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Emergence of 4′,4″-Aminoglycoside Nucleotidyltransferase in Enterococci

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Enterococcus faecium **BM4102 was resistant to macrolide-lincosamide-streptogramin B-type (MLS) antibiotics; tetracycline-minocycline; and high levels of kanamycin, neomycin, tobramycin, and dibekacin but not gentamicin. This aminoglycoside resistance phenotype is new in enterococci. The genes conferring resistance to aminoglycosides and MLS antibiotics in this strain were carried on a plasmid, pIP810, that was self-transferable to other *Enterococcus* strains. Resistance to tobramycin and structurally related aminoglycosides, kanamycin, neomycin, and dibekacin, was due to synthesis of a 4′,4″-aminoglycoside nucleotidyltransferase. Homology was detected by hybridization between pIP810 DNA and a probe specific for a gene encoding an enzyme with identical site specificity in staphylococci. The bacteriostatic activity of amikacin apparently was not affected by the presence of the enzyme, although it was modified in vitro. However, the bactericidal activity of amikacin and the synergism of this aminoglycoside with penicillin were abolished.**

Enterococci and streptococci are naturally resistant to low levels of aminoglycosides (with MICs of 4 to 250 μg/ml) (30). Enterococci are less susceptible than streptococci to penicillin (15). However, combinations of penicillin with aminoglycosides usually display bactericidal synergy against clinical isolates of enterococci (14, 27, 32). Synergism is abolished in isolates with high-level resistance to aminoglycosides (MICs, ≥2,000 μg/ml) (12, 34; R. Leclercq, S. Dutka-Malen, A. Brisson-Noël, C. Molinas, E. Derlot, M. Arthur, and P. Courvalin, Rev. Infect. Dis., in press). Resistance to high levels of aminoglycosides is generally due to acquisition of genes encoding aminoglycoside-modifying enzymes (3). The resistance determinants are most often part of self-transferable or mobilizable plasmids (7, 13, 25) or of conjugative transposons (5) and have spread in recent years in numerous species of streptococci and enterococci (Leclercq et al., in press).

High-level resistance to tobramycin not associated with resistance to gentamicin has, to our knowledge, not been detected in enterococci-streptococci. In this study, we describe an *Enterococcus faecium* strain that is resistant to high levels (>1,000 μg/ml) of tobramycin and susceptible to gentamicin. The plasmid-borne resistance gene was compared with that gene (20) which is responsible for a similar phenotype in *Staphylococcus aureus* (16). The consequences of the presence of the enzyme on the bacteriostatic and bactericidal activities of aminoglycosides alone or in combination with penicillin against the bacterial host were determined.

MATERIALS AND METHODS

Bacterial strains. The properties of the strains used in this study are listed in Table 1. *E. faecium* BM4102 was isolated in France in 1985 from a sputum sample. *E. faecium* BM4105 is a wild-type strain that is susceptible to antibiotics. Both isolates produced low levels of 6′-aminoglycoside acetyltransferase, which is generally present in strains of *E. faecium* (21, 33). They were identified as enterococci by Gram staining; absence of catalase; inability to produce gas; presence of Lancefield antigen group D; and growth on 40% bile, in 6.5% sodium chloride, in 0.1% methylene blue, and at pH 9.6. Species identification (9, 24) was based on the absence of potassium tellurite reduction and tests for acid production from 50 carbohydrates in API 50 CH galleries of tests (API System, La Balme-les-Grottes, France).

Media. Brain heart infusion broth and agar (Difco Laboratories, Detroit, Mich.) were used. Susceptibility tests were done in Mueller-Hinton broth and agar (Diagnostics Pasteur, Marnes-la-Coquette, France). All incubations were done at 37°C.

Antibiotic susceptibility testing. The method of Steers et al. (28), with 10^4 CFU per spot, was used to determine the MICs of antibiotics in solid medium. The MICs in liquid medium were determined with an inoculum of 10^5 to 10^6 CFU/ml, and the MBCs were at the 99.9% killing level. Time-kill curves were determined with an inoculum of 10^7 CFU/ml and 20 μg of penicillin, amikacin, or kanamycin per ml and 4 μg of tobramycin per ml. The concentrations of aminoglycosides were below the MICs for the strains studied and did not affect their growth. Synergy was defined as an increase in killing by the association after 24 h equal to or greater than 100-fold compared with that of the most effective antibiotic alone.

Transfer of antibiotic resistance traits. Mating on filters was performed as described previously (31). The antibiotic concentrations for selection of transconjugants were as follows: erythromycin, 10 μg/ml; and streptomycin and tobramycin, 1,000 μg/ml.

Preparation of DNA and agarose gel electrophoresis. Preparation of high-molecular-weight total DNA (8) and purification of plasmid DNA (6) were done as described previously. The DNA restriction fragments were separated by electrophoresis in horizontal slab gels (20 by 20 by 0.7 cm) containing 0.8% agarose type II or type VII (Sigma Chemical Co., St. Louis, Mo.). Fragments were extracted from low-temperature-gelling agarose type VII as described previously (18).

Assay for aminoglycoside-modifying enzymes. Bacterial ex-

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TABLE 1. Properties of the strains used in this study

Strain	Relevant characteristics and plasmid content[a]	Source or reference
E. faecalis JH2-2	Fus Rif	11
E. faecalis BM4110	Str	5
E. faecium BM4105		Wild strain
E. faecium BM4102	Tc; pIP810 Tra^+ Em Tm	Wild strain
E. faecalis BM4103	Str; pIP810 Tra^+ Em Tm	Conjugation, BM4102 × BM4110
E. faecalis BM4104	Str; pIP810 Tra^+ Em Tm	Conjugation, BM4102 × BM4110

[a] Tra^+, Self-transferable; Fus, fusidic acid resistance; Rif, rifampin resistance; Str, streptomycin resistance; Em, erythromycin resistance; Tc, tetracycline resistance; Tm, tobramycin resistance. The designation of the plasmid and its phenotypic characters are indicated. The tetracycline resistance is apparently chromosomal (see text).

tracts were prepared (7) and the enzymes were assayed by the phosphocellulose paper-binding technique (10) as described previously. The final concentration of aminoglycosides in the assay mixture was 66.7 μg/ml, and the reaction was allowed to proceed for 30 min at 30°C.

DNA-DNA hybridization. DNA of plasmid pUB110 (22) originating from *S. aureus* (16) was purified by a modification of the procedure of Birnboim and Doly (1), digested with *Hin*cII, and analyzed by agarose gel electrophoresis. The 473-base-pair (bp) *Hin*cII fragment internal to *aadC* (20) was extracted, purified, and cloned into pUC8 that was digested with *Hin*cII and dephosphorylated (18). The resulting recombinant plasmid, pAT16, was digested with *Bam*HI-*Pst*I; and the 491-bp fragment containing part of *aadC* was extracted, purified (18), and labeled with [α-^{32}P]dATP by nick translation (19). Dot blot hybridization under stringent conditions was done in 50% formamide at 42°C for 24 h, followed by three washings in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature for 15 min and by two washings in 0.2× SSC at 65°C for 1 h.

Enzymes and chemicals. Restriction endonucleases were obtained from Boehringer GmbH (Mannheim, Federal Republic of Germany) and were used according to the recommendations of the manufacturer. Lysozyme was from Sigma Chemical Co. RNase A (bovine pancreas) and proteinase K were from Calbiochem-Behring (La Jolla, Calif.). [1-^{14}C] acetylcoenzyme A, [α-^{32}P]ATP (triethylammonium salt), [γ-^{32}P]ATP (triethylammonium salt), and [U-^{14}C]ATP (ammonium salt) were obtained from the Radiochemical Centre (Amersham, England). The following antibiotics were provided by the indicated laboratories: gentamicins C1a, C1, A, B, and complex, sisomicin, and netilmicin, Schering Corp. (Bloomfield, N.J.); kanamycins A and B and amikacin, Bristol Laboratories (Syracuse, N.Y.); dibekacin and habekacin, Roger Bellon (Neuilly-sur-Seine, France); neomycins A and B, The Upjohn Co. (Kalomazoo, Mich.); paromomycin and butirosin, Parke, Davis & Co. (Detroit, Mich.); tobramycin and apramycin, Eli Lilly & Co. (Indianapolis, Ind.); lividomycin A, Kowa (Japan); fortimicin, Kyowa (Kokaï, Japan); streptomycin and tetracycline, Pfizer Inc. (New York, N.Y.); minocycline, Lederle Laboratories (Pearl River, N.Y.); erythromycin, Abbott Laboratories (North Chicago, Ill.); rifampin, Le Petit (Milan, Italy); and fusidic acid, Leo (Montigny-le-Bretonneux, France).

TABLE 2. MICs of various aminoglycosides against enterococcal strains

Strain	MIC (μg/ml)[a]						
	Gen	Kan	Neo	Tob	Dib	Ami	Hab
E. faecalis BM4110	8	64	64	16	16	64	16
E. faecium BM4102	8	>1,000	1,000	>1,000	128	64	16
E. faecalis BM4103	8	>1,000	1,000	>1,000	128	64	16
E. faecalis BM4104	8	>1,000	1,000	>1,000	128	64	16
E. faecium BM4105	8	256	64	64	64	64	16

[a] Abbreviations: Ami, amikacin; Dib, dibekacin; Gen, gentamicin complex; Hab, habekacin; Kan, kanamycin; Neo, neomycin; Tob, tobramycin.

RESULTS

Plasmid-mediated characters expressed by *E. faecium* BM4102. Strain BM4102 was resistant to high levels of kanamycin, neomycin, and tobramycin; dibekacin; macrolide-lincosamide-streptogramin B-type (MLS) antibiotics constitutively; and tetracyline-minocycline (Tables 1 and 2). The genes conferring resistance to aminoglycosides and MLS antibiotics were transferred en bloc by conjugation from *E. faecium* BM4102 to *E. faecalis* BM4110. Selection for transfer of tobramycin or erythromycin resistance produced cotransfer of the two characters. Two transconjugants, *E. faecalis* BM4103 and BM4104, were selected for further studies. Transfer of tetracycline resistance was not observed. In retransfer experiments, transconjugants could transfer the aminoglycoside and MLS antibiotic resistances by conjugation to *E. faecalis* JH2-2. The MICs of various aminoglycosides for the parental strain BM4102, the recipient, transconjugants, and BM4105, a susceptible *E. faecium* control strain, are given in Table 2.

Plasmid DNA from transconjugants BM4103 and BM4104 (Table 1) was purified by ultracentrifugation and analyzed by agarose gel electrophoresis before (data not shown) and after digestion with *Eco*RI or *Hin*dIII (Fig. 1). Strains BM4103 and BM4104 were aminoglycoside and MLS antibiotic resistant after acquisition of plasmid pIP810, which had a size of 24.9 kilobases.

Mechanism of resistance to aminoglycosides mediated by pIP810. *E. faecium* BM4102 and *E. faecalis* BM4110, BM4103, and BM4104 were examined for aminoglycoside-modifying activities. Strains harboring plasmid pIP810 were found to contain aminoglycoside adenylyltransferase activity but not phosphotransferase or acetyltransferase activity, except for BM4102, which also produced low levels of the 6′-aminoglycoside acetyltransferase that is common in *E. faecium* (21, 33) (data not shown). The substrate profiles of the adenylyltransferases extracted from the wild-type strain and the transconjugants were very similar. Those of BM4102 and BM4103 are shown in Fig. 2. Gentamicin A and B antibiotics, the kanamycin compounds, tobramycin, the neomycins, and butirosin were excellent substrates for the adenylyltransferase, whereas dibekacin, amikacin, paromomycin, and lividomycin were poor substrates. Gentamicins C, sisomicin, netilmicin, habekacin, apramycin, and fortimicin were not adenylylated. Based on this substrate profile and hybridization experiments (see below), the enzyme is a 4′,4″-aminoglycoside nucleotidyltransferase [ANT(4′)(4″)]. The fact that dibekacin (3′,4′-dideoxykanamycin B) was modified and habekacin [1-*N*-(4 amino-2 hydroxy-butiryl) dibekacin] was not confirms that the 4′- and 4″-hydroxyl groups are the sites of adenylylation.

Analysis of DNA by hybridization. Total DNA of *E. faecium* BM4102 and *E. faecalis* BM4110, BM4103, and

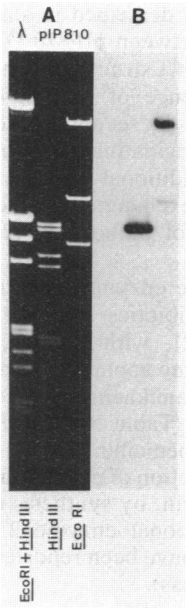

FIG. 1. Analysis of plasmid DNA by agarose gel electrophoresis (A) and hybridization (B). Plasmid pIP810 DNA was digested with the restriction endonucleases indicated at the bottom. Fragments obtained by digestion of λ c1857 DNA with *Eco*RI-*Hin*dIII (λ) were used as molecular size standards (23). The resulting fragments were fractionated by agarose gel electrophoresis, transferred to a nitrocellulose filter (26), and hybridized to an in vitro, ^{32}P-labeled (19) pUB110 473-bp *Hin*cII fragment.

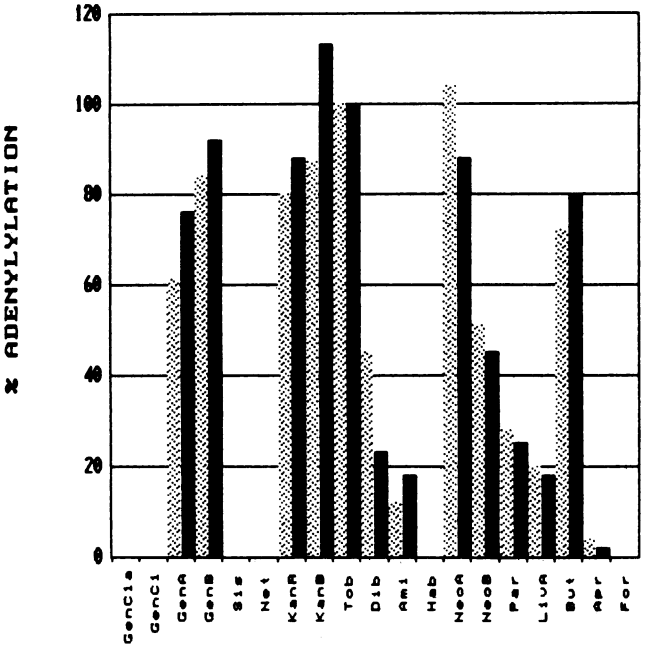

FIG. 2. Substrate profiles of enzymes extracted from *E. faecium* BM4102 (spotted bars) and *E. faecalis* BM4103 (solid bars) assayed by the phosphocellulose paper-binding technique (10). Adenylylation (average of three independent experiments) is expressed relative to that of tobramycin, which was set at 100%. No phosphorylation of the same aminoglycosides was detected in these strains, and no acetylation was detected in *E. faecalis* BM4103. No aminoglycoside-modifying activity was detected in the susceptible strain BM4110. *E. faecium* BM4102 and BM4105 produced low levels of 6′-aminoglycoside acetyltransferase (33) (data not shown). Abbreviations: Gen, gentamicin; Sis, sisomicin; Net, netilmicin; Kan, kanamycin; Tob, tobramycin; Dib, dibekacin; Ami, amikacin; Hab, habekacin; Neo, neomycin; Par, paromomycin; Liv, lividomycin; But, butirosin; Apr, apramycin; For, fortimicin.

BM4104 was transferred to a nitrocellulose filter and hybridized with a probe specific for *aadC*, the structural gene for ANT(4′)(4″) from *S. aureus* (Fig. 3). Homology was found between the 473-bp fragment of pUB110 and total DNA of the strains harboring pIP810. DNA of pIP810 was hybridized to the same probe, and one *Hin*dIII and two *Eco*RI fragments of the plasmid displayed homology (Fig. 1).

Influence of ANT(4′)(4″) on the bacteriostatic and bactericidal activities of aminoglycosides alone or combined with β-lactams. There was a good correlation, except for amikacin, between the in vitro substrate range of the enzyme (Fig. 2) and the resistance phenotype of the host (Table 2); antibiotics which were good substrates (e.g., kanamycin, tobramycin, and neomycin) had very high MICs and antibiotics which were poor substrates (e.g., dibekacin) had moderately elevated MICs, whereas the MICs of aminoglycosides which were not detoxified (e.g., gentamicins C and habekacin) remained unchanged. By contrast, the bacteriostatic activity of amikacin apparently was not affected by the presence of the enzyme, although, as mentioned above, it was significantly modified by the cell extracts. This in vitro-in vivo discrepancy was studied further by determination, in liquid medium, of the MICs and MBCs of amikacin against isogenic strains that differed by the presence or the absence of the modifying activity. As in solid medium (Table 2), the MICs of amikacin (128 μg/ml) were not altered by the presence of the enzyme, whereas the MBCs were dramatically increased (from 256 μg/ml for BM4110 to >2,048 μg/ml for BM4102 and BM4103).

The activities of combinations of penicillin with amikacin, kanamycin, and tobramycin were studied against the same pair of strains. The results are summarized in Table 3, and Fig. 4 provides part of this analysis. As expected, high-level resistance to kanamycin and tobramycin abolished the synergy with penicillin that these antibiotics display against susceptible strains. Most importantly, the synergism between penicillin and amikacin and the bactericidal activity of the combination were totally abolished by the presence of ANT(4′)(4″).

DISCUSSION

E. faecium BM4102 was resistant to high levels of tobramycin and structurally related antibiotics but not to gentamicin (Table 2). Aminoglycoside resistance in this strain was

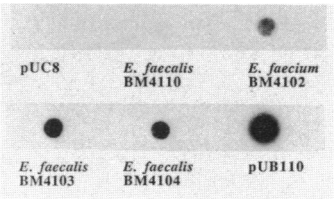

FIG. 3. Analysis of DNA by dot blot hybridization. Total or plasmid DNA was transferred to a nitrocellulose filter (26) and hybridized to an in vitro, ^{32}P-labeled (19) pUB110 473-bp *Hin*cII fragment. Total DNA from *E. faecalis* BM4110 and DNA of plasmid pUB110 served as negative and positive controls, respectively. The absence of hybridization with pUC8 DNA indicates that the probe was not contaminated with vector DNA.

TABLE 3. Effects of combinations of penicillin and aminoglycosides against *E. faecalis* BM4110 and BM4103

Antibiotics (concn [μg/ml])	Magnitude (log 10) of increased killing by combination relative to that by penicillin against:	
	BM4110	BM4103
Penicillin (20) + amikacin (20)	+2.2	−0.3
Penicillin (20) + kanamycin (20)	+2.5	−0.8
Penicillin (20) + tobramycin (4)	+2.2	+0.3

mediated by pIP810, a 24.9-kilobase plasmid (Fig. 1) that is self-transferable to other *Enterococcus* cells. Resistance to aminoglycosides was correlated with the presence of pIP810 DNA (Fig. 1 and 3).

Plasmid-encoded, high-level resistance to aminoglycosides in enterococci-streptococci has been described previously (7, 11, 13, 25). Resistance is frequently mediated by aminoglycoside-inactivating enzymes which are classified by the mechanism of their modification and the site of the antibiotic which they modify (4). High-level resistance to tobramycin not associated with resistance to gentamicin is novel in enterococci. We established, by in vitro substrate profile determination (Fig. 2) and DNA annealing studies (Figs. 1 and 3), that resistance to tobramycin-kanamycin in BM4102 is due to the synthesis of a ANT(4′)(4″), an enzyme that has already been described in staphylococci (17). The homology detected between pIP810 DNA and a probe specific for the ANT(4′)(4″) structural gene from staphylococci suggests that emergence of tobramycin-kanamycin resistance in enterococci is secondary to the acquisition of genetic information originating in staphylococci. This observation constitutes additional support for the notion that resistance genes are exchanged between these phylogenetically remote genera of pathogenic bacteria under natural conditions.

The presence of the enzyme conferred to the host resistance toward the antibiotics that were substrates in vitro (Table 2 and Fig. 2), with the exception of amikacin. Modification of the drug apparently did not affect the bacteriostatic activity of amikacin. However, the bactericidal activity of amikacin (Table 3) and the synergism of this aminoglycoside with penicillin (Fig. 4) were abolished. Similar pitfalls in the detection of plasmid-mediated resistance to amikacin and netilmicin, by synthesis of a 3′-phosphotransferase or the bifunctional enzyme 2″-phosphotransferase-6′-acetyltransferase, have been reported previously (2, 4, 29; Leclercq et al., in press).

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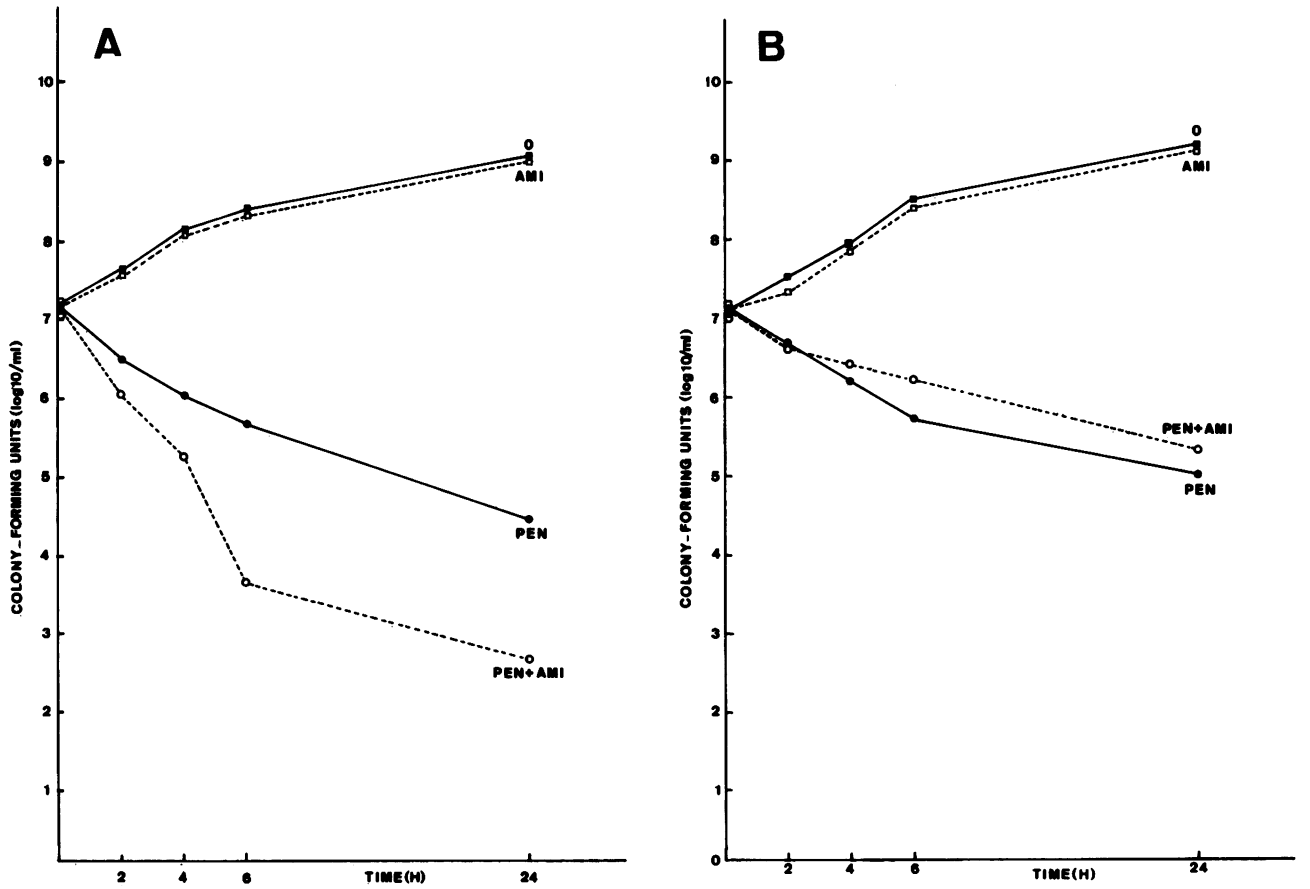

FIG. 4. Effect of combinations of penicillin and amikacin against *E. faecalis* BM4110 (A) and BM4103 (B). ■, Antibiotic-free medium; AMI, amikacin (20 μg/ml); PEN, penicillin (20 μg/ml).

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