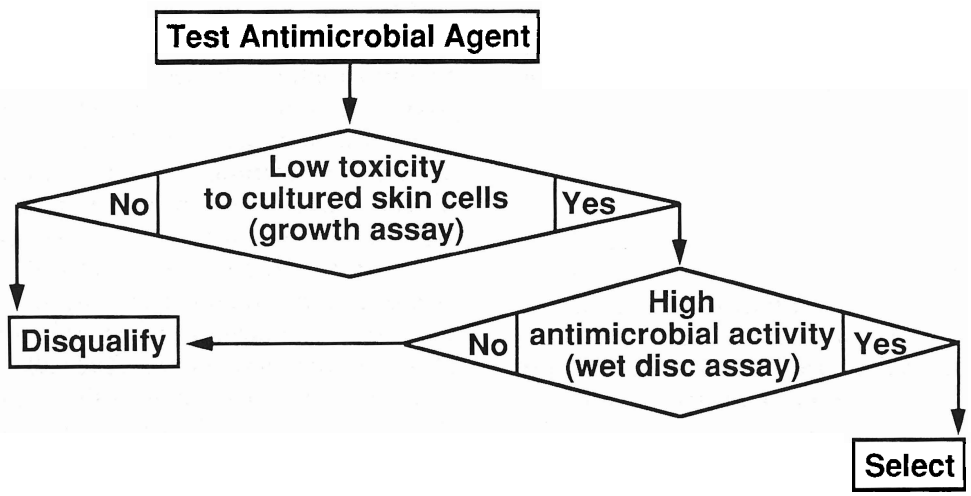

FIG. 1. Experimental design. Candidate agents for topical use on cultured skin grafts are subjected to combined assays of cytotoxicity to cultured human skin cells (epidermal keratinocytes and dermal fibroblasts), and antimicrobial activity to clinical isolates of organisms from burn wounds.

TABLE I
Antimicrobial Agents Tested

	Test Agent	Highest Test Dose	KM/FM pH	KM/FM Osm
Antiseptic:	1 Hibiclens*	50% (V/V)	7.07/7.70	727/693
		15% (V/V)	7.46/7.37	467/418
		5% (V/V)	7.53/7.56	390/344
Antibiotics:	2 Amikacin	150 μg/mL	7.60/7.76	353/309
	3 Piperacillin	300 μg/mL	7.59/7.85	354/312
	4. Norfloxacin	250 μg/mL	7.65/7.90	352/309
Antimycotic:	5 Nystatin	1000 U/mL	7.47/7.28	331/291
Combinations:	6 Amikacin	150 μg/mL	7.50/7.33	356/305
	Piperacillin	300 μg/mL		
	Nystatin	100 U/mL		
	7 Norfloxacin	25 μg/mL	7.51/7.42	352/309
	Nystatin (constant)	100 U/mL		

Abbreviations: KM, keratinocyte medium; FM, fibroblast medium; Osm, osmolarity.
* A commercial skin disinfectant product that contains 4% (v/v) chlorhexidine gluconate as the active antimicrobial ingredient.

was performed in triplicate, and each experiment was repeated once ($n = 6$; except Hibiclens, $n = 3$).

Wet Disc Assay

Four strains each of *S. aureus, P. aeruginosa, K. pneumoniae, Enterobacter sp., E. coli,* and *Candida sp.* were isolated from wounds of burn patients at the Shriners Burns Institute, Cincinnati Unit. Petri dishes (150 × 15 mm) containing Mueller-Hinton agar were inoculated by uniformly swabbing the plate surface with a suspension of each organism diluted to 0.5 McFarlane density units. Sterile 6-mm filter paper discs, placed on the microbial lawns, received 25 μL of the highest test dose of antimicrobial solution. Dishes were incubated at 37°C overnight, and the zone of clearing was scored.[15]

Data Collection, Analysis, and Interpretation

Data collected from human cell assays were tested for significance ($p < 0.05$, ANOVA) compared with cultures that received no drug. Antimicrobial agents tested in wet disc assays were scored as effective if the zone of clearing was ≥2 mm diameter. By evaluation of combined results from both assays, dosages of test agents that had no/low toxicity to cultured cells and were effective in the wet disc assay could be considered for topical use with cultured skin.

Results

Human Cell Assays

Figure 2 shows that PSF or increased osmolarity caused no significant reduction in growth of human keratinocytes and fibroblasts. Hibiclens (Fig. 3) at concentrations as low as 0.5 percent (V/V) inhibited growth of both fibroblasts and keratinocytes. Separate titrations of amikacin, piperacillin, or nystatin over two log dilutions caused no inhibition of growth of either fibroblasts or keratinocytes (data not shown). Figure 4 shows that combination of amikacin

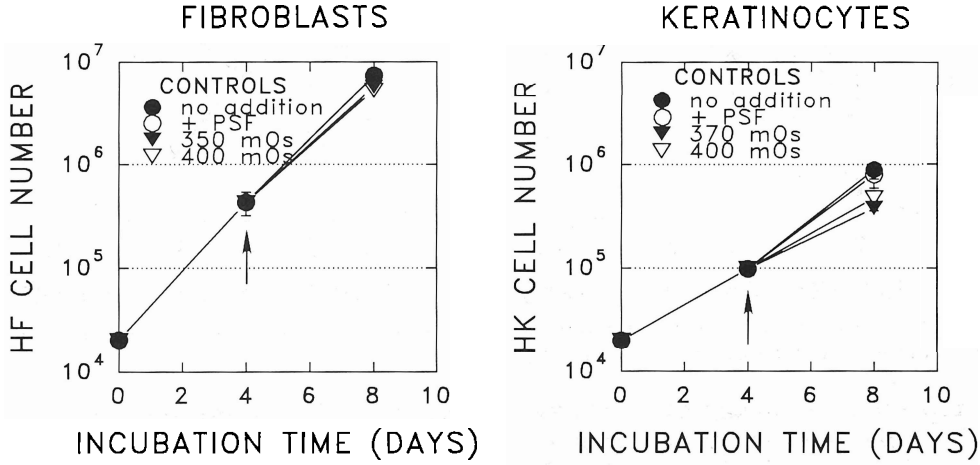

FIG. 2. Cell culture controls: increased osmolarity and penicillin-streptomycin-fungizone. Test conditions were initiated on culture day 4 (↑).

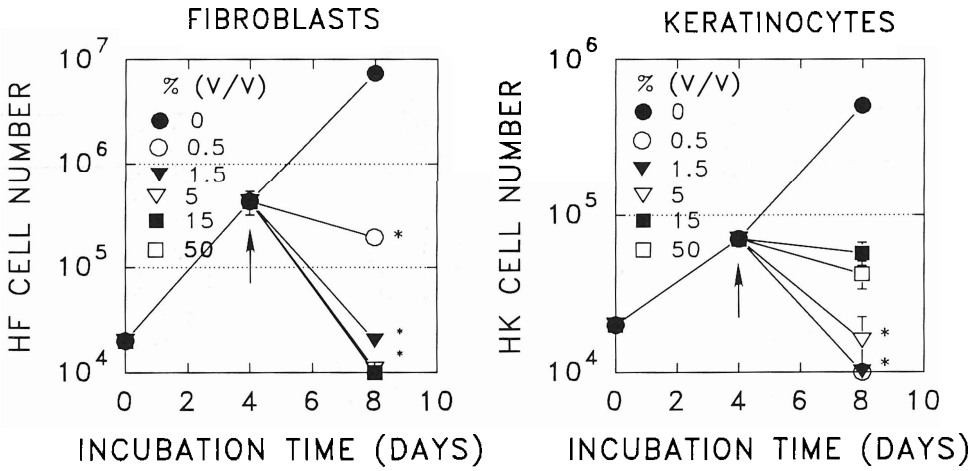

FIG. 3. Dose response of cultured human keratinocytes and fibroblasts to Hibiclens. Test conditions were initiated on culture day 4 (↑).

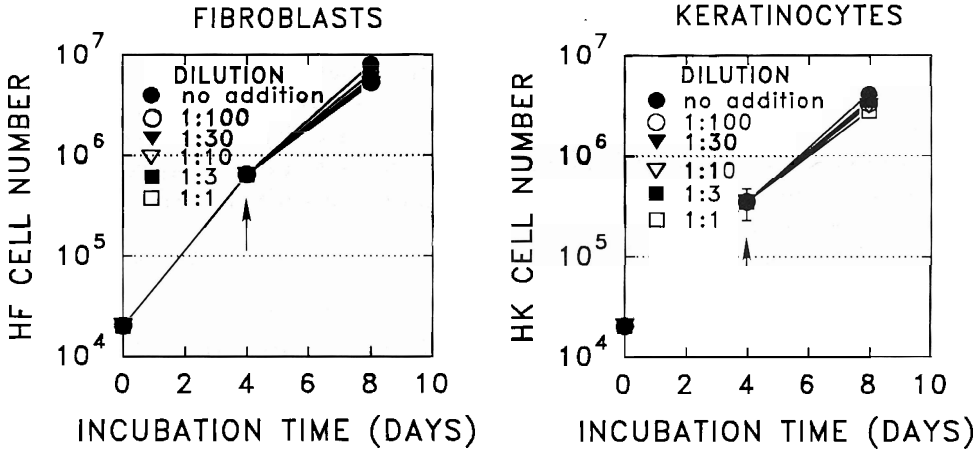

FIG. 4. Dose response of cultured human keratinocytes and fibroblasts to amikacin-piperacillin-nystatin. Test conditions were initiated on culture day 4 (↑).

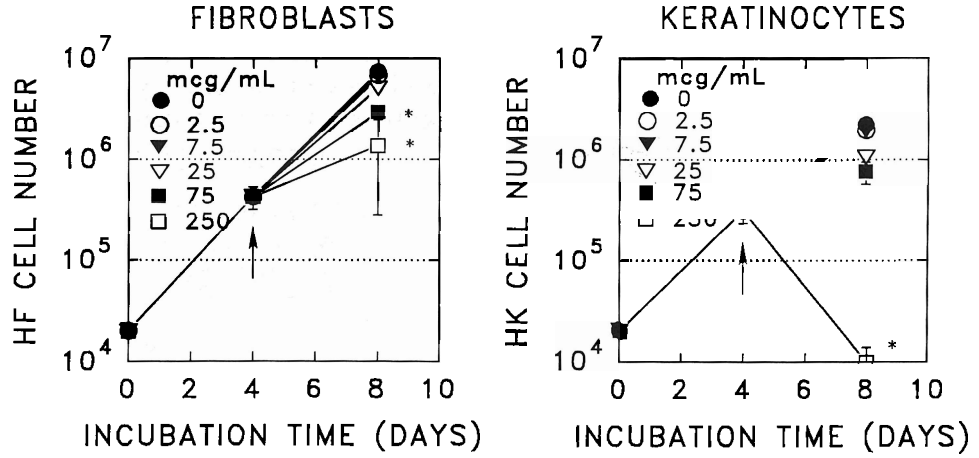

FIG. 5. Dose response of cultured human keratinocytes and fibroblasts to norfloxacin. Test conditions were initiated on culture day 4 (↑).

(150 μg/mL), piperacillin (300 μg/mL), and nystatin (100 U/mL) had no toxicity to either fibroblasts or keratinocytes. Norfloxacin (Fig. 5) inhibited significantly fibroblast growth at 250 μg/mL or 75 μg/mL but not at lower concentrations. Keratinocytes (Fig. 5) were destroyed by norfloxacin at 250 μg/mL in serum-free medium but were not significantly inhibited at

lower concentrations. Figure 6 shows that combination of 100 U/mL nystatin with norfloxacin at 25 μg/mL or less caused no significant inhibition of fibroblast or keratinocyte growth.

Figure 7 shows cultured human keratinocytes with no inhibition of growth (7*A*) or cytotoxic response (7*B*), and fibroblasts with no growth inhibition (7*C*) or after exposure to 5% Hibiclens (7*D*).

Wet Disc Assays

Table II summarizes results of wet disc assays of 24 clinical isolates of microorganisms from burn wounds. Hibiclens was uniformly effective (24 of 24) against all organisms tested. Amikacin (19 of 20) or piperacillin (15 of 20) was not uniformly effective against 20 bacterial strains but was uniformly effective (20 of 20) in combination. Nystatin was effective against *Candida* strains (4 of 4) both separately and in combination with amikacin plus piperacillin, or norfloxacin. Norfloxacin was uniformly effective against these bacterial strains (20 of 20). The combination of norfloxacin and nystatin was uniformly effective (24 of 24) against these test organisms.

Discussion

Although various models of cultured skin have been demonstrated to close wounds permanently,[1,4,7] all are more subject to destruction by common burn organisms than are conventional skin grafts. This general limitation of cultured skin results from the incomplete epidermal barrier at grafting, the absence of vascular plexus and immune cells, and the consequent protraction of engraftment by several days. These biologic deficiencies of cultured skin account for its vulnerability to microbial destruction and impose additional surgical and postsurgical requirements for protection of the cultured cells until epidermal tissue becomes keratinized. However, it has been shown[12,13,22] that topical antimicrobial agents (i.e., mafenide acetate, silver sulfadiazine, Hibiclens) that are used successfully with native skin grafts are toxic to cultured keratinocytes and lead to failure of cultured grafts. Therefore, quantitative assays are needed to select antimicrobial agents in appropriate dosages that have no or low toxicity to transplanted cells, and that retain a broad spectrum of antimicrobial activity. The results presented here demonstrate a combined assay system for determination of concentration ranges of candidate drugs with a low toxicity to human cells and a high susceptibility to burn wound microorganisms.

This assay system can both qualify and disqualify either individual agents or combinations of agents for topical use. Although Hibiclens is uniformly effective against a wide variety of burn organisms, it is disqualified from consideration because it is also highly cytotoxic to cultured keratinocytes and fibroblasts (Fig. 3, Table II). Conversely, norfloxacin is qualified as an antibiotic that has low toxicity to skin cells only within certain concentration ranges, but with retention of broad activity against burn organisms in those ranges (Fig. 5, Table II). However, subsequent testing of norfloxacin at 25 μg/mL or less has identified strains of *P. aureus* and *S. aureus* that are not susceptible. Furthermore, the wet disc assay for antimicrobial activity determined that amikacin and piperacillin separately

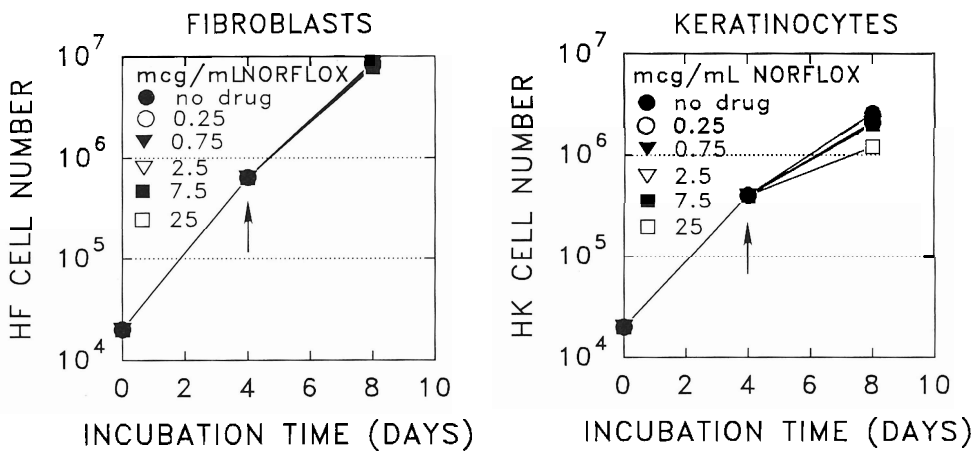

FIG. 6. Dose response of cultured human keratinocytes and fibroblasts to norfloxacin with a constant concentration of nystatin at 100 U/mL. Test conditions were initiated on culture day 4 (↑).

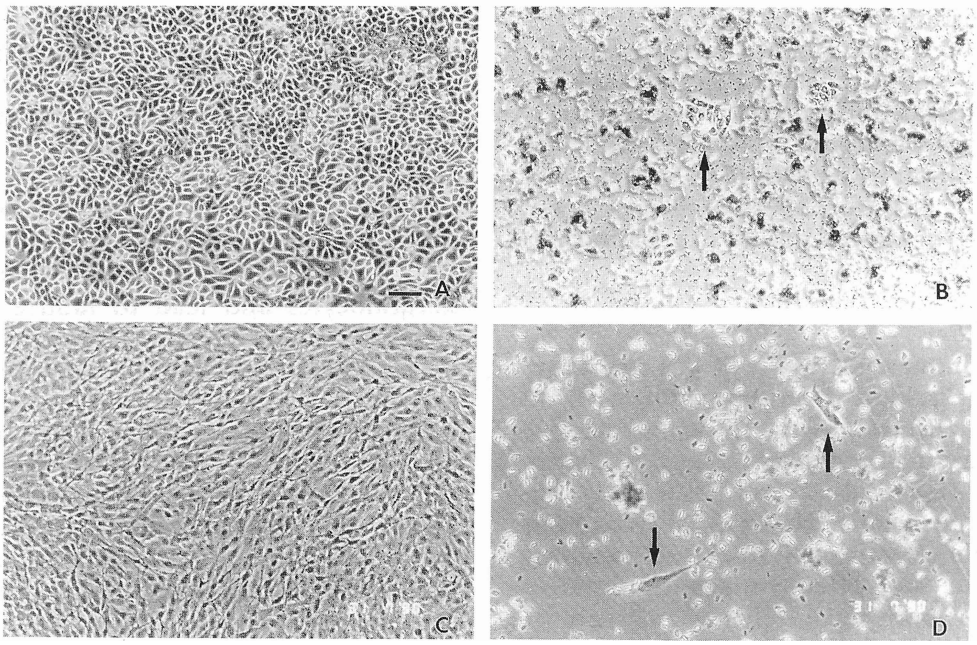

FIG. 7. Cytotoxic response of human keratinocytes and fibroblasts to antimicrobial agents. Actively growing control cultures of human keratinocytes (*A*) and fibroblasts (*C*). Cytotoxicity to keratinocytes (*B, arrows*) and fibroblasts (*D, arrows*) after exposure to 5% (V/V) Hibiclens.

TABLE II
Results: Wet DISC Assay

Organism*/Agent	HIBI	AM	PIP	NYS	AM-PIP-NYS	NORF	NORF-NYS
S. aureus	4/4†	3/4	4/4	0/4	4/4	4/4	4/4
P. aerug.	4/4	4/4	4/4	0/4	4/4	4/4	4/4
K. pneumo	4/4	4/4	2/4	0/4	4/4	4/4	4/4
Ent. sp.	4/4	4/4	4/4	0/4	4/4	4/4	4/4
E. coli	4/4	4/4	1/4	0/4	4/4	4/4	4/4
Candida sp.	4/4	0/4	0/4	4/4	3/4	0/4	4/4
Totals	24/24	19/24	15/24	4/24	23/24	20/24	24/24
Concentrations							
HIBI				50% (V/V)			
AM				150 μg/mL			
PIP				300 μg/mL			
NORF				25 μg/mL			
NYS				100 U/mL			

Abbreviations: HIBI, Hibiclens; AM, amikacin; PIP, piperacillin; NORF, norfloxacin; NYS, nystatin.
* Four different clinical isolates of each were tested: *S. aureus, P. aeruginosa, K. pneumoniae, Enterobacter sp., E. coli, Candida sp.*

are not uniformly effective against assorted burn organisms but are effective together. These combined cytotoxicity-antimicrobial assays also identified that addition of an antimycotic agent, nystatin, to antibiotic formulations does not increase cytotoxicity to transplanted cells or reduce the antimicrobial activities of the individual agents. Therefore, these assays identify highly stringent requirements for selected antimicrobial agents for avoiding cytotoxicity to cultured skin. However, it is expected that as cultured skin substitutes are developed with more biologic homology to native skin (i.e., improved keratinization and barrier function), stringency for clinical management of cultured and native skin grafts may also become more homologous.

Some other factors not addressed in this study that are important to clinical management of microbial contamination of cultured skin include: (1) nonoverlapping antimicrobial activity with parenteral treatment of burn sepsis; (2) delivery of adequate levels of antimicrobial formulations to the site of the microbial contamination; and (3) monitoring of contaminations of each patient to determine type and sensitivity of organisms to certain formulations. Amikacin and piperacillin are parenteral drugs used for treatment of sepsis and are not proposed here for

topical use. They were included in this study to demonstrate the process by which candidate compounds are qualified for individual or combined administration. Norfloxacin at 25 μg/mL or less, in combination with 100 U/mL nystatin, meets this additional criteria for topical use.[14,23] Delivery of novel formulations to the site of contamination is a complex question that requires dedicated studies to address. To understand and regulate drug delivery, future studies should address woundbed preparation and pretreatment, vehicles and protocols for administration of novel formulations, penetration and half-life of compounds in the wound, and effective dose at the graft-wound interface. Finally, monitoring of patient wounds to determine type and sensitivity of microbial contamination is a routine clinical procedure with which the cytotoxicity-antimicrobial assays described here can be easily coordinated. Any novel formulations of topical antimicrobials[24,25] qualified by the assays described here may be added to the sensitivity testing of organisms for identification of whether the formulations are suitable for use with cultured skin in particular patient cases.

In conclusion, this study demonstrates a direct and inexpensive assay system by which topical antimicrobial agents may be selected for use with cultured skin grafts for burn wound treatment. Application of these findings may improve engraftment and survival of cultured skin for burns and may assist in the identification of qualified formulations of antimicrobial agents for management of other surgical infections.

Steven T. Boyce, Ph.D.
Shriners Burns Institute
Research Department
3229 Burnet Avenue
Cincinnati, Ohio 45229-3095

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