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Attachment of an Aminoglycoside, Amikacin, to Implantable Collagen for Local Delivery in Wounds

STEVEN T. BOYCE,[1,2]* ANDREW P. SUPP,[1] GLENN D. WARDEN,[1,2] AND IAN A. HOLDER[1,3]

Shriners Burns Institute,[1] and Departments of Surgery[2] and Microbiology,[3] University of Cincinnati, Cincinnati, Ohio 45229

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Cultured skin substitutes consisting of implantable collagen (COL) and cultured human skin cells often fail clinically from destruction by microbial contamination. Hypothetically, addition of selected antimicrobial drugs to the implant may control microbial contamination and increase healing of skin wounds with these materials. As a model for drug delivery, bovine skin COL (1 mg/ml) and amikacin (AM; 46 μg/ml) were modified by covalent addition of biotin (B-COL and B-AM, respectively) from B-*N*-hydroxysuccinimide and bound together noncovalently with avidin (A). B-COL was incubated with A and then with B-peroxidase (B-P) or by serial incubation with B-AM and B-P, before P-dependent chromogen formation. Colorimetric data (n = 12 per condition) from spot tests on nitrocellulose paper were collected by transmission spectrophotometry. Specificity of drug binding in spot tests was determined by (i) serial dilution of B-COL; (ii) reactions with COL, AM, or P that had no B; (iii) removal of A; or (iv) preincubation of B-COL–A with B before incubation with B-P. Binding of B-AM was (i) dependent on the concentration of B-COL; (ii) specific to B-COL, A, and B-P ($P < 0.05$); and (iii) not eluted by incubation in 0.15 or 1.0 M NaCl. B-AM was found to block binding of B-P to the B-COL–A complex and to retain bacteriocidal activity against 10 clinical isolates of wound bacteria in the wet disc assay. Antimicrobial activity of B-AM was removed from solution by treatment with magnetic A and a permanent magnet. These results suggest that selected antimicrobial drugs can be biotinylated for attachment to COL-cultured cell implants without loss of pharmacologic activity. Because this chemistry utilizes a common ligand, any molar ratio of agents may be administered simultaneously and localized to the site of implantation.

Local contamination and infection of wounds is a common complication of recovery from invasive surgery or traumatic skin loss from burns (18). Skin wounds are treated routinely with topical antimicrobial agents with high anti-infective activity, low histotoxicity, and minimum overlap of activity with parenteral antimicrobial agents (4). Advances in skin repair have resulted in transplantation of cultured epidermal cells (12) or composite grafts of cultured skin cells and biopolymers (3). These materials are avascular and have greater susceptibility to burn wound microorganisms than conventional split-thickness skin grafts (3, 12, 14, 20). Cell-biopolymer composites that contain collagen (COL) also provide an advantageous surface for attachment and proliferation of bacteria (24). Cultured epithelia are also incompletely keratinized when grafted and therefore are more sensitive to cytotoxic activity of topical antimicrobial agents than fully keratinized grafts of native skin (10). Localized delivery of antimicrobial agents that are effective and nontoxic may reduce wound contamination and improve wound healing. This prospective advantage must also be balanced against potential disadvantages of local delivery, such as accelerated selection of resistant organisms (13). Mechanisms for localized delivery of drugs include, but are not limited to, conjugation with implantable polymers (11), microencapsulation in liposomes (19) or polylactic acid (9), addition to polyanhydrides (22) or antibodies (7), iontophoresis (23), and release from hydrogels (17).

Previous studies have reported precision fabrication of COL-glycosaminoglycan implants (2); population of the implants with cultured human keratinocytes, fibroblasts, and melanocytes (5); biochemical modification of implantable COL with biotin (B) for drug delivery (6); attachment by B-avidin (A) chemistry of peptide growth factors to COL and retention of biological activity after modification (21); and healing of burn wounds with cultured cell-biopolymer grafts (3, 14). Cultured skin substitutes are currently used as an adjunctive therapy for closure of burn wounds that involve very large percentages of the body surface (8). The present study describes covalent addition of B to an aminoglycoside, amikacin (AM), and to implantable COL and subsequent attachment with A of the drug to the modified COL. Attachment of AM to COL is dependent on the concentration of biotinylated COL (B-COL) and is specific to B-A binding chemistry. Antimicrobial activity of AM is retained after biotinylation and is completely abolished by removal of the biotinylated AM (B-AM) from solution with magnetic A (M-A) and a magnet. These results suggest that modification of antimicrobial agents with B is an experimental alternative for local delivery of active antimicrobial agents on biocompatible implants for control of infections associated with wounds from invasive surgery or burns.

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MATERIALS AND METHODS

Materials. Comminuted bovine COL was obtained from USDA Eastern Regional Facility (Philadelphia, Pa.). The following reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.): B-*N*-hydroxysuccinimide (BNHS; H1759); A (A9275); horseradish peroxidase (P; P8375); B-P (P9272); bovine serum albumin (BSA; A6793); d-B (B4501); hydrogen peroxide (H_2O_2; H1009); pepsin (P7012); *N*,*N*-

* Corresponding author.

dimethylformamide (DMF; D8654); Tween 20 (P1379); and 4-chloro-1-naphthol (4CN; C8890). M-A (74620) was purchased as a suspension of iron oxide particles, 0.5 to 1.5 μm in size, covalently bound to A in phosphate-buffered saline (PBS; pH 7.5) from Collaborative Biomedical Products (Bedford, Mass.). The permanent magnet (MPC-1) was from Dynal (Great Neck, N.Y.). Dialysis tubing, MWCO 3500 (132720), was obtained from Spectrum Medical Industries (Los Angeles, Calif.). The nitrocellulose (NC) transfer membrane used was 0.22-μm-pore-size Nitro ME (E02HY315F5) from Micron Separations, Inc. (Westboro, Mass.). AM sulfate was obtained as a sterile solution (250 mg/ml) from Bristol-Myers (Evansville, Ind.). The equipment used for both preparation and experimental analysis was the following: lyophilizer, Virtis Unitop 800L (Gardiner, N.Y.); centrifuge, Beckman J2-MI (Palo Alto, Calif.); filtration manifold, Schleicher & Schuell Minifold I (Keene, N.H.); and a 96-well plate reader, Molecular Devices THERMOmax (Menlo Park, Calif.).

Biotinylation of COL and AM. Bovine skin COL and AM were modified by the covalent attachment of B from the *N*-hydroxysuccinimido group of BNHS, to primary amino groups present on the protein and the antibiotic. Pepsin-digested bovine COL was biotinylated as previously described (2, 6), with the BNHS used in an approximately 100:1 molar excess to ensure complete biotinylation of available sites. B-AM was prepared from a solution of 3.2×10^{-3} M AM (10 mg of AM in 4 ml of 0.05 M Tris-HCl buffer, pH 7.6), containing approximately 1.28×10^{-5} mol of AM total. The AM was reacted with 360 μl of a 0.125 M BNHS solution (85.35 mg of BNHS in 2 ml of DMF = 4.5×10^{-5} mol of BNHS) to produce a 3.5:1 molar excess of BNHS. The BNHS acted as the reaction-limiting reagent because AM has four primary amino groups (biotinylation sites) per molecule. Parallel preparations of AM were reacted with BNHS at molar ratios of 3.0, 2.5, 2.0, and 1.5. The mixtures were reacted for 1 h in an ice bath with repeated agitation. The resulting solutions (2.29 mg/ml) were stored at 4°C and diluted with 0.05 M Tris-HCl buffer to a working concentration of 46 μg of B-AM per ml. A stock of nonbiotinylated AM (2.29 mg/ml) was prepared to serve as a control in the following assays.

Spot test. The spot test assay used in this study was modified from procedures previously reported (1, 2, 6, 21). Tests were performed in a 96-well format with a vacuum filtration manifold. B-COL and nonbiotinylated COL (1 mg/ml) were diluted 1:10, 1:100, and 1:1,000 with 0.05 M Tris-HCl buffer and dispensed (75 μl of each dilution) into wells of the manifold under gentle vacuum to facilitate attachment to NC membranes. Membranes were removed from the manifold and allowed to air dry for 1 h at room temperature before being blocked for 2 h at room temperature with 2% (wt/vol) BSA in a 0.15 M PBS solution, pH 7.6. NC membranes were rinsed three times (10 min each) with 75 ml of 0.05% (vol/vol) Tween 20-PBS with gentle aspiration to remove each rinse solution and then returned to the manifold for application of subsequent reagents. The volume of all reagents used was 75 μl per well, with the following concentrations: A, 1 mg/ml; B-AM, nonbiotinylated AM, and free B, 46 μg/ml; and B-P and P, 50 μg/ml. Each reagent was incubated for 30 min at room temperature with no vacuum applied to ensure a surface attachment only. The membrane was removed from the manifold again and rinsed with Tween 20-PBS (as described above) between each reagent application. The addition sequence was as follows: (i) B-COL or COL; (ii) ± A; (iii) ± B-AM, AM, or B; and (iv) + B-P or P. After the membrane was rinsed, following the P addition, the bound P was reacted for 5 min with 20 ml of cold 4CN in methanol (3 mg/ml) and 100 ml of 0.018% (vol/vol) H_2O_2-Tris buffer to form a blue chromogen on the NC paper (15). This reaction mixture was incubated for 5 min at room temperature in the dark (to reduce nonspecific color development) before being quenched by an excess of Tween 20-PBS and then rinsed with Tween 20-PBS as described above. Reacted membranes were allowed to air dry overnight in the dark prior to data collection.

Elution of B-AM. Reaction complexes of B-COL (1 mg/ml) ± A ± B-AM were incubated in isotonic (0.15 M) or hypertonic (1.0 M) salt solutions to determine whether either of the B-A bonds would be disrupted by salt solutions. To test the B-COL/A bond, NC membranes were submerged and incubated for 1 h at 37°C in 0.15 M NaCl or in 1.0 M NaCl following the 30-min incubation of A. NC membranes were then rinsed and incubated with B-P, and stained with 4CN-H_2O_2 solution, and data were collected as described below. The A/B-AM bond was tested in the same manner following the 30-min incubation of B-AM. Both reaction complexes (± B-AM) were incubated for 1 h at room temperature in 0.15 M PBS as a control for the elution studies.

Bioassays. Wet disc assays (16) were performed to demonstrate retention of antimicrobial activity by AM after biotinylation. Clinical isolates from burn patients of two strains each of AM-sensitive *Pseudomonas aeruginosa* and *Enterobacter cloacae* and three strains each of *Klebsiella pneumoniae* and *Staphylococcus aureus* were prepared in petri dishes (150 by 15 mm) containing Mueller-Hinton agar. Twenty-five microliters of AM or B-AM (2.29 mg/ml) was applied to 6-mm-diameter filter paper discs in the dishes. After a 2-h incubation at 37°C, 25 μl of the respective solutions was reapplied to the discs and incubated overnight.

Wet disc assays were also performed to confirm that the B was covalently bound to AM and not just in solution with the drug. AM and B-AM (2.29-mg/ml) samples were diluted to a working concentration of 46 μg/ml to facilitate reaction with M-A. Four milliliters of each sample dilution was prepared and separated into two aliquots. M-A (6.25 mg/ml) was resuspended with one of the AM or B-AM (46-μg/ml) aliquots, gently vortexed, and allowed to react for 30 min at 4°C. When the reaction was complete, the tube was exposed to a permanent magnet for 10 min, and the supernatant was decanted and collected. Wet disc assays and direct applications of compounds to bacterial lawns were performed in parallel on a single strain of *K. pneumoniae* ($n = 6$ per condition). Loss of antimicrobial activity from the B-AM–M-A samples indicated that the AM was biotinylated and subsequently removed from solution by the M-A.

The B-COL/A/B-AM complex was evaluated on cultures of *K. pneumoniae*, as follows: 0.264 ml of B-COL (6.1 mg/ml) was reacted with 1.7 ml of A (10 mg/ml) for 10 min at room temperature. The unbound A was removed by centrifugation at 4,500 × *g* two times for 1 h each at 4°C by using a Millipore Ultrafree CL filter unit (100,000 nominal molecular weight limit). The B-COL/A complex was then resuspended with 1.72 ml of B-AM (0.229 mg/ml) and allowed to react for 30 min at room temperature to facilitate attachment. The unbound B-AM was removed by centrifugation, as above, by using an Ultrafree CL filter unit (5,000 nominal molecular weight limit). The B-COL/A/B-AM complex was resuspended in 1.0 ml of Tris-Cl buffer and transferred to a microcentrifuge tube for isolation. The complex was pelleted by centrifugation in a microcentrifuge for 30 min at 2,850 ×

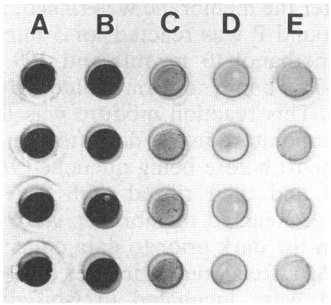

FIG. 1. Spot test for binding of B-P to COL on NC paper. Spots in columns are quadruplicate assays of identical conditions. Lanes (left to right): serial dilution of B-COL at 1.0 (A), 0.1 (B), 0.01 (C), and 0.001 (D) mg/ml demonstrates concentration-dependent binding of B-P. Lane E, nonbiotinylated COL produces only background chromogen formation.

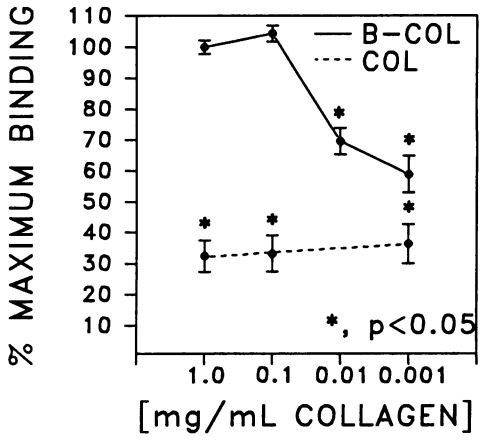

FIG. 2. Plot of substrate concentration versus percent maximum binding. Concentration-dependent binding of B-P to B-COL and nonspecific background staining of nonbiotinylated COL. Statistically significant ($P < 0.05$) reductions of binding of B-P compared with 100% control are indicated with asterisks.

g (6,500 rpm). The supernatant was aspirated, and the pellet was resuspended in 0.5 ml of Tris-Cl buffer, and 50 μl of the B-COL/A/B-AM complex was applied to the bacterial lawn and incubated as in the wet disc assay.

Data collection and analysis. Data from the spot tests were collected by transmission spectrophotometry at 550 nm by using a 96-well plate reader, after clarification of NC membranes with light mineral oil. Data for spot tests are expressed as percent maximum binding (mean ± standard error of the mean) for each experimental reaction condition compared with the chromogen formation of the B-COL/A/B-P complex as the 100% control. All experimental conditions in spot tests were performed in quadruplicate in triplicate experiments to yield $n = 12$ per condition. Analysis of variance was performed to determine differences ($P < 0.0001$) among the conditions. Honest significant difference between pairs of conditions (experimental versus 100% control) was determined at a 95% confidence level ($P < 0.05$). If analysis of variance demonstrated significance, pairwise comparisons were performed by Tukey's Studentized range test. Data from bioassays were collected by measuring the zone of clearing that developed around the applied compound with vernier calipers. Data from wet disc assays (antimicrobial activity) are expressed as effective if a clear zone of ≥2 mm in diameter developed in the lawns after overnight incubation at 37°C.

RESULTS

Results from spot tests demonstrate concentration-dependent and reaction-specific binding of B-AM to B-COL. Figure 1 is a photograph of a representative spot test that shows chromogen formation as a concentration-dependent function of B-COL on NC paper. Chromogen intensity is directly proportional to binding of A/B-P to serial dilution of B-COL (Fig. 1, lanes A to D). Specificity of binding of A/B-P to B-COL is demonstrated by comparison of reaction with COL (Fig. 1, lanes A and E). Quantitative comparison of binding of A/B-P to B-COL or COL is expressed in Fig. 2 as percent maximum binding in the absence of AM or B-AM. Serial dilution of B-COL from 1.0 to 0.001 mg/ml corresponds to reduction in chromogen formation from 100 to 59%. In comparison, nonbiotinylated COL generates 32 to 36% maximum binding and was statistically different from B-COL at all concentrations tested.

Figure 3 shows concentration-dependent inhibition of binding to <10% of maximum by reaction of the B-COL/A complex with B-AM before B-P. Serial dilution of B-AM restores maximum binding of B-P. Reduction by B-AM of B-P binding identifies competitive inhibition of chromogen formation by B-AM solution. However, because AM is approximately the same molecular weight as B, biochemical separation of any unreacted compounds is difficult. Consequently, covalent addition of B to AM is not demonstrated in this experiment but is shown below (see Figure 8).

Figure 4 shows specificity of each component of the biotin-avidin chemistry, beginning with control conditions in the leftmost bar. Reaction specificity is demonstrated (from left to right) as follows: A, nonbiotinylated COL reduces binding to 32.4% ± 5.0% of maximum; B, nonbiotinylated P results in reduction to 12.2% ± 2.1%; C, preincubation of B-COL/A with free B gives 2.0% ± 0.3%; D, deletion of A gives 0.8% ± 0.3%; E, B-AM (46 μg/ml) after B-COL/A reduces binding to 8.7% ± 2.8%; and F, nonbiotinylated AM

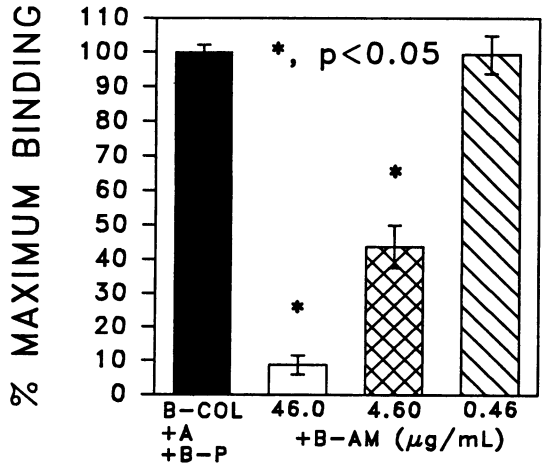

FIG. 3. Plot of concentration-dependent inhibition of P binding by B-AM. Dilution of B-AM restores binding of B-P. Statistically significant ($P < 0.05$) reductions of binding of B-P compared with 100% control are indicated with asterisks.

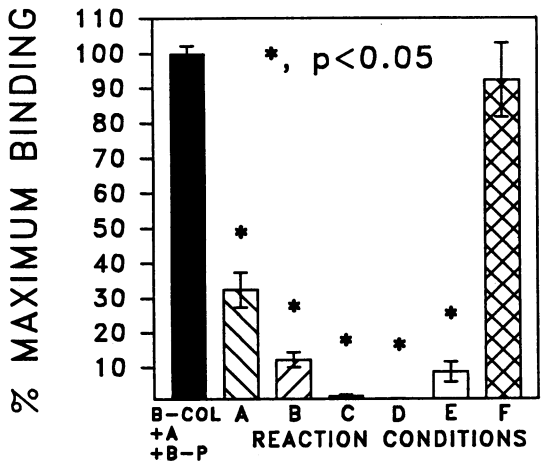

FIG. 4. Plot of reaction specificity of binding of B-AM to B-COL. Left bar, control binding of B-P to B-COL. Modified reaction conditions with nonbiotinylated COL (A), nonbiotinylated P (B), preincubation of B-COL/A with free B before B-P (C), removal of A (D), preincubation of B-COL/A with B-AM before B-P (E), and preincubation with nonbiotinylated AM before B-P (F). Statistically significant ($P < 0.05$) reductions of binding of B-P compared with 100% control are indicated with asterisks.

gives 92.2% ± 10.7% maximum binding. All of the reactions in Fig. 4, except incubation with nonbiotinylated AM, are statistically significant reductions in B-P binding.

Stability of the biotin-avidin chemistry to salt elution is demonstrated in Fig. 5. B-COL was reacted sequentially with A, ± B-AM, and incubated at room temperature in reaction buffer (control) or at 37°C for 1 h in 0.15 or 1.0 M NaCl. After incubation, all conditions were reacted with B-P and chromogen. No changes from control binding were detected in the presence or absence of B-AM. This result demonstrates the high-affinity binding and stability of the biotin-avidin complex to ionic displacement.

Figure 6 summarizes results of wet disc assays of AM or

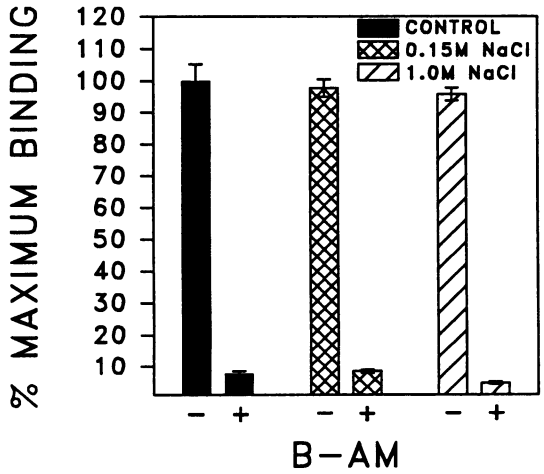

FIG. 5. Plot of reaction stability to salt elution. Prior to reaction with B-P and chromogen, B-COL/A ± B-AM was incubated 1 h in 0.05 M Tris-HCl buffer (control) at room temperature, 0.15 M NaCl at 37°C, or 1.0 M NaCl at 37°C. No changes in chromogen formation occurred as a function of salt concentration.

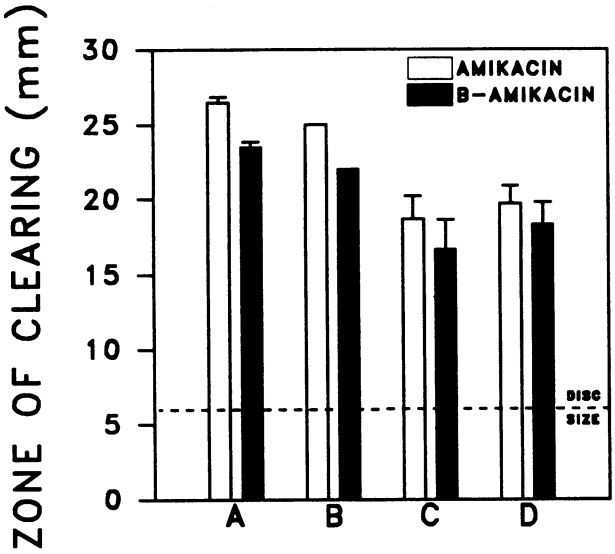

FIG. 6. Plot of zone of clearing produced by AM or B-AM on gram-negative and gram-positive bacteria in the wet disc assay. Test organisms include individual clinical isolates of *P. aeruginosa* (n = 2) (A), *E. cloacae* (n = 2) (B), *S. aureus* (n = 3) (C), and *K. pneumoniae* (n = 3) (D). B-AM produces smaller absolute zones of clearing than AM, but retention of antimicrobial activity by B-AM is demonstrated on all test organisms.

B-AM on 10 clinical isolates of four strains of gram-negative and gram-positive organisms. No differences in antimicrobial activity were detected between AM or B-AM. These results demonstrate that antimicrobial activity of AM is retained after incubation with BNHS. However, biotinylation of AM is not demonstrated in this experiment.

Figure 7 shows concentration-dependent deactivation of AM as a function of BNHS concentration. As the molar ratio of BNHS increases in the reaction mixture, antimicrobial activity decreases. This result is consistent with conversion of primary amino groups in AM to substituted amide bonds by addition of B from BNHS and with the antimicrobial activity of AM residing in the primary amino groups.

Figure 8 plots results from treatment of AM or B-AM ± M-A and a magnet. Antimicrobial activity is retained by AM

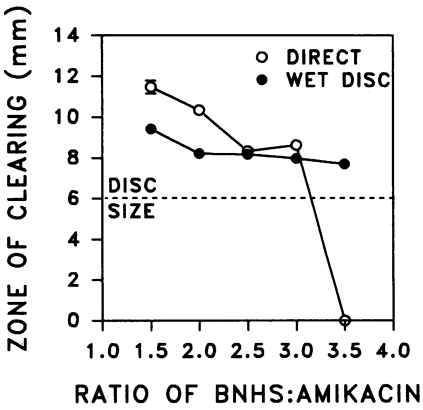

FIG. 7. Plot of zone of clearing versus ratio of BNHS to AM in the biotinylation reaction. B-AM was applied to lawns of *K. pneumoniae* either directly or in the wet disc assay. Antimicrobial activity of B-AM decreases as the ratio of BNHS to AM increases.

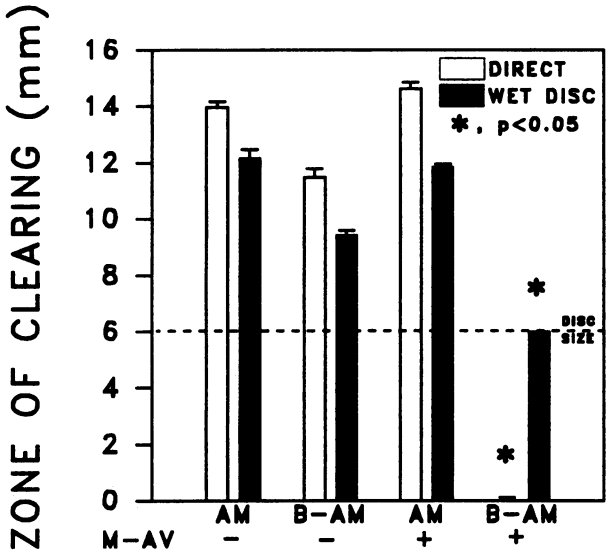

FIG. 8. Plot of specific activity of B-AM applied directly or in the wet disc assay. AM or B-AM was incubated ± M-A and exposed to a magnet, and supernatant fluid was tested for antimicrobial activity (zone of clearing, >8 mm) on *K. pneumoniae*. AM ± M-A and B-AM without M-A demonstrated antimicrobial activity. Activity of B-AM was completely abolished by exposure to M-A and a magnet. This result verifies the covalent addition of B to AM.

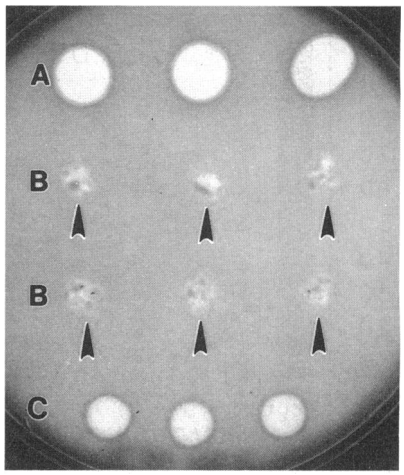

FIG. 9. Photograph of a backlighted petri dish containing a culture of *K. pneumoniae* which was inoculated directly with 25 μl of AM (46 μg/ml) (A); precipitate from reaction of B-COL, A, and B-AM (B); or 25 μl of B-AM (46 μg/ml) (C). Localized clearing of the microbial culture at the site of application of the precipitate indicates that antimicrobial activity is retained by the drug-COL complex. Application of B-COL plus A was negative (not shown).

and B-AM that is not incubated with M-A and a magnet. All antimicrobial activity of B-AM is abolished by incubation with M-A and a magnet, but it has no effect on the AM. This result verifies that B is bound covalently to AM by BNHS and that B-AM is biologically active.

Figure 9 shows antimicrobial activity of the B-COL/A/B-AM complex. Mixture of the three reagents generates a precipitate, as expected, by extensive cross-linking of B-COL by A. Therefore, no diffusion of antimicrobial activity is observed. Rather, antimicrobial activity is observed directly at the site of application of the drug-COL precipitate. Clearing of the microbial lawn is irregular, corresponding to the distribution of the drug-COL complex.

DISCUSSION

Data presented here demonstrate that an aminoglycoside antibiotic, AM, can be modified by covalent addition of B and bound to B-COL without loss of effective antimicrobial activity. The condensation reaction for addition of B from BNHS requires a primary amino group (25). AM was selected for this study because it has four primary amino groups as sites for covalent addition of B. However, other antimicrobial agents with primary amino groups could also have B added by this reaction. Furthermore, other condensing reagents are available for covalent addition of B to other functional groups, including hydroxyl, carboxyl, and carbonyl. Therefore, few limitations exist for chemical modification with B of virtually any antimicrobial agent.

To consider the prospective application of this chemistry for local drug delivery, it is critical to retain the biologic and pharmacologic activities of the drug after biotinylation. This study shows retention of an antimicrobial activity in vitro after chemical modification. This result agrees with an earlier study in which biotinylated growth factors retained mitogenic activity after modification with B (21). Although this is an important initial step toward prospective application, the present study did not address whether biologic activity of the biotinylated antibiotic is retained if it is bound to COL in vivo. To address these questions, future studies must demonstrate effective management of contaminated wounds with B-COL implants loaded with agents to which the experimental organisms are sensitive. In addition, determinations of kinetic and concentration-dependent parameters must be performed.

An advantage of this approach to delivery of antimicrobial agents is that multiple agents with unequal rates of release are attached to the delivery vehicle by a common species of biochemical ligand. This property allows preparation of assortments of routinely used agents that may be tested on microorganisms from individual patients and combined in formulations specific to each case according to type and sensitivity of isolated organisms (3, 4, 16). Release kinetics of bound antimicrobials were not tested in this study and must be better understood before clinical application of this system. Studies in vivo must answer questions of delivery rates, half-lives of bound agents, and immunogenicity of COL-drug complexes to determine safety and efficacy of this approach for local delivery of antimicrobial agents. Additional issues that require study before this model can be considered for clinical use include, but are not limited to, risk of developing resistance to implanted drugs and extrapolation of data from in vitro assays of antimicrobial activities to clinical applications that are far more complicated.

Findings of this study represent an initial advance toward localized and prescribed administration of antimicrobial agents for treatment of wounds resulting from surgery and trauma. Integration of this delivery system into composite implants of biopolymers and cells may improve regeneration of tissues at the site of implantation and contribute to more rapid recovery of tissue function after injury.

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