Agar well diffusion assay testing of bacterial susceptibility to various antimicrobials in concentrations non-toxic for human cells in culture

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Previously, we showed that microbial susceptibility to antimicrobials in concentrations non-toxic for human cells in culture could be tested using the wet disc topical antimicrobial assay. In this report, wet disc assay and agar well diffusion assay results were compared testing the susceptibility of Ps. aeruginosa *isolates from burn patients to concentrations of Polymyxin B non-toxic for cultured cells. Both assays were performed on the same agar plates. No differences in results were observed. Further agar well diffusion assay testing showed that susceptibility/resistance could be demonstrated when testing several antimicrobials in concentrations non-toxic for cultured cells against a variety of bacteria isolated from burn patients. Therefore, the more familiar agar well diffusion as well as the wet disc assay can be used to test microbial susceptibility to these concentrations of antimicrobials.*

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Introduction

Cultured skin grafts have become a potentially important therapy for the closure of burn wounds, chronic ulcers and sites of reconstructive surgery[1]. All cultured skin graft models contain keratinocytes, and some also contain fibroblasts and a biopolymer implant. All are avascular and partially keratinized, and therefore easily subject to microbial destruction[2–4]. Because of these biological deficiencies topical antimicrobial treatment used in conjunction with cultured skin grafts is necessary. A number of parenteral and topical antimicrobial agents are toxic for cultured cells, and because of this are excluded from use[5,6]. Conversely, a number of both parenteral and topical antimicrobial solutions have been shown in certain concentrations not to be toxic for cultured cells[7]. While this report suggested that those concentrations of antimicrobials could be used clinically, no demonstration of retention of antimicrobial activity was shown for the solutions at those concentrations.

Recent publications from this institution have addressed both of these issues and presented methods to determine concentrations of antimicrobial drugs non-toxic for cells in culture and to test whether these drug concentrations retained antimicrobial activity[8,9].

In our studies the method that we used for antimicrobial testing was the recently described wet disc topical antimicrobial assay (WDA)[10,11]. However, an agar well diffusion assay (AWDA) first described by Nathan et al. in 1978, has been the model most commonly used by burn units for testing topical antimicrobial activity[12–15].

Therefore, using antimicrobial concentrations non-toxic for human cells in culture, we compared these two methods to determine if the AWDA results were similar to results using the WDA. Susceptibility of *Ps. aeruginosa* isolates to the antimicrobial drug Polymyxin B was used for this comparison. After it was determined that results using the two methods were comparable, additional bacterial isolates were tested in the AWDA for susceptibility to other antimicrobials, alone or in combination. Results of these two studies are presented in this report.

Materials and methods

Bacteria

Fresh Gram-negative bacteria and *Staph. aureus* isolated from the wounds of patients at this institution were tested.

Antimicrobials

Mupirocin (MUP) and Sparfloxacin (SPAR) powders were supplied through the generosity of Smith Kline Beecham Pharmaceuticals (Philadelphia, PA, USA) and Parke-Davis Pharmaceutical Research Division (Ann Arbor, MI, USA), respectively. Polymyxin B (POLY B; 500 000 unit vials; Rorig/Pfizer & Co., New York, USA) was supplied by our pharmacy. All antimicrobial solutions were prepared in our hospital pharmacy using sterile saline as the diluent for MUP and SPAR. POLY B, 40 mg, was dissolved in 1 ml of polyethylene glycol before being diluted to 100 ml with sterile saline. After warming of the solution to effect complete solubilization of the POLY B, appropriate dilutions were prepared in sterile saline.

After testing in our recently described cellular cytotoxicity assay[8], it was found that 20 µg/ml SPAR, 400 U/ml POLY B and 32 µg/ml MUP caused no toxicity to either keratinocytes or fibroblasts[9]. These concentrations were used in susceptibility test assays in vitro to determine their activity against bacteria isolated from our patients. While SPAR has a spectrum of activity against both Gram-

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negative and Gram-positive bacteria[16] it was tested only against Gram-negative bacteria in this study.

Antimicrobial susceptibility test methods

Initially, susceptibility of *Ps. aeruginosa* isolates to POLY B was tested using both the WDA[10,11] and a modification of the AWDA[17], to determine if the results using the two procedures were comparable. For both methods, the test microorganism, grown up to a density of an 0.5 MacFarlane Standard in brain–heart infusion broth, was poured evenly over the surface of commercially available 150 mm Mueller–Hinton agar plates (BBL; Cockeysville, MD, USA). After the excess inoculum was decanted, the plate surface was dried by placing the plate in an incubator (35°C) for 10 min. For the WDA sterile filter paper discs (6 mm) wetted with 25 µl of antimicrobial solution were placed on the surface of the inoculated plate.

For the AWDA, a modification of the published method originally designed to test the efficacy of topical antimicrobial creams and ointments was used[17]. Plates inoculated with the test organism had 6-mm wells cut into the surface of the agar using a cork borer dipped in alcohol and flamed. The wells were filled with 100 µl of antimicrobial solutions. All plates were incubated (35°C) overnight.

After incubation, the diameters of any clear zones around the antimicrobial-containing discs or 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 test disc or agar well of ≥ 1 mm in radius (i.e. a total zone diameter measurement of ≥ 8 mm) would be taken as susceptibility of the test bacterial strain to the antimicrobial.

Experimental studies

Initially, a dose response experiment comparing the activities of various amounts of POLY B against 12 clinical isolates of *Ps. aeruginosa* was performed using both the WDA and the AWDA. 100-µl aliquots of POLY B were delivered to sterile 6-mm filter paper discs in a volume of 25 µl. Each disc was placed on the same plate, close to the well containing the comparable amount of POLY B, but far enough away that any zones of inhibition could be clearly read and measured. This experiment allowed us, simultaneously, to determine the optimal concentration of antimicrobial solution to use for testing and to determine if results using AWDA were comparable to results obtained using the WDA.

After it was determined that results using the AWDA were equivalent to those obtained using the WDA, an expanded study was performed using the AWDA to test the efficacy of SPAR, POLY B and MUP against a wide variety of clinical isolates of bacteria obtained from burn patients. One hundred microlitre volumes of the concentrations of these antimicrobials found to be non-toxic for cells in culture were used.

In addition, to determine whether mixtures of these antimicrobials would be synergistic, additive, antagonistic or neutral to each other, combinations of both SPAR and POLY B with MUP were prepared to contain concentrations of antimicrobial agents that were isomolar to the individual preparations. These antimicrobial mixtures have also been shown to be non-toxic for human cells in culture[18]. Results using mixtures were compared to results using individual antimicrobial solutions.

Stability study

Because testing was projected to require several weeks, the stability of the antimicrobial solutions over time was determined. Prior to routine testing, SPAR and POLY B were tested for 4 consecutive weeks using the WDA, against the same two susceptible isolates of *Ps. aeruginosa*; MUP was tested against the same two susceptible isolates of *Staph. aureus*. The antimicrobial solutions were refrigerated between tests. Stability was determined by comparing the zone diameters after each test. If the zone diameter was the same as the initial zone diameter ±1 mm, the solution was considered stable for that time period. Using this criterion, SPAR was stable only for 3 weeks. Therefore, fresh solutions for testing were prepared on a 3-week basis.

Statistical analysis

Results of the WDA versus AWDA dose response were compared using a one-between, one-within repeated measurements of analysis of variance. Differences were considered significant if $P \leq 0.05$.

Results

Dose response testing of POLY B for its antimicrobial activity against clinical isolates of *Ps. aeruginosa* was compared using WDA and AWDA techniques on the same test plates (*Table I*). With 10 U POLY B absorbed into the discs or placed in the wells, only 50–60 per cent of the isolates tested were susceptible. In contrast, all *Ps. aeruginosa* strains tested were susceptible when 20–40 U of POLY B were placed on discs or into wells. No significant differences in mean zone diameters were seen after any equimolar weight of POLY B was compared using the WDA versus the AWDA.

This comparison showed that results obtained using the AWDA were the same as results obtained using the WDA. Therefore, additional testing of antimicrobials (MUP, SPAR, POLY B) against a variety of clinical isolates of bacteria was conducted using the AWDA. Results of this testing are presented in *Table II*. Except for POLY B, alone or in combination with MUP tested against *Proteus mirabilis* strains and against one strain of *Serratia marcescens*, all bacteria were susceptible to the antimicrobial tested. Comparisons of single antimicrobial agents versus mixtures showed no additive, synergistic nor antagonistic actions. POLY B was totally inactive against *P. mirabilis*, and no change occurred when POLY B:MUP mixtures were tested. Gram-negative bacteria were uniformly susceptible to SPAR with or without the addition of MUP and all *Staph. aureus* isolates tested susceptible to MUP with or without SPAR or POLY B.

Table I. Comparative results of wet disc assay (WDA) and agar well diffusion assay (AWDA) testing: Polymyxin B versus *P. aeruginosa* tested on the same plate

POLY B (U) tested	*WDA*	*AWDA*
10	8.9±4.3 (7)*	12.2±1.0 (5)
20	10.4±2.4 (12)	11.2±2.4 (12)
30	12.4±2.2 (12)	11.2±5.0 (12)
40	12.9±1.8 (12)	13.2±3.2 (12)

*Mean zone diameter ±s.d. (no. susceptible out of 12).

Table II. Agar well diffusion assay results testing susceptibility of bacteria isolated from burn patients to Sparfloxacin*, Polymyxin B* and Mupirocin*, alone and in combination

Antimicrobial	*Ps. aeruginosa* (n = 33)	*Enterobacter cloacae* (n = 19)	*Klebsiella pneumoniae* (n = 19)	*Escherichia coli* (n = 9)	*Proteus mirabilis* (n = 8)	*Serratia marcescens* (n = 10)	*Acinetobacter baumannii* (n = 7)	*Staph. aureus* (n = 33)
MUP	NT	NT	NT	NT	NT	NT	NT	27.1 ± 1.5
SPAR	20.0 ± 4.3†	25.9 ± 2.0	24.9 ± 2.2	30.9 ± 1.7	24.3 ± 5.9	20.7 ± 3.0	28.4 ± 3.3	NT
SPAR:MUP	19.8 ± 4.0	26.3 ± 2.0	24.4 ± 2.3	31.3 ± 1.0	24.0 ± 4.0	21.1 ± 3.2	28.1 ± 3.0	26.3 ± 2.0
POLY B	12.4 ± 0.9	10.7 ± 0.7	10.9 ± 1.1	11.8 ± 0.4	0	12.0 ± 1.3‡	12.9 ± 1.7	NT
POLY B:MUP	12.3 ± 1.0	11.2 ± 0.7	11.6 ± 1.4	11.0 ± 0.3	0	12.0 ± 1.5‡	13.1 ± 2.1	26.8 ± 1.9

*100 µl of concentration, in either µg/ml or U/ml, found to be non-toxic for cells in culture per well.
NT, not tested.
†Mean zone diameter ± s.d. in millimetres.
‡One isolate = no zone.

Discussion

Our results demonstrate that both the WDA and the AWDA were equally valid for antimicrobial testing (*Table I*). The antimicrobial activity of POLY B was concentration dependent in both assays, since discs or wells containing only 10 U of POLY B inhibited the growth of only five to seven strains of *Ps. aeruginosa*, whereas discs or wells containing ≥ 20 U POLY B uniformly inhibited all 12 strains tested (*Table I*). Why seven of 12 strains tested susceptible in the AWDA, is not clear. Perhaps the kinetics of diffusion of the antimicrobial agents into the agar from a surface application (WDA) is different from diffusion through the agar (AWDA) when the antimicrobial is placed into a well. Alternatively, the fact that the antimicrobial solution was applied to discs in only 25 µl amounts while 100 µl of solution was placed in wells, might account for the difference. In any case, these results demonstrate that both assays discriminate between susceptible and resistant strains. When higher amounts of POLY B were used in either the disc or well format, all isolates were susceptible.

Susceptible versus resistant strains of bacteria were apparent using the AWDA, even when 100 µl of antimicrobial agents in concentrations found non-toxic for cells in culture were placed in wells (*Table I*). This is illustrated by comparing the 100 per cent susceptibility of *P. mirabilis* strains to SPAR, alone or mixed with MUP, with the total resistance of these same strains to POLY B or POLY B:MUP. In addition, one strain of *S. marcescens* was resistant to both POLY B and POLY B:MUP while nine others were susceptible. In all other cases each antimicrobial agent inhibited the in vitro growth of appropriate Gram-positive or Gram-negative bacteria enough to be considered effective against those organisms by the criteria set up in this study.

In summary, the data show that determinations of bacterial susceptibility/resistance to antimicrobial agents in concentrations non-toxic for cells in culture are directly comparable using the WDA or the AWDA. Furthermore, 100 µl of these concentrations of antimicrobial agents placed in the well in the AWDA discriminates between resistant and susceptible bacteria.

We conclude, therefore, that in addition to the WDA, the more familiar AWDA can be used to test susceptibility/resistance of microorganisms to concentrations of antimicrobial agents non-toxic for cells in culture.

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