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In Vitro Antibiotic Removal and Bacterial Recovery from Blood with an Antibiotic Removal Device

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The antibiotic removal device manufactured by Marion Laboratories (Kansas City, Mo.) is intended for treatment, before culture, of blood specimens from hospital patients being treated with antibiotics. Measurement of 13 antibiotics showed that the antibiotic removal device removed amikacin, ampicillin, carbenicillin, cefazolin, cephalothin, chloramphenicol, gentamicin, nafcillin, tetracycline, tobramycin, and vancomycin and reduced cefoxitin and ticarcillin to extremely low levels. Three combinations of antibiotics were similarly removed or reduced. Five species of anaerobic bacteria, one yeast species, and six species of facultative or aerobic bacteria were used to challenge the possibility that the antibiotic removal device would trap or inhibit microorganisms. All were recovered from the device in the same numbers as were inoculated.

It is theoretically possible for antibiotics given to a hospital patient for prophylaxis or treatment to inhibit the growth of bacteria in blood cultures made for diagnosis or therapy management. It was shown early in the antibiotics era that such inhibition could be decreased by adding concentrated penicillinase to blood cultures (1), leading to the current common practive of adding penicillinase to blood cultures from persons who have received a penicillin. The heparinoid anticoagulant sodium polyanetholesulfonate is widely used in blood cultures partly because it inactivates several aminoglycoside and polymyxin class antibiotics (3, 5). The report that this inactivation by sodium polyanetholesulfonate may be culture medium dependent (6) could mean that sodium polyanetholesulfonate effectiveness is diminished in some blood culture formulae. The only recourse for doing blood cultures for patients in the course of antibiotic therapy, beyond these systems, has been reliance upon the dilution effect of the 5:1, 10:1, or 20:1 ratio between culture broth and blood inoculum.

Marion Laboratories (Kansas City, Mo.) has developed a device to be used for removing antibiotics from blood specimens before inoculating them to broth. The device consists of a 50-ml serum bottle containing treated plastic resins to which a solution of 0.025% sodium polyanetholesulfonate in saline has been added. It is fitted with a rubber stopper with septum and has a filter in the neck to retain resin fines when the treated blood is removed. From 5 to 10 ml of blood is aseptically injected through the septum, and the bottle is tumbled end-over-end for 15 min on a simple mechanical tumbler at prescribed force and speed. The blood sample is then withdrawn by syringe and inoculated into any blood culture broth.

The numbers of microorganisms circulating in septicemia may be small. Our unpublished records show that more than half of blood specimens from patients with confirmed septicemia have a count of less than 1 colony-forming unit per ml.

In some applications resins similar to those in this antibiotic removal device (ARD) are used as microbial trapping filters. Evaluation of this device requires then that it be shown both to remove antibiotics and not to inhibit microorganisms in, or retain them from, blood specimens. We report the results of experiments addressed to both of these questions.

MATERIALS AND METHODS

Antibiotics challenge. Antibiotics were chosen after a survey of the drugs most prescribed for treatment of septicemia in several hospitals in this country. Assayed powder antibiotics were generously provided by manufacturers (amikacin and ampicillin, Bristol; carbenicillin, Roerig Division of Pfizer; cefazolin, Smith Kline & French; cephalothin, tobramycin, and vancomycin, Eli Lilly; chloramphenicol, Parke-Davis; gentamicin, Schering; nafcillin, Wyeth; ticarcillin, Beecham Division of Bristol; cefoxitin, Merck Sharpe & Dohme; trimethoprim and sulfamethoxazole, Burroughs-Wellcome).

All antibiotics were prepared in human serum to correct for protein binding. A measured amount of antibiotic was added to a measured amount of fresh human blood-bank blood. A volume of blood with antibiotic was retained for assay as base line, and 5 or

10 ml was injected into an ARD bottle and treated as already described.

Antibiotics assays. Antibiotics were assayed by the regular methods in use in this laboratory, which are derived directly from the *Code of Federal Regulations* (2). Alterations of these methods included the use of a 6-ml layer of seeded agar in petri dishes without an unseeded agar base, the use of 0.25-in. (ca. 0.64-cm) paper disks with 20 μl of sample for all antibiotics except chloramphenicol (stainless-steel cylinders with 100 μl of sample), and incubation for 6 or 8 h at 35°C, depending upon the assay organism. *Bacillus subtilis* ATCC 6633 was bought from Difco as spore suspension; all other assay organisms were prepared and standaridized in the laboratory. Assay organisms were: *Providencia stuartii* Bristol A 21471, kindly supplied by Bristol Laboratories, for amikacin and cefoxitin; *Micrococcus luteus* (*Sarcina lutea*) ATCC 9341 for ampicillin, chloramphenicol, and nafcillin; *Staphylococcus aureus* ATCC 6538P for cefazolin; *Staphylococcus epidermidis* ATCC 27626 for gentamicin; *Klebsiella pneumoniae* ATCC 27799 for tobramycin; and *B. subtilis* ATCC 6633 for carbenicillin, ticarcillin, vancomycin, trimethoprim, and sulfamethoxazole.

Trimethoprim and sulfamethoxazole could not be measured exactly but were estimated on Mueller-Hinton agar or on Mueller-Hinton agar containing 5% lysed sheep blood, respectively.

Assay designs. The antibiotics in Table 1 were used individually at about the expected clinical concentration to challenge the ARD. In addition, the ARD was challenged with elevated concentrations of ampicillin, chloramphenicol, gentamicin, tobramycin, and ticarcillin.

The system was also challenged with the following antimicrobial mixtures: trimethoprim + sulfamethoxazole, cefazolin + tobramycin, ticarcillin + tobramycin, and vancomycin + gentamicin.

Additional experiments included measurement of the effect of different tumbling times on antibiotic removal and tests for elution of antibiotics from ARD resins after prolonged holding of specimens in the device.

Preparation of bacteria. Eleven bacterial and one yeast species (see Table 4) were chosen either because they are frequently encountered in septicemia or because they have exacting growth requirements. *Peptococcus prevotii* was a clinical isolate. All others were American Type Culture Collection derived. Each organism was inoculated to a suitable medium and incubated for about 24 h immediately before use. Each was serially diluted to 10^{-2}, 10^{-4}, and 10^{-6} in sterile phosphate buffer or sterile saline; these dilutions were the inocula.

Bacterial challenge. A 1-ml volume of bacterial dilution, estimated by triplicate plate count to contain 200 to 2,000 bacteria, was inoculated to 200 ml of sterile fresh blood-bank blood and mixed (final bacterial density, 1 to 10 colony-forming units per ml). A portion of inoculated blood was reserved as control, and a second control was comprised of this blood diluted 1:2 in sterile saline. A 5-ml sample of inoculated blood was injected into each of a series of ARD bottles. Samples of 5 to 6 ml for bacterial count were taken from untreated control and control diluted at time 0 and 20, 60, and 120 min. ARD-treated blood was shaken briefly by hand, and then time zero samples were taken; cultures either received the prescribed tumbling and were sampled at 20, 60, and 120 min, or were held untumbled for 2 h and then tumbled and sampled.

Bacterial assays. Media and incubation times and conditions were those appropriate for each species. Of each 5- to 6-ml sample, 1 ml was used for a count by pour plate, 0.1 ml was used for a count by surface inoculation of an agar plate, and the remainder was inoculated to a commercial blood culture bottle. Plates were incubated for 24 to 72 h as suitable for the strain; bottles were incubated until growth was evident or for 7 days if no growth was seen. Subcultures were made from bottles with or without visible growth.

Identity of organisms recovered was confirmed by colony morphology and Gram stain.

TABLE 1. *Removal of antibiotics from human blood by ARD*

Antibiotic	Drug concn (μg/ml)			
	Replicate 1		Replicate 2	
	Not treated	Treated	Not treated	Treated
Amikacin	21	NAD[a]	17	NAD
Ampicillin[b]	23.2	NAD	20.0	NAD
Carbenicillin	14.5	NAD	14.5	NAD
Cefazolin	20	NAD	33	Trace[c]
Cefoxitin	36	Trace	22	NAD
Cephalothin	7	NAD	7.2	NAD
Chloramphenicol[b]	68	NAD	62	NAD
Gentamicin[b]	11.6	NAD	10.8	NAD
Nafcillin	10.0	NAD	8.9	NAD
Tetracycline	11	NAD	23.6	NAD
Ticarcillin[b]	62	3.0	58	Trace
Tobramycin[b]	13.6	NAD	14.0	NAD
Vancomycin	23	NAD	27	NAD

[a] NAD, No activity detected.

[b] Antibiotics were tested at elevated concentrations; all lower concentrations were removed by ARD.

[c] Trace, Slight activity detected but too low for measurement or estimation.

RESULTS

Single antibiotics challenge. Precision of these assay systems was ±10% for gentamicin and tobramycin, ±15% for amikacin, and ±15% of control for others. The detection limit, defined as the lowest concentration at which even slight activity is consistently detected, was about one-half the lowest concentration on the standard curve for amikacin, chloramphenicol, tobramycin, and tetracycline and one-twentieth to one-tenth the lowest standard concentration for all others. Table 1 shows the amount of each of 10 antibiotics reduced to apparent zero activity by ARD treatment and 2 reduced to a very low level. Ampicillin, chloramphenicol, gentamicin,

ticarcillin, tobramycin, and vancomycin results are shown at concentrations much higher than would usually be seen in therapy, since earlier experiments showed that all lower concentrations were removed. Cephalothin, carbenicillin, nafcillin, and amikacin were tested only at the concentrations shown in Table 1. Because cefazolin added at 75 and 120 μg/ml was not completely removed, experiments were done to determine the extent of removal. Quadruplicate ARD bottles were challenged with 171 μg of cefazolin per ml. Activity was reduced to 0.8 to 1.7 μg/ml, values below the standard curve, allowing the calculation that about 99.01 to 99.55% of cefazolin was removed.

Mixed antibiotics. Results are shown in Table 2. Comparison of these results with the values in Table 1 for cefazolin, ticarcillin, and vancomycin demonstrates that the ARD system was not compromised by combining antibiotics. Trimethoprim and sulfamethoxazole each interfered in the assay for the other, so a precise value in the assay system for their removal could not be assigned. However, it is clear that there was sufficient activity of each before ARD treatment to permit the conclusion that ARD treatment removes both.

Time required for removal. Table 3 demonstrates that the prescribed tumbling motion is necessary for binding by ARD and that holding the bottle for up to 2 h after blood is added does not provoke elution of bound antibiotics. Other experiments, not shown, demonstrated that cefazolin activity did not reappear if ARD bottles containing blood were held at room temperature for 25 h. Finally, in experiments (not shown) which measured antibiotic activity after 15 s and 5, 10, and 15 min of tumbling of ARD bottles, ampicillin, chloramphenicol, and tobramycin were reduced to a trace after 5 min and to zero after 10 min. However, the full prescribed 15 min was required to reduce gentamicin to zero and cefazolin to its lowest value.

Recovery of bacteria and yeasts. Results from one experiment dealing with recovery are shown in Table 4. Colony counts made at the beginning and end of one test are shown and do not differ demonstrably from the results of repetition of this experiment. The only instance of failure of growth in a blood culture bottle was with *Peptococcus aerogenes*, for which the challenge inoculum was only 0.02 colony-forming unit per ml. The numbers of colonies recovered in all the challenges were essentially equal to the numbers recovered from controls consisting of blood inoculated with bacteria and not ARD treated. Similarly, the number of colonies of any of the isolates recovered after 2 h in the ARD bottle was the same as the number recovered from the control and from ARD-treated blood sampled at time zero. Every challenge inoculum was recovered either as countable colonies and growth in the blood culture bottle or, for one replicate each of *Candida albicans*, *Escherichia coli*, *Neisseria meningitidis*, *Streptococcus pyogenes*, and *P. aerogenes* (data not shown), as

TABLE 2. *Removal of mixed drugs from human blood by ARD*

Combination	Assay values (μg/ml)			
	Drug A		Drug B	
	Untreated	Treated	Untreated	Treated
Trimethoprim (A) + sulfamethoxazole (B)	7–10[a]	NAD[b]	100–>100[a]	NAD
	10–>10[a]	NAD	40–60[a]	NAD
	7–10[a]	NAD	100–>100[a]	NAD
Cefazolin (A) + tobramycin (B)	16.5	Trace	12.8	NAD
	18.0	NAD	12.6	NAD
Ticarcillin (A) + tobramycin (B)	46.0	3.0[c]	7.8	NAD
	50.0	1.0[c]	11.6	NAD
Vancomycin (A) + gentamicin (B)	60.0	2.0[c]	11.0	NAD
	60.0	Trace	11.0	NAD

[a] Trimethoprim and sulfamethoxazole each interferes in the assay for the other; when both are present, real concentrations cannot be measured. The values reported are the concentrations of a drug standard producing the next-smaller and next-larger zones than those of the test samples.

[b] NAD, No activity detected.

[c] Value is an estimate; activity was below that of the lowest standard.

TABLE 3. *Elution of antibiotics from ARD resins*

Antibiotic	Concn (μg/ml) before ARD treatment	Concn (μg/ml) after ARD treatment[a]			
		A	B	C	D
Cefazolin	20	NAD	NAD	3[b]	NAD
	33	Trace	NAD	6[b]	Trace
Gentamicin	4.1	NAD	NAD	Trace	NAD
	3.3	NAD	NAD	Trace	NAD
Ampicillin	2.6	NAD	NAD	Trace	NAD
	2.6	Trace	NAD	Trace	NAD
Chloramphenicol	11.5	NAD	NAD	NAD	NAD
	18.0	NAD	NAD	NAD	NAD

[a] Treatment: A, bottle tumbled for 15 min; B, bottle tumbled for 15 min, then held for 2 h at room temperature; C, bottle held for 2 h, not tumbled; D, bottle held for 2 h, then tumbled for 15 min. NAD, No activity detected; Trace, slight activity detected, but too low for measurement or estimation.

[b] Value is estimated; activity was below that of the lowest standard.

growth in the bottle but without countable colonies.

DISCUSSION

The antimicrobial agents used for challenge in these studies represent those most used for treatment of septicemia and included one or more representatives of each of the major antibiotics classes, as well as the combination trimethoprim-sulfamethoxazole. The device is described for use in blood cultures only from patients receiving antimicrobial treatment for serious infections. The ARD system either removes each of the tested drugs to a level below the limit of its assay system (apparent zero) or reduces it to a very low level. For example, if the reduction of 171 μg of cefazolin per ml by at least 99% in our experiment is extrapolated to an expected clinical concentration of about 50 μg/ml, that clinical concentration would be reduced to 0.05 μg/ml or less. The minimum inhibitory concentration of cefazolin against most gram-negative bacteria is greater than 1 μg/ml, and that against gram-positive bacteria is about 0.5 μg/ml and only very rarely as low as 0.05 μg/ml. This ought to mean that 0.05 μg of cefazolin per ml, reduced by dilution to 0.005 μg/ml in most blood culture systems, is not sufficient to inhibit in vitro growth of most bacteria surviving in the blood of a treated person. Similar reasoning could be applied to vancomycin and ticarcillin, the other antibiotics found to have trace residual activity after ARD treatment.

The removal device is effective against two penicillins, two new semisynthetic penicillins, three aminoglycosides, one tetracycline, and sulfamethoxazole. The mechanisms by which this device removes antimicrobial activity are probably nonpartitional absorption or cationic exchange by the resins and inactivation by sodium polyanetholesulfonate. Although the ARD was not challenged with every antibiotic, we are willing to speculate, from these results and the nature of the ARD function, that it very likely will be found effective against other members of the antimicrobial "families" tested. Of course many of the antibacterial agents not tested are not ordinarily used for treating systemic infections.

We have not measured the capacity of this device to remove immune globulins, complement, or bacterial metabolic products, such as polysaccharide or lipid. Such removal, if it occurs, might be either irrelevant to or possibly an additional advantage in bacterial growth. Finally, capacity of this device to remove antifungal drugs as well as antibacterial ones remains to be determined.

The device did not inhibit or kill bacteria used for challenge. Efforts were made to select for challenge numbers of each test organism low enough to simulate the numbers expected in bacteremia yet high enough to permit the counting of colonies. Comparison of inocula from Table 4 with respective control counts exemplifies that colony counts are only approximately reproducible in this number range. Low reproducibility is an intrinsic flaw of colony count experiments with bacterial numbers in this range, but we believe our conclusion is validated by similar results in the four replicates shown and others not shown.

The somewhat fastidious or fragile organisms, such as *C. albicans*, *Haemophilus influenzae*, *Fusobacterium nucleatum*, and *Peptococcus prevotii*, survived as well in these experiments

TABLE 4. *Recovery of bacteria and yeasts from human blood with and without ARD treatment*

Strain	Time (min)[a]	CFU[b]/ml inoculated	CFU/ml recovered	
			Control[c]	After ARD treatment[d]
Bacteroides fragilis	0	1.4	10	8, 4, 3, 5
	120		10	10, 5, 9, 4
Clostridium sporogenes	0	11.9	9	3, 3, 8, 6
	120		4	2, 4, 4, 6
Fusobacterim nucleatum	0	7.1	2	1, 1, 2, 6
	120		3	1, 3, 2, 4
Peptococcus aerogenes	0	0.02	1	0, 0, 1, 2
	120		4	1, 2, 1, 0[e]
Peptococcus prevotii	0	7.7	5	1, 3, 4, 3
	120		3	3, 3, 2, 1
Candida albicans	0	1.5	2	1, 0, 0, 1
	120		1	1, 1, 0, 1
Escherichia coli	0	1.0	4	1, 1, 2, 4
	120		3	0, 0, 1, 2
Haemophilus influenzae	0	1.8	10	1, 9, 1, 8
	120		3	1, 3, 4, 2
Neisseria meningitidis	0	0.7	1	2, 2, 1, 0
	120		6	4, 1, 0, 3
Bacillus subtilis	0	2.8	11	10, 11, 7, 8
	120		6	8, 5, 8, 7
Staphylococcus aureus	0	1.7	2	2, 13, 2, 1
	120		1	1, 7, 1, 1
Streptococcus pyogenes	0	1.7	5	2, 1, 3, 1
	120		2	1, 0, 1, 1

[a] Time interval between addition of test organism to blood and collection of samples for counts and inoculation to blood culture bottles.

[b] CFU, Colony-forming units.

[c] Organism inoculated to blood, not ARD treated.

[d] Number of colony-forming units per milliliter recovered from four replicate challenges. Companion bottles in all replicates showed growth, unless indicated otherwise.

[e] Three of four bottles inoculated showed growth.

as did the sturdier *E. coli*, *B. subtilis*, *S. aureus*, *Streptococcus pyogenes*, and *Bacteroides fragilis*. Of particular importance is the fact that anaerobic bacteria survived well. These experiments demonstrate that, in vitro, this device does not trap, inhibit, or kill bacteria.

The final test of the usefulness of the ARD must show that there actually is an increase of microbial isolations from antibiotic-treated clinical specimens subjected to ARD treatment as compared with matched or similar specimens not ARD treated. Appleman and colleagues (M. D. Appleman, R. Swinney, and P. N. R. Heseltine, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, C260, p. 318) reported that 17 of 38 positive blood cultures were from ARD-treated specimens only, whereas only 3 of the 38 were negative from ARD specimens. McLimans et al. (C. A. McLimans, M. M. Hall, and R. L. Thompson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, C259, p. 317) reported that of 47 cultures from treated patients with *S. aureus* infections 21 were negative with and without ARD treatment, 4 were positive both with and without ARD treatment, and 22 were positive with ARD treatment but negative without ARD treatment. Wallis and colleagues (7) and Melnick (4) report similar results.

In conclusion, it seems clear that the ARD does remove antibiotics and does not interfere with bacterial growth from ARD-treated blood specimens. Continued effectiveness of this device will require that it be challenged with new antimicrobial agents as these are developed.

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