Electric Transmembrane Potential Mutation and Resistance to the Cationic and Amphiphilic Antitumoral Drugs Derived from Pyridocarbazole, 2-*N*-Methylellipticinium and 2-*N*-Methyl-9-hydroxyellipticinium, in *Streptococcus pneumoniae*

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$\Delta\psi$ -reduced *amiA* mutants of *Streptococcus pneumoniae* were shown to be resistant to the positively charged antitumoral drugs 2-*N*-methylellipticinium (NME) and 2-*N*-methyl-9-hydroxyellipticinium (NMHE). Conversely, mutants selected for their resistance to NMHE were mapped within the *amiA* locus and exhibited the pleiotropic *AmiA*[−] phenotype. This shows that $\Delta\psi$ is a critical parameter in determining resistance to these drugs in *S. pneumoniae* and suggests that they are accumulated within this bacterium in response to $\Delta\psi$. As a consequence NME and NMHE appear to be valuable tools for selecting $\Delta\psi$ -reduced mutants in *S. pneumoniae*.

INTRODUCTION

The antitumoral drugs derived from pyridocarbazole, viz. ellipticine and its derivatives 9-hydroxyellipticine, 2-*N*-methylellipticinium (NME) and 2-*N*-methyl-9-hydroxyellipticinium (NMHE), have been shown to interact with DNA (Festy *et al.*, 1971; Le Pecq *et al.*, 1974) as well as with model (Lempereur *et al.*, 1984; Tercé *et al.*, 1983) and natural (Tercé *et al.*, 1983) membranes *in vitro*. Their mechanism of action in both eukaryotic and prokaryotic systems is, however, poorly understood. NME and NMHE are amphiphilic and cationic; therefore a possibility could be that they penetrate cells in response to the electric transmembrane potential, $\Delta\psi$.

The chemiosmotic hypothesis predicts that, for cationic substrates such as NME or NMHE, transport can be driven by $\Delta\psi$ (interior negative). If this is the case, $\Delta\psi$ -reduced mutants should be resistant to NME and NMHE and, conversely, among NME- and NMHE-resistant mutants one would expect to find $\Delta\psi$ -reduced strains. The *amiA* mutants of the Gram-positive bacterium *Streptococcus pneumoniae* selected for their resistance to methotrexate (MTX) have been characterized as $\Delta\psi$ -reduced mutants (Trombe *et al.*, 1984). These mutants thus constitute a valuable tool for determining the effect of $\Delta\psi$ on the transport and the toxicity of NME and NMHE.

METHODS

Media and organisms. These have been described by Trombe (1984).

Mutant isolation. Fifty tubes containing 1 ml growth medium were inoculated with 10^3 wild-type bacteria and incubated at 37 °C until the cell density reached 10^7 bacteria ml^{−1}. Each culture was plated in agar medium containing 2×10^{-3} M-NMHE. A single resistant mutant was retained from each culture.

Resistance determination. Freshly grown bacteria were plated in media containing increasing concentrations of the drugs. The minimal inhibitory concentration (MIC) of drug was determined for each strain. Fresh NMHE solutions were used.

Abbreviations: NME, 2-*N*-methylellipticinium; NMHE, 2-*N*-methyl-9-hydroxyellipticinium (Celiptium); MTX, methotrexate.

Fig. 1. Structure of NME (R = H) and NMHE (R = OH).

Table 1. MICs of ellipticine and its derivatives 9-hydroxyellipticine, NME and NMHE for the wild-type and *amiA9* strains

The data represent the minimal concentrations which do not allow colony formation on plates after 18 h incubation at 37 °C.

	MIC (M)	
	Wild-type	<i>amiA9</i>
Ellipticine	7.5×10^{-6}	7.5×10^{-6}
9-Hydroxyellipticine	1.5×10^{-6}	1.5×10^{-6}
NME	1.5×10^{-5}	1.5×10^{-5}
NMHE	5×10^{-4}	3×10^{-3}

Mapping experiments. These were done according to Sicard (1984). Mutants sensitive to an imbalance in the concentrations of the three branched amino acids, valine, leucine and isoleucine (sensitive to an excess of isoleucine, Iso^S) were transformed, either by chimaeric plasmids bearing wild-type sequences of the *amiA* locus, or by DNA isolated from other mutants with the same phenotype. Wild-type recombinants, resistant to an excess of isoleucine (Iso^R), were selected on plates. Recombination frequency was calculated by reference to the mutation *Str41* (Sicard, 1964). One recombination unit represents 30 DNA base pairs (Claverys *et al.*, 1979).

Chemicals. Ellipticine and 9-hydroxyellipticine were synthesized and purified by P. Lecoigne (Centre de Pharmacologie et Toxicologie Fondamentales du CNRS, Toulouse, France). NME and NMHE (Fig. 1) were synthesized and purified by J. Chenu (SANOFI and Centre de Pharmacologie et Toxicologie Fondamentales du CNRS, Toulouse, France). Methotrexate (MTX) was kindly provided by Specia (16 rue Clisson, 75646 Paris, France).

RESULTS

*Specific resistance of the $\Delta\psi$ -reduced *amiA9* mutant of *S. pneumoniae* to the positively charged derivatives of ellipticine, NME and NMHE*

The MICs of ellipticine, 9-hydroxyellipticine, NME and NMHE were determined for the nonsense mutant *amiA9* (Gasc *et al.*, 1979) and the wild-type strain (Table 1). For both strains the quaternary ammonium derivatives (NME and NMHE) were less inhibitory than ellipticine and 9-hydroxyellipticine. Similar results have been obtained in eukaryotic systems (Paoletti *et al.*, 1979). Mutant *amiA9*, which has a reduced $\Delta\psi$ compared with the wild-type (Trombe *et al.*, 1984), was selectively resistant to NME and NMHE (Table 1). This could reflect the

Table 2. Characteristics of NMHE-resistant mutants compared with the wild-type and *amiA9* strains

	No. of NMHE-resistant mutants	Max. concn (M) allowing 100% colony formation			
		MTX	NMHE	NME	Isoleucine*
Wild-type	—	5×10^{-7}	2.5×10^{-4}	7.5×10^{-6}	2×10^{-2}
<i>amiA9</i>	—	5×10^{-6}	2×10^{-3}	2×10^{-4}	10^{-3}
Group I	13	5×10^{-6}	3×10^{-3}	2×10^{-4}	10^{-3}
Group II	15	5×10^{-7}	3×10^{-3}	2×10^{-4}	2×10^{-2}

* Isoleucine concentrations allowing colony formation in plates of synthetic medium containing 5×10^{-5} M valine and 5×10^{-5} M leucine.

involvement in the resistance mechanism of a decreased drug uptake in response to a decreased $\Delta\psi$. Indeed, NMHE is mutagenic for *S. pneumoniae* (A. M. Sicard, personal communication), implying that the drug penetrates this bacterium. This is reminiscent of data presented by Charcosset *et al.* (1984) which showed that NME uptake by L1210 cells depended on $\Delta\psi$.

Interestingly, *amiA* mutants are also resistant to another antitumoral drug, MTX, which is a folate analogue (Trombe & Sicard, 1975; Trombe *et al.*, 1979, 1984; Trombe, 1984). We therefore determined whether resistance to NME and to NMHE, which was observed for the MTX-resistant *amiA9* strain, was related to folate analogue resistance in general. Isogenic MTX-resistant strains bearing mutations in the different genes involved in folate analogue resistance, i.e. dihydrofolate reductase (Sirotiak *et al.*, 1964; Trombe & Sicard, 1975), thymidilate synthase (Friedman & Ravin, 1972) or *amiA* (Trombe & Sicard, 1975; Trombe *et al.*, 1979, 1984; Trombe, 1984), were tested for their resistance to NME and NMHE. The results obtained (not shown) indicated that resistance to NME and NMHE was not correlated with MTX resistance in general but was specific to the *amiA* mutants. Indeed all *amiA* mutants tested, including point mutations (*amiA9*, *amiA25*, *amiA5*, *amiA19*, *amiA24* and *amiA3*), and deletions (*amiA502* and *amiA504*) (Claverys *et al.*, 1980), which cover the entire locus (Fig. 2), were resistant to NME and NMHE (not shown).

Isolation of NMHE-resistant mutants

To determine whether mutants selected for resistance to NMHE also exhibited the *AmiA*⁻ phenotype, i.e. MTX resistance and sensitivity to an imbalance in the branched amino acids valine, leucine and isoleucine (Trombe *et al.*, 1984), 28 spontaneous, independent NMHE-resistant mutants isolated from the wild-type parent were analysed. All these mutants were as sensitive as the *amiA9* mutant to the four ellipticine derivatives tested (Table 2). These mutants formed two groups with respect to their resistance to MTX and sensitivity to an imbalance in branched amino acids. The results in Table 2 show that 13 of the 28 NMHE-resistant mutants were also MTX-resistant and were as sensitive to an imbalance in branched amino acids as the *amiA9* mutant (group I), while the others, like the wild-type strain, were MTX-sensitive and isoleucine-resistant (group II). In addition, NMHE resistance and MTX resistance of the group I mutants were both transferred by a single transformation event. This suggests that both characters are the result of a single mutation.

Mapping of the pleiotropic NMHE-resistant mutants within the *amiA* locus

To localize the group I mutations described above we transformed each strain with a chimaeric plasmid derived from pBR325 carrying a 5.7 kb *Pst*I/*Eco*RI fragment belonging to the wild-type *amiA* locus (Méjean *et al.*, 1981) (Fig. 2). All but three strains gave wild-type recombinants resistant to an imbalance in branched amino acids. This suggests that all the NMHE-resistant mutants belonging to group I, except these three, lie within this restriction fragment. The mutations were then localized more precisely with plasmids bearing subrestriction fragments. Nine out of a total of ten mutations were located within a 1.8 kb

Fig. 2. Genetic and physical map of the *amiA* locus. Numbers in ordinary type represent mutations selected using MTX; numbers in **bold** type represent mutations selected using NMHE. 502 and 504 are deletions (Claverys *et al.*, 1980).

Table 3. Genetic distances between the mutations located near the *Pst*I restriction site

Genetic distances between two markers are expressed as percentage recombination frequency according to Sicard (1964) (see Methods); 1% recombination represents 30 base pairs (Claverys *et al.*, 1979).

Receptor	Donor			
	<i>amiA1109</i>	<i>amiA1101</i>	<i>amiA1110</i>	<i>amiA142</i>
<i>amiA1109</i>		1.4	1.9	1.2
<i>amiA1101</i>	2.8		5.4	4.3
<i>amiA1110</i>	1.9	1.6		1.1
<i>amiA142</i>	2.4	3.6	1.6	

*Eco*RI/*Eco*RI fragment, the A fragment (Fig. 2); one was within a 1.8 kb *Hind*III/*Eco*RI fragment to the right (Fig. 2). The three mutants which did not recombine with the 5.7 kb *Pst*I/*Eco*RI fragment were crossed with several mutants bearing point mutations which lie outside that fragment. The results (Table 3) show that these three mutants are linked to the mutation *amiA142* located near a *Pst*I site (Vasseghi & Claverys, 1983) on the left of the locus (Fig. 2). Recombination frequencies (Table 3) imply that the four mutations *amiA142*, *amiA1101*, *amiA1109* and *amiA1110*, which are external to the *Pst*I/*Eco*RI fragment, define a fragment of about 0.15 kb. Therefore mutants selected for their NMHE resistance, belonging to group I, are *amiA* mutants. We have previously shown that the high MTX resistance of the *amiA* mutants could not be entirely explained by a decreased uptake of the drug resulting from their reduced $\Delta\psi$ (Trombe, 1985). It appears, rather, that the MTX resistance of the *amiA* mutants is correlated with the resistance of their membrane to hyperpolarization by MTX (Trombe, 1984). Therefore, the *amiA* function is involved in $\Delta\psi$ -generation (Trombe *et al.*, 1984) and in the response of the cytoplasmic membrane to MTX (Trombe, 1984). Interestingly, the distribution of mutations selected using NMHE is quite different from those selected using MTX (Fig. 2). NMHE may thus constitute a tool for the preferential selection of mutants in specific regions of the *amiA* locus. It is possible that these regions code for domains in a protein or for polypeptides specifically involved in the generation of $\Delta\psi$.

DISCUSSION

In the present report we have investigated the role of $\Delta\psi$ in resistance to antitumoral drugs derived from ellipticine, using *S. pneumoniae* as a model system.

The $\Delta\psi$ -reduced *amiA* mutants of *S. pneumoniae* (Trombe *et al.*, 1984) were selectively resistant to the positively charged derivatives of ellipticine NME and NMHE, but were as sensitive to ellipticine and 9-hydroxyellipticine as the wild-type parent (Tables 1 and 2). In addition, a set of NME- and NMHE-resistant mutants exhibited the same phenotype as the *amiA* mutants (Table 2) and mapped within the *amiA* locus (Fig. 2). This shows that $\Delta\psi$ is a critical parameter for NME and NMHE inhibition of *S. pneumoniae* growth, and suggests that

$\Delta\psi$ -driven transport of NME and NMHE is required for these compounds to have an effect. Indeed, experiments with [^3H]NMHE demonstrate a strong accumulation in wild-type cells and significantly less accumulation in the mutants (unpublished results). NME and NMHE have been shown to interact both with DNA (Festy *et al.*, 1971; Le Pecq *et al.*, 1974) and with membrane lipids (Lempereur *et al.*, 1984; Tercé *et al.*, 1983) *in vitro*. Indeed, NMHE is mutagenic for *S. pneumoniae* (A. M. Sicard, personal communication), suggesting interaction with DNA *in vivo*. Such an interaction has been demonstrated in *Salmonella typhimurium* using the fluorescent ellipticine derivative Val-NMHE (René *et al.*, 1985). Experiments are in progress with our model system to determine whether other targets for these compounds exist *in vivo*. In any case, the results in this report show that NME and NMHE constitute valuable tools to screen $\Delta\psi$ -reduced mutants of *S. pneumoniae*.

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