Incidence of an aminoglycoside 6'-N-acetyltransferase, ACC(6')-1b, in amikacin-resistant clinical isolates of gram-negative bacilli, as determined by DNA-DNA hybridisation and immunoblotting

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Summary. Seventy amikacin-resistant clinical isolates of gram-negative bacteria belonging to nine genera were examined by immunoblotting and by DNA-DNA hybridisation for the presence of ACC(6')1b enzyme, previously called AAC(6')-4, or its encoding gene *aacA1b*. The organisms mostly had resistance profiles compatible with AAC(6') production and were from South and North America, the Far East and Europe. Polyclonal (rabbit) anti-AAC(6')-1b antisera and an intragenic *aacA1b* (*aacA4*) probe derived from the multiresistance plasmid pAZ007 were used. The *aacA1b* gene was found to be widespread. Positive hybridisation, and immunologically cross-reactive proteins, were observed in 44% of the isolates examined. They were present most frequently ($\geq 70\%$) in isolates of *Klebsiella*, *Escherichia* and *Enterobacter* spp., but less often ($\leq 25\%$) in *Serratia*, *Citrobacter*, *Acinetobacter* and *Pseudomonas* spp. The strains that reacted with the probe produced enzymes that varied in their apparent mol. wts between c. 24 000 and 26 000. The existence of multiple electrophoretic forms of amikacin-acetylating enzymes of the ACC(6')-1b type may be useful in epidemiological surveys of AAC(6')-mediated amikacin resistance.

Introduction

Resistance to amikacin in clinical isolates of gram-negative bacteria is most often attributable to the presence of aminoglycoside 6'-N-acetyltransferases of type 1, alternatively also called type 4.¹⁻⁴ Enzymes of this type, which typically modify amikacin, tobramycin and netilmicin, but not gentamicin,⁵ have been found to be plasmid-mediated and encoded by transposable elements^{6,7} or to be specified by a chromosomal gene.⁸ The nucleotide sequences of three AAC(6')-1 genes, isolated from plasmids pAZ007,⁹ pBWH100,¹⁰ and pU0490¹¹ have been established. They show no substantial homology but confer the same resistance phenotype.

However, amikacin resistance does not always involve 6'-N-acetyltransferases. Resistance in *Acinetobacter* spp. may depend upon the synthesis of a phosphotransferase, APH(3')-VI.¹² The possible role of other enzymes such as APH(3')-II^{13,14} or AAD(2'')¹⁵ in clinical resistance remains uncertain.

In the present study we have used an intragenic probe for the *aacA1b* (previously called *aacA4*) gene carried by plasmid pAZ007^{9,16} and anti-AAC(6')-1b (previously called AAC(6')-4) antibodies¹⁷ to estimate the incidence of this gene and its product amongst

amikacin-resistant clinical strains from various geographic areas.

Materials and methods

Bacterial strains, plasmids and growth conditions

A total of 70 gram-negative bacterial isolates that were resistant to amikacin was examined. These had been isolated in Europe, North America, South America and the Far East (table I). The strain used as an AAC(6')1b-positive control was *E. coli* BM694(pAZ007). Plasmid pAZ007, encoding resistance to amikacin, streptomycin, ampicillin, sulphonamides and trimethoprim, was obtained initially from enterobacteria isolated at a hospital in Chile.¹⁵ The derived plasmid pAZ505⁹ served as a source of the intragenic probe for the *aacA1b* gene.

Cultures were grown routinely on Mueller-Hinton agar, or in Mueller-Hinton broth, at 37°C.

Susceptibility tests

Minimal inhibitory concentrations (MICs) were determined on agar plates containing serial dilutions of antibiotic. These were inoculated with a Steers-type replicator delivering c. 10^5 cfu/spot.

DNA preparation for dot and Southern blots

Total DNA was prepared from 1.5-ml volumes of overnight cultures. The cells were harvested by centrifugation, then resuspended in 100 μ l of a buffer containing 10 mM edetic acid, 50 mM glucose, 25 mM Tris-HCl, pH 8.0. Lysozyme was added to a final concentration of 5 mg/ml. After incubation for 10 min on ice, 1 μ l of the same buffer containing 1 μ g of proteinase K was added. The sample was left for 15 min at room temperature. Subsequently, 33 μ l of sodium lauroyl sarkosinate 3% w/v in 375 mM edetic acid pH 8.0, was added. Lysis was allowed to proceed for 60 min at 65°C. The mixture was then extracted with 150 μ l of phenol, and the DNA was precipitated with ethanol and resuspended in 400 μ l of TE buffer (10 mM Tris-HCl, 1 mM edetic acid, pH 8.0).¹⁸

For dot blot analyses, 10 μ l of 1M HCl was added to 30 μ l of the DNA preparation described above. The samples were left at room temperature for 10 min, after which the DNA was denatured by adding 40 μ l of a solution containing 1M NaOH and 3M NaCl and heating to 95°C for 5 min. Neutralisation was then achieved by adding 240 μ l of 500 mM Tris-HCl, pH 7.5, containing 1.5 M NaCl. The denatured DNA (300 μ l) was finally spotted on to a nitrocellulose filter by means of a 96-well dot-blot apparatus, washed with 20 $\times$ SSC (3 M NaCl, 340 mM sodium citrate) pH 7.0,¹⁸ and baked at 80°C for 2 h.

For Southern blot analysis, total undigested DNA in 30 μ l of TE buffer was separated on an agarose 0.6% gel, and transferred to a nitrocellulose filter, as described previously.¹⁸

Hybridisation experiments

The probe used for hybridisation studies was a 344-bp *Dde*I-*Ava*I intragenic fragment of the *aacA1b* gene from plasmid pAZ505, which did not include the region of homology with the *bla* gene of transposon Tn3 or the *aacC3* gene.⁹ This fragment is contained within the c. 500-bp *Dde*I-*Hae* III fragment, the specificity of which, as a probe, has been established previously.¹⁷ The fragment was isolated, after digestion of plasmid pAZ505 with the appropriate enzymes and electrophoretic separation, from a polyacrylamide 4.5% w/v gel. It was labelled with ³²P-CTP (3000 mCi/ml) by means of a Multiprime Kit (Amersham International, Amersham, Bucks).

Pre-hybridisation and hybridisation were carried out for 6 h and 12 h, respectively, under conditions of high stringency at 42°C in formamide 50%, 5 $\times$ SSC, and 5 $\times$ Denhardt's solution.¹⁸ Washes were performed at 68°C in 2 $\times$ SSC.

Immunoblot analysis

Total protein extracts were prepared from bacteria that had been grown to an OD₆₅₀ of approximately 0.6. The cells from 1 ml of culture were sedimented,

resuspended in 400 μ l of sample buffer,¹⁹ and boiled for 10 min. Samples (25 μ l) were electrophoresed on a polyacrylamide gel containing acrylamide 12%, *N,N*-methylene-bis-acrylamide 0.3%, and sodium dodecyl sulphate (SDS) 0.1%. The buffer system was that described by Laemmli.¹⁹ Proteins were transferred to nitrocellulose filters as described previously.²⁰ The filters were treated, as described,²¹ with anti-AAC(6')-1b antiserum, which had been prepared in rabbits immunised with the purified enzyme.¹⁷ The antiserum and the peroxidase-labelled anti-rabbit IgG (Diagnostics Pasteur, 92430 Marne-La-Coquette, France) were used at a 1000-fold dilution.

Results

Susceptibility to aminoglycosides

All the strains studied were resistant to amikacin (table I), kanamycin and netilmicin (data not shown). The MIC ranges were, respectively, 8–>256, 32–>256 and 8–>256 mg/L; 20% of the strains additionally were resistant to gentamicin (MICs 16–>256 mg/L). Four strains of *Acinetobacter* spp. and two of *Providencia* spp. were resistant to amikacin but susceptible to tobramycin and, therefore, did not exhibit the aminoglycoside-resistance phenotype associated with aminoglycoside 6'-*N*-acetyltransferase type 1. Resistance to amikacin in strains of *Acinetobacter* spp. isolated in France recently has been reported to be conferred by a novel aminoglycoside phosphotransferase, APH(3')-VI.¹² The two *Providencia* strains hybridised with an APH(3')-VI gene probe (data not shown).

Hybridisation experiments with the intragenic probe for *aacA1b*

An autoradiograph obtained from the dot-blot experiment is shown in fig. 1, and the full results are analysed in tables II and III. Weak hybridisation in dot blots, which was not confirmed by Southern blotting, was not considered to be a positive result. Hybridisation seemed to occur more frequently with DNA from gentamicin-susceptible strains, particularly those from South America, than with DNA from gentamicin-resistant organisms (table II). The results showed also the distribution of the *aacA1b* gene amongst the gram-negative bacilli from the different geographical areas. Approximately 44% of the strains (31 of 70) apparently contained an *aacA1b* gene identical, or closely related, to that of plasmid pAZ007. This gene was particularly prevalent in some genera. Thus, DNA from all the resistant strains of *Klebsiella* spp. hybridised with the probe, as did that from most ($\geq 70\%$) of the *E. coli* and *Enterobacter* strains. The DNA of both strains of *Proteus* spp. and that of the only strain of *Achromobacter* spp. also hybridised with the probe. On the other hand, hybridisation was less frequent ($\leq 25\%$) with DNA from the members of the genera *Serratia*, *Citrobacter*, *Pseudomonas* and *Acine-*

Table I. Incidence of AAC(6')-1b-producing strains, and resistance patterns, amongst the genera studied

Genus	Number of strains	Number of strains that reacted with <i>aacA1b</i> probe/anti-AAC(6')-1b antibody when resistance pattern was		
		Ami ^R Tob ^R Gen ^S	Ami ^R Tob ^R Gen ^R	Ami ^R Tob ^S Gen ^R
<i>Achromobacter</i>	1	0	1/1	0
<i>Acinetobacter</i>	8	0	2/4	0/4
<i>Citrobacter</i>	8	1/7	1/1	0
<i>Escherichia</i>	11	4/4	4/7	0
<i>Enterobacter</i>	10	5/6	2/4	0
<i>Klebsiella</i>	6	4/4	2/2	0
<i>Pseudomonas</i>	10	0	1/10	0
<i>Proteus</i>	2	2/2	0	0
<i>Providencia</i>	4	0/1	0/1	0/2
<i>Serratia</i>	10	1/2	1/8	0

Ami, amikacin; Tob, tobramycin; Gen, gentamicin.

tobacter (table III), and was not seen with that from the four *Providencia* strains.

Southern blotting, with undigested DNA (fig. 2) showed that most of the *aacA1b*-positive strains yielded a band of DNA that hybridised with the probe

Fig. 1. Autoradiograph of a dot-blot filter, showing reaction of the *accA1b* probe with DNA extracted from: row A: well 1, *Achromobacter*; 2-9, *Acinetobacter*; A, 10 and B, 1-7, *Citrobacter*; B, 10 and C, 1-10, *Escherichia*; D, 1-10, *Enterobacter*; E, 1-6, *Klebsiella*; F, 1-10, *Pseudomonas*; G, 1 and 2, *Proteus*; 3-6, *Providencia*; H, 1-10, *Serratia*. The weak signals in positions B2, 6, C6, 7, F3, 4 and G3, 4, 5 were not considered to represent positive-hybridisation, because no signals were observed in Southern blotting experiments.

and that could not be separated from the randomly fragmented chromosomal DNA, even after prolonged electrophoresis. Some strains of *E. coli* (fig. 2; lanes 11, 12, 13), *Pseudomonas* spp. (lane 14), *Klebsiella* spp. (lanes 22, 23, 24) and *Serratia* spp. (lanes 30, 31) contained additional DNA of various mol. wts that also hybridised with the probe. In these cases, the *aacA1b* gene was probably carried on plasmids that migrated in different forms, some of which apparently co-electrophoresed with the chromosomal DNA.

Immunoblot analysis

All the strains were tested for the production of AAC(6')-1b by immunoblotting. Representative results, obtained with *E. coli* strains, are shown in fig. 3. There was a good correlation between the immunoblot and the hybridisation analyses. All the strains that contained a protein which reacted with the antiserum also hybridised with the *aacA1b* gene probe, and *vice versa*. The intensity of the bands detected during immunoblotting varied amongst the organisms. In some strains, minor bands were observed in addition to the major reactive band (fig. 3). These could correspond to modification products of the enzyme, since similar satellite bands have been observed with the purified AAC(6')-1b.⁹ The apparent mol. wts cited in table III are those estimated for the major bands. There were at least two, but possibly more, categories

Table II. Occurrence of the aminoglycoside resistance phenotypes according to the geographical origin of the strains, and fraction of probe-positive strains

Resistance phenotype	Number of strains that reacted with <i>aacA1b</i> probe/total number of strains from				All areas
	Europe	North America	South America	Far East	
Ami ^R Gen ^S Tob ^R	6/7	2/6	5/6	3/5	18/24
Ami ^R Gen ^R Tob ^R	11/35	3/5	0	1/2	15/42
Ami ^R Gen ^R Tob ^S	0/4	0	0	0	0/4
Total	17/46	5/11	5/6	4/7	31/70

Table III. Occurrence of the 6'-*N*-acetyltransferases of type 1b of different sizes in gram-negative bacilli from various geographical areas

Genus	Number of strains from				Number of strains with enzyme of apparent mol. wt	
	Eur	NAm	SAm	FEa	24 000	26 000
<i>Escherichia</i> (8/11)*	4	2	1	1	2	2
<i>Klebsiella</i> (6/6)	1	2	1	2	1	1
<i>Enterobacter</i> (7/10)	4	1	1	1	4	1
<i>Serratia</i> (2/10)	1		1		1	1
<i>Citrobacter</i> (2/8)	1		1		1	1
<i>Proteus</i> (2/2)	2					2
<i>Acinetobacter</i> (2/8)	2					2
<i>Pseudomonas</i> (1/10)	1				†	
<i>Achromobacter</i> (1/1)	1				†	

Eur, Europe; NAm, North America; SAm, South America; FEa, Far East.

* Fraction of isolates that reacted with the *aacA1b* probe and the anti-AAC(6')-1b antibodies.

† These strains contained multiple cross-reactive proteins of mol. wt between c. 19 000 and c. 30 000.

of proteins detected. One type had a mol. wt of c. 24 000, the other had a mol. wt of c. 26 000 (fig. 3). In one strain of *Pseudomonas* and in the strain of *Achromobacter* there were additional cross-reactive proteins with mol. wts of c. 19 000 and 30 000, but these were not studied further. No obvious correlation was observed between the mol. wt of the AAC(6')-1b enzyme, the species and whether the corresponding gene was located on either a plasmid or in the chromosome.

Discussion

We studied the occurrence of the *aacA1b* gene and its product, AAC(6')1b, amongst 70 amikacin-resist-

ant isolates of gram-negative bacilli from South and North America, the Far East and Europe. DNA-DNA hybridisation and immunoblot analyses, showed that the *aacA1b* gene, which was first described from plasmid pAZ007^{9,17} or closely-related genes were widespread geographically as well as amongst different gram-negative genera. The presence of this gene could be correlated with resistance to amikacin in c. 44% of the strains examined. Although the gene was most prevalent in resistant members of the genera *Klebsiella*, *Escherichia* and *Enterobacter* (tables II and III), no strict genus-specificity was observed. This is not surprising since an *aacA1b* gene almost identical to that originally analysed⁹ has subsequently been recognised on the transposon Tn1331.²²

Recently, the gene coding for an AAC(6')-II enzyme

Fig. 2. Autoradiograph of a Southern blot filter showing reaction of the *aacA1b* probe with total undigested DNA from strains of: Lane 1, *Achromobacter*; 2 and 3, *Acinetobacter*; 4 and 5, *Citrobacter*; 6-13, *Escherichia*; 14, *Pseudomonas*; 15-21, *Enterobacter*; 22-27, *Klebsiella*; 28 and 29, *Proteus*; 30 and 31, *Serratia*. The position of the chromosomal DNA (Chr) is indicated. The bands in lanes 2 and 3 were weak, but were clearly visible on the original autoradiograph. Within each genus, the probe-positive strains are arranged in the same order as in fig. 1.

Fig. 3. Immunoblot of total proteins from seven hybridisation-positive *E. coli* strains after reaction with antibody to AAC(6')-Ib. The apparent mol. wts, indicated to the right, were estimated from Coomassie Blue-stained gels containing SDS-PAGE mol. wt standards range 14 400–97 200 (BioRad, 75014 Paris, France). The strains analysed in lanes 1–7 correspond, respectively, to those of lanes 6, 7, 8, 10, 11, 12 and 13 in fig. 2.

from *Pseudomonas aeruginosa* has been sequenced. This gene is common in *Pseudomonas* spp. Its product confers resistance to gentamicin but not amikacin. The deduced amino-acid sequence of AAC(6')-II was c. 80% homologous with that of AAC(6')-Ib.⁴ The AAC(6')-Ib gene probe cross-hybridised with the AAC(6')-II gene²³ and the anti-AAC(6')-Ib antiserum cross-reacted with the AAC(6')-II enzyme. Therefore, it is possible that one of the bands observed in the only *Pseudomonas* strain (table III) that reacted with the anti-AAC(6')-Ib antibody corresponded to AAC(6')-II, rather than AAC(6')-Ib.

The mechanism of resistance in the strains for which no hybridisation was detected, and which did not contain cross-reaction protein, were not investigated. Resistance in these organisms could have been mediated by other, structurally unrelated, 6'-*N*-acetyltransferases such as AAC(6')-Ia¹⁰ or by the chromosomally-encoded acetyltransferase AAC(6')-Ic, which occurs in *Serratia* spp.⁸ AAC(6')-Ia and -Ic are not recognised by the anti-AAC(6')-Ib antibody used

previously¹⁷ and in this study. As mentioned earlier, an APH(3')-VI enzyme might be responsible for amikacin resistance in *Acinetobacter* and in *Providencia* strains.

The perfect correlation observed between the hybridisation and immunoblotting results indicates that the *aacA1b* genes are consistently expressed in the different species. The differences in intensity of the bands containing cross-reactive protein could result either from variations in the level of *aacA1b* gene expression or from variations in the immunological relatedness of the particular enzymes, or both.

Comparison of the three *aacA* nucleotide sequences reported to date,^{9,10,11} in conjunction with previous results¹⁷ and those presented here, reveals two kinds of variation. There is a major variation, reflected in the absence both of cross-hybridisation between DNAs and of immunological cross-reactivity between enzymes of some AAC(6') producers. On the other hand, there is minor variation within at least one set of closely related acetyltransferases, of type Ib, which is reflected in the differences of the apparent mol. wts (fig. 3).

Previously, we have characterised a gene fusion at the 5' end of the *aacA1b* (*aacA4*) gene of plasmid pAZ007. This places expression of the *aacA1b* gene under the control of the regulatory sequences of the β -lactamase gene of Tn3.⁹ This fusion leads to the synthesis of a protein with apparent and deduced mol. wts of c. 24 000 and of 22 400, respectively. The fact that the *N*-terminal region of this acetyltransferase is encoded by an unrelated gene implies the existence of an "original" enzyme which differs from that encoded by plasmid pAZ007 in at least this region. Therefore, variation of the *N*-terminal sequence could be considered as one possible source for the minor variation in mol. wt described here. The occurrence of such heterogeneous enzymes should allow a more precise epidemiological survey of amikacin resistance due to AAC(6')-Ib production when immunoblotting rather than DNA-DNA hybridisation is used.

We are grateful to R. S. Hare, G. H. Miller and K. J. Shaw for discussions and for providing an AAC(6')-II-producing strain. This study was supported by a grant from the Caisse Nationale de l'Assurance Maladie des Travailleurs Salariés (87-3-22-03-E) and from the Institut National de la Santé et de la Recherche Médicale (CJF-89-01). We thank C. Harcour for secretarial assistance.

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