Elimination by Ethidium Bromide of Antibiotic Resistance in Enterobacteria and Staphylococci

By D. H. BOUANCHAUD, M. R. SCAVIZZI AND Y. A. CHABBERT

Pasteur Institute, Paris (XV), France

(Accepted for publication 6 August 1968)

SUMMARY

Ethidium bromide, a trypanocidal drug affecting nucleic acid synthesis, was found to be a powerful agent in eliminating some antibiotic resistance in bacteria. In staphylococci, penicillinase production was eliminated in mercury-resistant organisms, but not in mercury-sensitive ones. Among enterobacteria, two resistance factors showing the same resistance pattern were differently eliminated, and correlation between elimination and transfer of resistance factors was not always observed. F'-lac⁺ factor was also eliminated by ethidium bromide in *Escherichia coli* K12. Elimination of antibiotic resistance was observed generally at high frequency, and could be better reproduced than with acridine dyes.

INTRODUCTION

Many genetic determinants of antibiotic resistance in Enterobacteriaceae and Staphylococcus have been shown to belong to extrachromosomal elements; genetic studies on this subject have been reviewed by Mitsuhashi (1965, 1967), Watanabe (1967) and Novick (1967). These drug-resistance determinants are transmissible to sensitive bacteria independently of chromosomal genes and like the sex factor they can be eliminated by acridine dyes, but generally at low frequencies. Recently, Ikeda, Iijima & Tajima (1967) reported that the sex factor in a male strain of *Escherichia coli* K12 was eliminated by sarkomycin, which was however less active than acridine orange. Elimination of antibiotic resistance at high frequency is of interest to assert extrachromosomal location of genetic determinants, and drugs with better eliminating or 'curing' effect are needed. Previous investigations have shown that ethidium bromide binds to DNA and RNA, and inhibits DNA-polymerase and RNA-polymerase (Waring, 1966). Like the acridines, ethidium bromide was intercalated between base-pairs of DNA. The complex between ethidium bromide and nucleic acids has been described by Lepecq & Paoletti (1967), and biochemical effects of this drug on bacteria were reported by Tomchick & Mandel (1964). The present paper summarizes the effects of ethidium bromide on antibiotic resistance in some multiresistant enterobacteria and staphylococci.

METHODS

Bacteria and culture media. Resistant enterobacteria (obtained from International Centre for Salmonella, Pasteur Institute, and Pasteur Hospital laboratory) were as described by Chabbert & Baudens (1966) and Baudens & Chabbert (1967). *Escherichia coli* K12 54117 F-lac⁺ and *E. coli* K12 C600 F-lac⁻ were provided by Dr E. L. Wollman, and *E. coli* K12 × 200 PS lac⁻ carrying F'-lac⁺ factor by Dr F. Jacob (Pasteur Institute,

Paris). The conjugation conditions used were as described by Watanabe & Fukasawa (1961). Nine strains were used for the present work: *Salmonella oranienburg* LA39.R4 (resistant to Sm Km Cm Tc Su); *S. paratyphi B* LA43.R8 (Sm Cm Su); *S. panama* LA46.R11 (Am Km Tc); *S. paratyphi B* LA47.R13 (Am Sm Cm Tc Su); *E. coli* (111:B4) LA49.R15 (Am Sm Cm Tc Su); *Shigella flexneri* LA56.R22 (Am Sm Cm Tc Su); *S. stanleyville* LA 68.R32 (Am Sm Km Cm Tc Su); *S. oranienburg* LA69.R33 (Am Sm); *S. derby* LA71.R35 (Am Sm Su); *E. coli* KI2 54117.R22; C600.R22; 54117.R15; C600.R15 were obtained by transfer of factors R22 and R15 from *Sh. flexneri* LA56.R22 and *E. coli* LA49.R15 to sensitive strains of *E. coli* KI2.

Eight strains of *Staphylococcus aureus* (coagulase-positive) isolated from clinical material were used. Drug-resistance patterns were: LA72: P Hg Sm Tc Em; LA75: P Sm Km Tc Em; LA78: P Hg Tc Em; LA80: P; LA82: P Hg Sm Tc Em; LA86: P Hg Em; LA87: P Sm Tc Em; LA89: P Hg Sm Km Tc Em. Penicillin resistance determinant was transduced from LA75 to *S. aureus* 209P (ATCC: 65.38P) by phage 53 (NCTC 8406), as previously described (Chabbert, Baudens & Gerbaud, 1964). Code key: Am, ampicillin; Sm, streptomycin; Km, Kanamycin; Cm, chloramphenicol; Tc, tetracycline; Su, sulphonamide; P, benzylpenicillin; Em, erythromycin; Hg, HgCl₂.

Tryptic soy broth (0370 Difco) and tryptic soy agar (0369 Difco) were routinely used, and the effects of different pH values were separately studied.

Chemicals. Ethidium bromide (3,8-diamino-5-ethyl-6-phenyl-phenanthridinium) was a gift from Boots Pure Drug Co., Ltd., Nottingham, Great Britain. Benzylpenicillin, dihydrostreptomycin sulphate and sulphamethoxypyridazine were obtained from 'Specia' Rhône-Poulenc, Paris; ampicillin from Delagrange Laboratories, Paris; chloramphenicol and erythromycin from Roussel Laboratories, Paris; tetracycline hydrochloride from Pfizer-Clin Laboratories, Paris.

Determination of ethidium bromide activity on bacterial growth. Minimal inhibitory concentrations of ethidium bromide were determined by the spot-technique on serial dilutions of drug in nutrient agar, and expressed in molarity. Growth curves in nutrient broth were read at 620 m μ where light adsorption by ethidium bromide was minimal (Tomchick & Mandel, 1964).

Determination of antibiotic resistance. Bacteria (10⁴) were inoculated by the spot-technique, using a modification of Steers's apparatus (1959), on nutrient agar supplemented with the following concentrations of antibiotics: benzylpenicillin, 0.1 i.u./ml.; ampicillin, 20 μ g./ml.; streptomycin, 32 μ g./ml.; chloramphenicol, 20 μ g./ml.; tetracycline, 16 μ g./ml.; erythromycin, 10 μ g./ml. kanamycin, 12.5 μ g./ml.; sulphamethoxypyridazine, 100 μ g./ml.

Resistance to mercuric ion was determined with the sensitivity discs described by Novick (1967).

Elimination of antibiotic resistance. A small inoculum (10⁴ bacteria/ml.) was grown overnight at 37° in nutrient broth containing a subinhibitory concentration of ethidium bromide, giving incomplete inhibition (from 6 to 10 $\times$ 10⁻⁶ M for staphylococci, and from 60 to 2500 $\times$ 10⁻⁶ M for enterobacteria). The culture was plated on agar, and isolated colonies tested for antibiotic resistance. In other cases, 10⁵ bacteria were plated on nutrient agar containing serial dilutions of ethidium bromide and incubated overnight. The plates were flooded with 2 ml. broth. Bacterial suspension were homogenized, and bacteria were re-isolated on agar and tested for drug resistance.

Effect of ethidium bromide on induced synthesis of penicillinase in staphylococci. Exponentially growing staphylococci (strain LA78) were induced for penicillinase synthesis with 1.5×10^{-6} M-methicillin (Novick & Richmond, 1965) and ethidium bromide was added 30 sec. later to give final concentrations 0, 5, 10, 20, 50 and 100×10^{-6} M. Synthesis of penicillinase was stopped at 30 min. by adding chloramphenicol (50 μ g./ml.) to all samples. Bacteria were centrifuged down (20,000 g for 5 min. at 4°), washed and suspended in phosphate buffer (0.1 M, pH 5.8). The extinctions of bacterial suspensions were adjusted at 620 m μ , and various dilutions were assayed iodometrically for penicillinase activity (Perret, 1954).

RESULTS

Elimination of antibiotic resistance in enterobacteria. Typical results are shown in Table 1, and others will be reported separately. All drug-resistance determinants in *Salmonella oranienburg* LA39.R4, *Shigella flexneri* LA56.R22, *Escherichia coli* K12 54117.R22 and *E. coli* K12 C600.R22 were eliminated at a high frequency. Spontaneous loss of complete drug resistance was low, and no loss of isolated resistance characters was observed. No significant differences in the elimination of resistance factor R22 were observed in the originally resistant *Sh. flexneri* LA56.R22, and *E. coli* K12 54117.R22 and C600.R22. In *S. derby* LA71.R35 (resistant to ampicillin, streptomycin, sulphonamide) ampicillin resistance was suppressed in 100 % of the organisms by ethidium bromide treatment, but streptomycin and sulphonamide resistances were not modified. The determinant for ampicillin resistance was easily transferred to *E. coli* K12 54117, but determinants for streptomycin and sulphonamide resistance were not transferred.

Kanamycin resistance was significantly eliminated in *Salmonella panama* LA46.R11, change in tetracycline resistance was not significant, and loss of ampicillin resistance was not observed. It has been reported that kanamycin resistance was significantly eliminated by acriflavine in this strain (Baudens & Chabbert, 1967). Nevertheless, all resistance characters were transferred jointly or separately to sensitive bacteria.

With *Salmonella stanleyville* LA68.R32, *S. paratyphi B* LA47.R13, and *S. oranienburg* LA69.R33, spontaneous loss of single resistance characters was observed, but the frequency was not significantly increased by ethidium bromide treatment. In *S. paratyphi B* LA43.R8 and *Escherichia coli* LA49.R15, no elimination of antibiotic resistance was observed.

Elimination of F'-lac⁺ factor by ethidium bromide. *Escherichia coli* K12 $\times$ 200 PS lac⁻ carrying F'-lac⁺ factor was treated by ethidium bromide, and organisms were isolated on Drigalski medium. Ethidium bromide, like acridine dyes (Hirota & Iijima, 1957), eliminated F'-lac⁺ factor: 20 % of tested colonies were unable to ferment lactose. No spontaneous loss of this factor was observed in controls (104 colonies).

Elimination of antibiotic resistance in staphylococci. Frequencies of loss of resistance in nine strains of staphylococci are shown in Table 2. Penicillin resistance was eliminated at high frequency (8 to 100 %) in five multiresistant strains. These five strains were resistant to mercuric chloride, and mercury resistance was co-eliminated in all penicillin-sensitive colonies. Induced synthesis of penicillinase in *Staphylococcus aureus* LA78 was partially inhibited at 50×10^{-6} M and completely inhibited at 100×10^{-6} M-ethidium bromide. With four strains, no elimination of penicillin resistance was ob-

Table 1. *Elimination by ethidium bromide of antibiotic resistance from enterobacteria*

Organisms	Lost characters	Frequency				Control		
		Plus ethidium bromide						
		Conc. (10 ⁻⁶ M)	No. exa- mined colonies	No. sensi- tive colonies	%	No. exa- mined colonies	No. sensi- tive colonies	%
<i>S. oranienburg</i> LA39. R4 (Sm Km Cm Tc Su)	Sm Km Cm Tc Su	625	154	75	49	309	0	0
<i>Sh. flexneri</i> LA56. R22 (Am Sm Cm Tc Su)	Am Sm Cm Tc Su	60	156	120	77	104	3	2.9
<i>E. coli</i> K12 54117. R22 (Am Sm Cm Tc Su)	Am Sm Cm Tc Su	1300	104	34	32	134	1	0.7
<i>E. coli</i> K12 C600. R22 (Am Sm Cm Tc Su)	Am Sm Cm Tc Su	75	104	73	71	104	4	3.9
<i>S. derby</i> LA71. R35 (Am Sm Su)	Am	2500	104	104	100	96	2	2.0
<i>S. panama</i> LA46. R11 (Am Km Tc)	Km Tc Km Tc	625 390 390	390 390 390	0 61 5	0 15.6 1.2	554 554 554	5 44 5	0.9 7.9* 0.9

* Corrected $\chi^2 = 10.0$ significant ($P = 0.01$).Table 2. *Elimination by ethidium bromide of antibiotic resistance from mercury-resistant and mercury sensitive staphylococci*

Strains and original drug resistance	No. exa- mined colonies	Lost characters		Control*		Strains and original drug resistance	No. exa- mined colonies	Lost characters	Control*
		P	Hg	P	Hg				
LA72 (P Hg Sm Tc Em)	130	97 (75 %)	97 (75 %)	0	0	LA75 (P Sm Km Tc Em)	104	0	0
LA78 (P Hg Tc Em)	104	104 (100 %)	104 (100 %)	0	0	LA80 (P)	134	0	0
LA82 (P Hg Sm Tc Em)	252	82 (32 %)	82 (32 %)	0	0	209P (ATCC 65. 38) (P)	104	0	0
LA86 (P Hg Em)	138	75 (54 %)	75 (54 %)	0	0	LA87 (P Sm Tc Em)	104	0	0
LA89 (P Hg Sm Km Tc Em)	52	4 (8 %)	4 (8 %)	0	0				
							Mercury sensitive		
							Mercury resistant		

* 104 colonies were tested for all controls.

served. These strains were multiresistant or resistant to benzylpenicillin only, but all mercury sensitive.

Relations between inhibitory activity and resistance elimination by ethidium bromide. As observed with acridine and other basic dyes, staphylococci were more sensitive to ethidium bromide than were the enterobacteria examined. All strains of *Staphylococcus aureus* were inhibited at low concentrations (8 to 12×10^{-6} M), but very important differences in minimal inhibitory concentrations were observed with the enterobacteria: 75×10^{-6} M for *Shigella flexneri* LA56.R22, and 3125×10^{-6} M for *Salmonella derby* LA46.R33. Subinhibitory concentrations with maximal eliminating effects are given in Table 1. The relation between ethidium bromide concentration, inhibition of growth and elimination of resistance in *S. aureus* LA78 and *Sh. flexneri* LA56.R22 are shown in Fig. 1.

Fig. 1. Relations between ethidium bromide concentration, inhibition of growth and percentage of antibiotic sensitive colonies in *Staphylococcus aureus* LA78 and *Shigella flexneri* LA56.R22. O --- O, Percentage of normal growth in overnight culture (turbidimetric reading at $620\text{ m}\mu$); x --- x, percentage of sensitive organisms.

Effects of light, manganese concentration and pH value on growth and elimination of resistance. The inhibitory activity of ethidium bromide on growth was significantly increased when organisms were incubated in serial concentrations of ethidium bromide in the presence of light (40 cm. from a white lamp of 100 W); the growth of *Staphylococcus aureus* LA78 was inhibited at 7×10^{-6} M (11×10^{-6} M in absence of light). At subinhibitory concentration of ethidium bromide in the presence of light, 50 to 100 % of the bacteria lost antibiotic resistance. Although it has been shown that 0.33 mM-manganese sulphate antagonized ethidium bromide induced growth inhibition (Tomchick & Mandel, 1964), no effect of manganese was observed on the elimination of antibiotic resistance in *Shigella flexneri* LA56.R22. Effects of different pH values on growth and elimination of resistance are shown in Table 3.

Absence of ethidium bromide selective effect. The possibility of selection by ethidium bromide of spontaneously sensitive bacteria was studied in *Shigella flexneri* LA56. Growth of an originally resistant strain carrying factor R22 and of a 'cured' sensitive

strain was inhibited at exactly the same concentration of ethidium bromide (75×10^{-6} M). There was no significant difference in growth rates of these strains with 60×10^{-6} M ethidium bromide, which eliminates factor R22 (Fig. 1). Identical results were obtained by Watanabe & Fukasawa (1961) with acridine dyes and according to them, a selective effect of ethidium bromide can be excluded.

Table 3. *Effects of variations of pH value on elimination by ethidium bromide of resistance to penicillin and mercury from Staphylococcus aureus LA78*

Nutrient broth was adjusted to various pH values with N-HCl or N-NaOH; ethidium bromide was added at final concentration of 8 and 5×10^{-6} M; 104 colonies were tested in each case.

pH value	Ethidium bromide, 8×10^{-6} M		Ethidium bromide, 5×10^{-6} M	
	Growth*	% of sensitive colonies	Growth*	% of sensitive colonies
6.8	+	4	+	0
7.0	±	20	+	0
7.2	±	50	+	0
7.4	±	35	+	0
7.6	±	20	+	0
7.8	0	—	±	4

* Normal growth, +; partial growth, ±; no growth, 0.

DISCUSSION

It is reported here that ethidium bromide eliminates some antibiotic resistance determinants and the F'-lac⁺ factor. Elimination of R factors by acridine dyes in Enterobacteriaceae has been reported by Mitsuhashi, Harada & Kameda (1961), and Watanabe & Fukasawa (1961); the frequencies of elimination were generally low, particularly in *Escherichia coli*. In our experiments, factors R4 and R22 were eliminated by ethidium bromide at high frequencies, from originally resistant strains and from two strains of *E. coli* K12 to which factor R22 had been transferred. The bacteria became sensitive to all the drugs tested, and in contrast to the results of Mitsuhashi *et al.* (1961), no difference was observed in elimination of factor R22 in *Shigella flexneri* and *E. coli* K12. With *Salmonella derby* LA71, only one isolated resistance character was eliminated at a very high frequency (100%). In this strain, only this suppressible character was transferred to sensitive bacteria. Conversely, no elimination of resistance characters was observed with *E. coli* LA49.R15 and *S. paratyphi B* LA43.R8 carrying R factors transferable to sensitive *E. coli* K12. Factor R15 was successively transferred to *E. coli* K12 C600 F'-lac⁻ and to *E. coli* K12 54117 F'-lac⁺; 90 to 100% of sensitive recipient cells became resistant in each case. Although factor R15, transferred at very high frequency was apparently extra-chromosomal and de-repressed (Meynell & Datta, 1967), ethidium bromide did not eliminate factor R15 from originally resistant *E. coli* LA49.R15, or from *E. coli* K12 54117 and C600 carrying R15. The influence of recipient strains can be excluded in this system, because factor R22 (showing the same resistance pattern as R15) was eliminated at high frequency from originally resistant *Sh. flexneri* and *E. coli* K12 54117.R22 and C600.R22.

It has been previously shown that with some strains of *Staphylococcus aureus*, penicillin resistance and erythromycin resistance were eliminated by acridine dyes, separately or jointly, at generally low frequencies: 0.1 to 3.5 % (Hashimoto, Kono & Mitsuhashi, 1964), 0.2 to 0.5 % (Harmon & Baldwin, 1964) and 0.6 to 3.9 % (Baudens, Gerbaud & Chabbert, 1965) for penicillin resistance; 2 to 8 % (Mitsuhashi, Morimura, Kono & Oshima, 1963) for erythromycin and other macrolides resistance; 0.1 to 3.5 % (Mitsuhashi, Hashimoto, Kono & Morimura, 1965) for joint elimination of penicillin and erythromycin resistances. Novick (1963) reported that no elimination of antibiotic resistance by acriflavine was observed with his strains.

Penicillin resistance was eliminated by ethidium bromide in five multiresistant strains of *Staphylococcus aureus*, at higher frequencies (8 to 100 %); 'curing' concentrations of ethidium bromide (5 to 10×10^{-6} M) were lower than acriflavine concentrations reported by different workers (50 to 100×10^{-6} M). Elimination of penicillin resistance was observed at a concentration which did not inhibit penicillinase synthesis. This finding agrees with previous results showing that protein formation was relatively unaffected by ethidium bromide (Tomchick & Mandel, 1964). Also, in our experience, penicillin sensitivity remained stable in absence of ethidium bromide.

The five strains of *Staphylococcus aureus* in which penicillin resistance was eliminated were mercury resistant, and mercury resistance was always co-eliminated with penicillin resistance. It has been reported that these two markers were co-transduced and co-eliminated spontaneously or by acriflavine (Richmond & John, 1964; Asheshov, 1966). In four strains of *S. aureus*, penicillin resistance was not eliminated. These strains were resistant to penicillin or to several antibiotics, but were sensitive to mercury salts. Chromosomal location for penicillin resistance determinants, not eliminated by acriflavine or high temperature, has been suggested (Asheshov, 1966).

Although it has been reported by Miller & Harmon (1967) that genetical determinants controlling penicillin and mercury resistance in one strain of *Staphylococcus aureus* were co-transduced with chromosomal determinant responsible for methionine synthesis, it seems that in our strains, mercury resistance is generally associated with 'curable' and probably extra-chromosomal penicillin resistance determinants.

In strains of *Staphylococcus aureus* where penicillin and mercury resistances were eliminated at high frequencies (8 to 100 %), no elimination of erythromycin resistance was observed. There is no close linkage of penicillin and erythromycin resistance determinants in these strains, which seem to offer a different system from those previously described by Novick (1967).

The partially selective effect of acriflavine and ethidium bromide on extra-chromosomal DNA synthesis remains unclear. It was reported (Lepecq & Paoletti, 1967) that there was no difference in ethidium bromide fixation on DNA of different origin, but that the linear or circular form of DNA affects drug fixation (Hudson & Vinograd, 1967). Although there are some differences in drug penetration in different strains of Enterobacteriaceae, resistant organisms grown on high concentrations of ethidium bromide contained large amounts of drug, and it can be suggested that differences in DNA polymerase and RNA polymerase sensitivities are responsible for differences in ethidium bromide sensitivity of bacterial strains.

The present work has shown that ethidium bromide is a powerful drug in eliminating

some resistance factors at high frequency with excellent reproducibility, and would appear to be a useful tool for further study of the lack of elimination of other resistance factors.

The authors thank Mr G. R. Gerbaud for excellent technical assistance.

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