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Noncytotoxic Combinations of Topical Antimicrobial Agents for Use with Cultured Skin Substitutes

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Cultured skin grafts are destroyed more easily than split-thickness skin grafts by common burn wound organisms, including gram-negative and gram-positive bacteria and fungi. To increase the survival and engraftment of cultured skin grafts, formulations of antimicrobial agents were tested for cytotoxicity to cultured human keratinocytes and fibroblasts and for activity against common organisms from burn wounds. On the basis of previous studies, a base formulation containing neomycin (40 μg/ml), polymyxin B (700 U/ml), and mupirocin (40 μg/ml) was prepared, to which ciprofloxacin (20 μg/ml) or norfloxacin (20 μg/ml) and amphotericin B (0.25 μg/ml) or nystatin (100 U/ml) were added. Toxicity to cultured human cells was determined by the growth response of cell cultures (n = 6) to each drug combination over 4 days. Activity against clinical isolates (n = 40) of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, other gram-negative bacteria, and *Candida* spp. was determined by the wet disc assay. Analysis of variance testing showed no significant differences in the growth of keratinocytes or fibroblasts under control or experimental conditions. Medium without antimicrobial agents was not effective against any of the 40 microbial strains tested. The base formulation was effective against all bacterial strains tested but against none of the fungi, while all experimental formulations were effective against all microbial strains tested. These findings suggest that neomycin, mupirocin, and polymyxin B may be combined with a quinolone and an antimycotic agent to provide broad antimicrobial activity for a formulation for topical use with cultured skin on burns. However, the formulations described here are strictly experimental and are not recommended for clinical use without further evaluation.

The management of the microbial contamination of burns to prevent sepsis is a routine requirement of acute care that has led to the development of a variety of therapeutic agents for topical use (30). Individual agents or combinations of agents for microbial management must provide effective stasis or activity against microbial growth until the host immune system regains function and access to the site of colonization. Major advances in topical antimicrobial therapy were made by the discovery of the antimicrobial activity of silver nitrate (31), which led to the development of other silver salts, including silver sulfadiazine (13). This compound remains in common use at present for the control of bacterial colonization of burn eschar before and after excision (30). Other topical agents including mafenide acetate (sulfamylon), bismuth tribromophenate (Xeroform), Dakin's solution (0.25% [vol/vol] sodium hypochlorite), bacitracin zinc, neomycin plus polymyxin B and bacitracin (Neosporin), mupirocin (Bactroban), gentamicin sulfate, and nystatin have been used individually and in combination to control microbial growth in burn wounds and on healing meshed skin grafts (21, 27, 35). All of these agents are considered effective in the control of burn microorganisms, and each has a spectrum of activity against gram-negative or gram-positive bacteria and/or fungi. Furthermore, all are considered relatively noncytotoxic with regard to the healing of viable tissue and skin grafts. However, the advent of alternative materials for grafting and healing of excised burns has introduced lower thresholds for the tolerance of cytotoxicity, particularly for the grafting of skin cells propagated in culture (18).

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Cultured skin substitutes are avascular and require longer periods than split-thickness skin grafts to engraft onto excised full-thickness wounds (1, 12, 14, 19, 22, 26, 33). This extended period of ischemia and nutrient deprivation provides an opportunity for microbial colonization and destruction of grafts containing cultured skin cells. Increased susceptibility to microbial contamination is a common complication that has been reported with the use of cultured epithelial autografts (1, 12, 14, 33) and collagen-cultured cell composites (2, 19, 26). Fewer complications with microbial contamination were reported in a study of DermaGraft, a polyglycolic-polylatic acid fabric (Vicryl) populated with allogeneic fibroblasts (20). Although the mechanism by which contamination is reduced with DermaGraft is not clear, the pH of the granulation tissue surrounding the hydrolyzing Vicryl may be sufficiently acidic to suppress microbial growth. However, polyglycolic-polylatic acid sutures and fabrics are also designed to promote inflammation, granulation tissue, and scar to add strength to wounds.

Increased contamination of cultured skin substitutes has stimulated qualitative and quantitative studies of antimicrobial agents that are effective against common burn organisms and permissive to survival and growth of transplanted skin cells but that do not overlap in activity with parenteral antimicrobial agents. Among the available agents, McCauley et al. (29) have found that low concentrations of silver are not toxic to fibroblasts, despite the formation of silver chloride precipitate in physiologic solutions. Cooper et al. (11) reported that sulfamylon and Dakin's solution were lethally toxic to cultured human keratinocytes but found that polymyxin B, bacitracin, neomycin, and gentamicin sulfate were not cytotoxic to epidermal cells. Lineweaver et al. (28) performed parallel evaluations of drug toxicities for fibroblasts and bacteria to identify the concentrations of drugs that provided effective antimicrobial activity but that were not cytotoxic to cells. More recently, a

TABLE 1. Antimicrobial agents tested

Formula[a]	Antimicrobial agent			
	Amphotericin B (0.25 μg/ml)	Nystatin (100 U/ml)	Ciprofloxacin (20 μg/ml)	Norfloxacin (20 μg/ml)
Formula 1	+	−	+	−
Formula 2	+	−	−	+
Formula 3	−	+	+	−
Formula 4	−	+	−	+

[a] All formulae contained a base of neomycin (40 μg/ml), polymyxin B (700 U/ml), and mupirocin (40 μg/ml).

general process for the evaluation of individual agents or combinations of drugs has been described in which the drugs are first tested on separate cultures of keratinocytes and fibroblasts to determine any growth inhibition (5). Compounds that do not inhibit cell growth significantly are tested by the wet disc assay for effectiveness against common burn organisms (7, 23). These studies identified certain concentration ranges of polymyxin B, mupirocin, a quinolone (norfloxacin or sparfloxacin), and nystatin that qualified the drugs individually as candidates for topical use with cultured skin substitutes.

The present study tests combinations of these qualified agents for any inhibition of the growth of cultured keratinocytes or fibroblasts and for broad activity against gram-negative and gram-positive bacteria and fungi isolated from burn patients. Antimicrobial agent formulations include polymyxin B (700 U/ml), neomycin (40 μg/ml), and mupirocin (40 μg/ml) in combination with an antimycotic agent (0.25 μg of amphotericin B per ml or 100 U of nystatin per ml) and a quinolone antibiotic (20 μg of norfloxacin per ml or 20 μg of ciprofloxacin per ml).

(Results of this work were presented at the 26th annual meeting of the American Burn Association on 21 April 1994.)

MATERIALS AND METHODS

Experiment design. The procedural plan by which candidate antimicrobial agents were qualified as having no or low toxicity to cultured human skin cells and activity against common burn wound organisms has been described elsewhere (5). After determination of the antimicrobial agent combinations that are not cytotoxic to cultured keratinocytes and fibroblasts, candidate formulations were tested for activity against microbial strains that were selected at random after isolation from burn wounds of hospitalized patients at the Shriners Burns Institute, Cincinnati Unit.

Antimicrobial agents. Table 1 lists the antimicrobial agents tested and the formulations of combined agents. The base formulation contained neomycin (40 μg/ml; Schein Pharmaceutical, Port Washington, N.Y.), polymyxin B (700 U/ml; Roerig-Pfizer, New York, N.Y.), and mupirocin (40 μg/ml; generously provided by SmithKline-Beecham), to which ciprofloxacin (20 μg/ml; Miles, Inc., Westhaven, Conn.) or norfloxacin (20 μg/ml; Sigma Chemical, St. Louis, Mo.) and amphotericin B (0.25 μg/ml; E. R. Squibb and Sons, Princeton, N.J.) or nystatin (100 U/ml; Grand Island Biological Co., Grand Island, N.Y.) were added. The qualitative and quantitative composition of the base formulation was determined from concentration-response titrations of individual agents in previous studies (5, 7, 11). All agents tested in the cultured-cell or microbial assays were formulated as aqueous solutions in serum-free culture medium for human cells. For human cell assays, each combination formulation was tested at the highest concentrations of individual agents that were noncytotoxic by previous determinations (5, 7, 11). For antimicrobial agent assays, combination formulations were tested on 40 clinical isolates of microorganisms isolated from full-thickness skin wounds of burn patients (Table 2).

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 in serum-free medium (3, 4) which contained 0.2 mM calcium and increased amino acids (33) and which was supplemented with 0.5% bovine pituitary extract, 1 ng of epidermal growth factor per ml, 5 μg of insulin per ml, and 0.5 μg of hydrocortisone per ml. Dermal fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1 ng of epidermal growth factor per ml, 5 μg of insulin per ml, and 0.5 μg of hydrocortisone per ml. Keratinocytes or fibroblasts were inoculated into 60-mm-diameter petri dishes at 2×10^4 cells per dish. The dishes contained the appropriate culture medium described above plus penicillin (100 U/ml)-streptomycin (100 μg/ml)-amphotericin B (Fungizone) (0.25 μg/ml). Cells were incubated for 4 days at 37°C in 5% CO_2 with saturated humidity. Cell counts were performed with a Coulter Model Z_{BI} particle counter. On day 4, the media were changed to remove the penicillin-streptomycin-amphotericin B, baseline cell counts were performed, and test compounds were titrated into the cell cultures. Cells were incubated for 4 additional days, harvested, and counted. Six individual cultures were assayed for each experimental condition.

Wet disc assay. Drug-susceptible strains of gram-positive (*Staphylococcus aureus*, 10 strains) and gram-negative (*Pseudomonas aeruginosa*, 10 strains; *Klebsiella oxytoca*, 2 strains; *Escherichia coli*, 3 strains; *Enterobacter cloacae*, 4 strains; and *Serratia marcescens*, 1 strain) bacteria and fungi (*Candida albicans*, 7 strains; and *Candida parapsilosis*, 3 strains) were isolated from wounds of burn patients. Petri dishes (150 by 15 mm) containing Mueller-Hinton agar were inoculated by uniformly swabbing the plate surface with a suspension of each organism diluted to 0.5 McFarland density units. Sterile 6-mm-diameter filter paper discs were placed on the microbial lawns and wetted with 25 μl of each antimicrobial agent formulation prepared in serum-free medium for epidermal keratinocytes. Dishes were incubated at 37°C overnight, and the zone of clearing was scored. Antimicrobial agents tested in wet disc assays were scored as effective if the zone of clearing was ≥2 mm in diameter.

Data collection, analysis, and interpretation. Data collected from human cell assays were tested for significance ($P < 0.05$, analysis of variance) compared with those from cultures that received no drug. By an evaluation of the combined results of both assays, dosages of test agents that had no significant growth inhibition for cultured cells and were effective in the wet disc assay could be selected for consideration for topical use with cultured skin.

TABLE 2. Comparison of the in vitro antimicrobial activities of formulae of topical antimicrobial agents prepared for use with cultured skin grafts

Species (no. of isolates)	Clear zone diam (mm) (mean ± SD)				
	Base formula[a]	Formula 1[b]	Formula 2[c]	Formula 3[d]	Formula 4[e]
S. aureus (10)	26.0 ± 5.4	28.0 ± 2.9	28.6 ± 2.7	24.4 ± 2.0	24.8 ± 2.2
P. aeruginosa (10)	12.6 ± 1.0	19.1 ± 2.9	17.7 ± 1.6	31.1 ± 2.5	21.1 ± 3.7
E. cloacae (4)	13.3 ± 0.5	28.8 ± 1.7	32.3 ± 1.3	32.3 ± 1.7	26.6 ± 1.9
E. coli (3)	14.7 ± 1.2	30.3 ± 3.5	28.3 ± 1.5	33.7 ± 2.3	29.3 ± 0.6
K. oxytoca (2)	14.5 ± 0.7	23.5 ± 0.7	23.5 ± 0.7	26.0 ± 0.0	22.5 ± 0.7
S. marcescens (1)	12.0	31.0	30.0	32.0	28.0
C. albicans (7)	No zone	8.9 ± 1.2	9.4 ± 1.2	12.1 ± 0.9	11.9 ± 1.1
C. parapsilosis (3)	No zone	9.3 ± 0.6	8.7 ± 0.6	12.7 ± 0.6	12.0 ± 0.0

[a] Neomycin (40 μg/ml), polymyxin B (700 U/ml), and mupirocin (40 μg/ml).
[b] Base formula plus amphotericin B (0.25 μg/ml) and ciprofloxacin (20 μg/ml).
[c] Base formula plus amphotericin B (0.25 μg/ml) and norfloxacin (20 μg/ml).
[d] Base formula plus nystatin (100 U/ml) and ciprofloxacin (20 μg/ml).
[e] Base formula plus nystatin (100 U/ml) and norfloxacin (20 μg/ml).

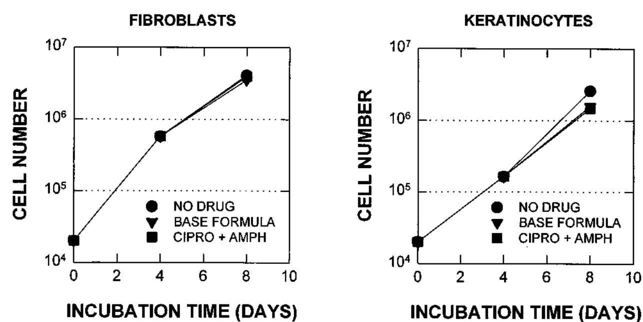

FIG. 1. Plots of cell numbers versus time for the growth of human keratinocytes and fibroblasts exposed to 20 μg of ciprofloxacin (CIPRO) per ml and 0.25 μg of amphotericin B (AMPH) per ml in combination with polymyxin B, neomycin, and mupirocin as described in Materials and Methods. No significant inhibition of cell growth occurs with either drug formulation during the 4-day incubation.

RESULTS AND DISCUSSION

Human cell assays. No significant inhibition of the growth of keratinocytes or fibroblasts occurred when they were incubated under control conditions with 40 μg of neomycin per ml, 700 U of polymyxin B per ml, and 40 μg of mupirocin per ml. Figure 1 shows that the addition of amphotericin B (0.25 μg/ml) and ciprofloxacin (20 μg/ml) to the medium caused no significant inhibition of growth for either keratinocytes or fibroblasts. Formulae 2, 3, and 4 (Table 1) also did not significantly inhibit the growth of human keratinocytes or fibroblasts (data not shown).

Wet disc assays. Table 2 summarizes the results of the wet disc assays of 40 clinical isolates of microorganisms from burn wounds. The base formula was effective against all gram-positive and gram-negative bacterial strains tested but was not effective against 10 strains of *Candida* species. Addition of an antimycotic agent, amphotericin B or nystatin, and a quinolone antibiotic, ciprofloxacin or norfloxacin, resulted in zones of clearing scored as effective (>8 mm total diameter), no change of activity against gram-positive organisms, and in an approximately 50% increase in the diameter of the zone of clearing for gram-negative organisms. All combination formulae were effective against all organisms tested.

Discussion. Data presented in this report demonstrate that formulations of antimicrobial agents that are noncytotoxic to human keratinocytes and fibroblasts in culture retain effective growth inhibitory activity against common microorganisms from burn wounds. The cytotoxicity assays performed in this study used conservative levels of drugs to which grafts of cultured keratinocytes or skin autografts may be exposed. Because cultured skin grafts are partially keratinized (8, 16) and native skin grafts are fully keratinized at the time of surgery, proliferative basal keratinocytes are not directly exposed to the total concentration of the topical agent. This protective function of the keratinized epithelium is supported by the high engraftment of native skin autografts by irrigation with 5.0% mafenide acetate (27, 37), which is lethal to cultured keratinocytes in vitro (11). Therefore, it is probable that higher concentrations of individual or combined agents may be nontoxic to cells on wounds after the formation of keratinized epithelium. Identification of drug concentration limits for skin substitutes in vivo requires further study in an experimental model with wounds contaminated with mixed microbial populations.

Fluoroquinolone compounds (ciprofloxacin, norfloxacin, and others) are administered systemically to provide broad coverage against gram-negative and gram-positive bacteria. Therefore, the use of quinolones as topical agents is not consistent with the general recommendation that topical agents not overlap in activity with parenteral antimicrobial agents. In addition, the probability of generating organisms resistant to quinolones may be greater than with other topical agents. Quinolones were tested in this study because at the local clinics, quinolones are not prescribed for the treatment of bacteremia, septicemia, or sepsis. However, the potentially serious risks of generating resistant organisms, particularly gram-negative organisms, by the topical use of quinolones are recognized. Resistance may be further promoted by repeated administrations over days or weeks. Despite this potential complication, norfloxacin and silver-norfloxacin have been reported to be candidate agents for topical use (17, 24). Nonetheless, the findings reported here are qualified as strictly experimental, and continuing studies are directed toward the elimination of quinolones from combination formulations by substitution with alternative agents to provide redundant coverage of bacterial types.

Survival and engraftment of cultured skin requires a vascular supply which delivers immune protection and nutritional support. Because dermal substitutes for composite skin substitutes are avascular, epidermal substitutes are separated from wounds by the thickness of the dermal substitute, and an extended period is required for epithelial engraftment. Separation of cultured keratinocytes from the wound for a protracted period presents a risk of both microbial colonization and malnutrition of the grafted cells. Both of these complications may be considered secondary to the slow vascularization of skin substitutes. Therefore, qualitative and quantitative determinations of appropriate antimicrobial agents alone do not promote survival and engraftment of cultured epithelium. Malnutrition of cultured keratinocytes may be corrected by administration of topical nutrients which have been shown to promote complete engraftment and repigmentation of composite cultured skin in preclinical models (6). The addition of peptide growth factors was not required for improved engraftment. Increased engraftment of cultured epithelial autografts has been reported to result from applications onto well vascularized, deepidermized allogeneic dermis (9, 12). Presumably, this improvement may be attributed, in part, to providing grafted keratinocytes with a nutrient source from the blood. Therefore, two deficiencies of composite cultured skin, susceptibility to microbial colonization and nutrient deprivation, must be corrected for improved engraftment of composite skin substitutes that are applied in a single procedure.

Routine swab cultures of wounds allow the identification of organisms in sites treated with cultured skin or skin autografts. However, if sheet grafts are applied (19, 25) and swab cultures are performed on the surface of the graft, the swab cultures may not show any contamination until the organisms beneath the graft have destroyed the graft and emerged at the surface. For this reason, prophylactic use of topical antimicrobial agents will be expected to provide the greatest protection for grafts (30). Effective delivery of antimicrobial drugs is another important requirement for the clinical use of composite cultured skin. Because wound contamination may be presumed to be localized at the wound surface, organisms are distributed between the wound and the graft after its placement. Therefore, antimicrobial agents may be most effective if targeted to the interface between the wound and the graft. Direct irrigation of widely meshed skin grafts delivers drugs to wound surfaces (25, 37). Alternatively, fibrin sealant has been used both as an adhesive for skin grafts (34) and as a vehicle for drug delivery (15). This alternative has promise, but whether

sufficient duration of delivery can be accomplished has not been demonstrated. Other possible mechanisms for the delivery of drugs to wounds include but are not limited to encapsulation in liposomes, chemical bonding to implanted biopolymers, and direct mixture into the implanted polymer matrix (10, 32, 36). However, each of these alternatives must meet the criteria for broad coverage of mixed microbial contaminants, and must sustain effective concentrations during the entire period of engraftment.

Drug combinations can neutralize activities or generate new toxicities. This formulation uses concentrations of individual compounds that are 10 to 1,000 times lower than those of other topical formulations. Mupirocin (as Bactroban) is 2% (wt/wt [20 mg/g]), whereas in this formulation, 40 μg of mupirocin per ml is effective. Concentrations of mupirocin of up to 160 μg/ml have been shown not to be inhibitory to the growth of cultured keratinocytes (7). The levels of neomycin and polymyxin B in the formulation presented here are also approximately 100-fold less than those in Neosporin. Amphotericin B for parenteral use has recommended limits of 1.5 mg/kg of body weight/day. Therefore, the levels shown here as effective and nontoxic are well below those limits. Nystatin is commonly used as a topical agent at concentrations of 100,000 U/g of vehicles but is effective in vitro at concentrations 1,000-fold lower. Quinolones represented by ciprofloxacin in this formula are also effective at low concentrations by comparison with those acceptable in blood. Two factors account for these great differences. First, application as a solution rather than with a viscous vehicle accounts, in part, for more rapid drug delivery. Second, conditions presented here for the wet disc assays do not include plasma factors that may neutralize or bind agents in these formulations. Although not significant statistically, results of cellular assays suggest that a 10% serum level in the nutrient medium for fibroblasts only detoxifies antimicrobial agents at high concentrations. Therefore, the conditions of this assay provide greater sensitivity to the toxicity of individual or combined antimicrobial drugs than would be expected in vivo. It is also possible that the addition of serum to the formulations used in the wet disc assays may reduce the activities of agents tested here and allow increases in the concentrations of individual agents in the formulation without increasing cytotoxicity.

Results of this study suggest strongly that the formulations reported on here may be considered for preclinical testing in vivo on contaminated wounds grafted with cultured skin. Although it is expected that the concentrations of individual components may be increased for use in vivo, the data presented here qualify selected combinations of antimicrobial agents for broad-spectrum coverage of mixed contaminations of gram-positive and gram-negative bacteria and fungi. On the basis of tests with these formulations, reduced contamination of grafts of cultured skin attributable to use of these formulations may be expected to result in increased survival of skin cells applied therapeutically to wounds. Together with parallel advances in the provision of nutritional support to grafted skin cells, topical use of these formulations of antimicrobial agents may increase the efficacy of cultured skin for the treatment of catastrophic burns and for scar reconstruction.

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