Curing Gonococci of B-Lactamase production with Ethidum, Acridius orange or Acriflavine

Pages with reference to book, From 171 To 174

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Abstract

In 12 B-lactamase producing strains of N gonorrhoeae tested, the coding for enzyme production was sensitive, to varying degrees, to one or more of the three agents - ethidium bromide, acridine orange, or acriflavine. The substances tested were able to eliminate the plasmid responsible for B-lactamase production but the time required varied. The Far East type of gonococci (plasmid 4.4 Md) were cured in under a week whereas the Liverpool/Ghana type (plasmid 3.2 Md) required more than 7 days treatment. (JPMA35 171, 1985).

Introduction

The recognition of B-lactamase producing Neisseria gonorrhoeae[1,3] gave rise to worldwide interest and led to the recognition of similar strains in many different countries. The way in which gonococci acquired the ability to produce B-lactamase is yet to be determined. However, all the f B-lactamase producing strains of gonococci studied so far possess plasrnids of either 3.2 Md or 4.4 Md[4,5]
It is now well established that antibiotic resistance factors are often lost following exposure of cultures to either acridine dyes or ethidium bromide. Resistance factors in Escherichia coli were eliminated by treatment with acridine dyes.[6] Harmon & Baldwin[7] showed that treatment of B-lactamase producing strains of staphylococci with acridine orange removed the resistance factors, as did acriflavine[8].
Ethidium bromide, a trypanocidal drug was found to be a powerful agent in removing some bacterial antibiotic resistance factors[9]. Jyssum[10] reported that both ethidium bromide and acriflavine were mutagenic, and both seemed to act preferentially at the replication point of the chromosome.
The effects of these three known active compounds. ethidium bromide, acridine orange and acriflavine, on B-lactamase producing strains of N gonorrhoeae have been studied and are reported here.

Material and Methods

Strains. Twelve B-lactamase producing strains of N gonorrhoeae were studied and included both the Liverpool/Ghana and Far East/ American types. Strains B_1, B_2 B_4 and B_6 were provided by Serum Institute, Copenhagen; R_1, R_{47} and HKI by Jephcott, Public Health Laboratory, Bristol; L_6, L_7 and strain by Public Health Laboratory, Liverpool. Finally, AN31 a fully sensitive strain was isolated and only became a B-lactamase producer after exposure to low concentrations of ethidium bromide. [11]
Strains were received as freezedried cultures, constituted with phosphate buffered saline (PBS) pH 7.2, cultured on solid media and stores in the vapour phase of liquid nitrogen.
Confirmation of identity and of production of B- lactamase. Confirmation of the strains examined as N. gonorrhoeae was obtained by Gram's stain, oxidase reaction and carbohydrate utilization.
The production of B-lactarnase was tested for with nitrocefin.[12]
Media. The initial cultures and the subsequent subcultures from ANM[13] were made on G.C. medium

base (Difco) with 2% (v/v) defined supplement.[14]
Reagents. Their source was as follows: benzyl penicillin (Glaxo Ltd.), nitrocefin (Glaxo Ltd.), ethidium bromide (Boots Ltd.), acridine orange (BDH) and acriflavine (BDH). All were of the highest grade of purity available.
Preparation of testsuspensions. The test suspension for each strain was prepared from a supplement G.C. medium agar culture harvested after 18 to 20 h incubation at 36°C in an atmosphere of 10% carbon dioxide and enhanced humidity, suspended in PBS pH 7.2 and the suspension standardized to Brown's opacity tubç $No._8$. The suspension thus prepared contained 108 colony forming units ml^{-1} and 0.01 ml formed the inoculum for 10 ml ANM.
Assessment of plasmid elimination
From the stock solutions containing 4pg ml^{-1} ANM of either ethidium bromide, acridine orange or acriflavine a series of concentrations, i.e. 4,2, 0.8, 0.2 and 0.1 p g ml^{-1}, was prepared. These concentrations together with ANM alone, as control, were dispensed in 10 ml amounts into universal containers and sterilized by autoclaving at 10 lb p.s.i. (115°C) for 10 mm. For each strain tested a set of dilutions of each substance together with ANIM controls were inoculated in duplicate with 0.01 ml test suspension and all were incubated at 36°C. for 40 days. One set was studied by the following regime while the other set was cultured only when curing was observed in the first set.
Day 30 transfer- subcultured
daily for 10
days; i.e.serial cultures rejuvenated every 10th day
At intervals of 10 days 1 ml from each tube in a series of concentrations was transferred to the corresponding concentration in a fresh series of 9 ml vol.
The broths were sub-cultured every 24 hours onto solld medium using a standard loop (0.02 ml) to give isolated colonies and the resulting growths were tested for B-lactamase production by flooding the plates with nitrocefmn and examining individual colonies for the development of brick red colour for B-lactamase producing colonies while failure to develop the colour was taken as cured. The control cultures gave a positive reaction in all the individual colonies, while the cured strains gave 10% negative reaction to nitrocefin a day prior to giving 100% cure. The second set was cultured when 10% cure was observed and gave results identical to those obtained with the first set. All the controls and cured strains were analysed for plasmids.
The cured cultures were tested for penicilin sensitivity by the plate dilution method[15]
Plasmid Analysis. The plasmid profile of the 12 original and the cured strains of N gonorrhoeae was determined by the agarose gel electrophoretic method.[16]

Results and Discussion

The time taken to eliminate the plasmid responsible for the production of B-lactamase ranged from 3 to 35 days, and the concentration of the agents required varied from 0.1 to 2pg ml^{-1} Ethidium bromide was most effective in eliminating the B-lactamase producing plasmid of N gonorrhoeae; 9/12 strains being cured by this agent compared with 5/12 by acridine orange and 2/12 by acriflavine. Of the 12 strains only strain R_1 could not be cured by either ethidium bromide, or acridine orange, it was however cured by acriflavine. Strain B6 was cured by all three agents (Table 1).

Table Elimination of N. gonorrhoeae of the Plasmid coding for β–Lactamase by Ethidium Bromide Acridine Orange and Acriflavine.

Strain	Origin	Type	Time (days) and concentration ($\mu g ml^{-1}$) required for curing by					
			Ethidium bromide		Acridine orange		Acriflavine	
			Time	Conc'n	Time	Conc'n	Time	Conc'n
L6	LIVERPOOL	LG*	24	0.4	40	NC‡	40	NC
L7	Liverpool	LG	14	0.8	10	2	40	NC
RI	Liverpool	LG	40	NC	40	NC	35	2
B7	Liverpool	LG	40	NC	12	0.4	40	NC
AN31	Sheffield	LG	14	2	40	NC	40	NC
BI	Switzerland	LG	10	0.2	40	NC	40	NC
B2	Sweden	LG	4	0.1	4	0.2	40	NC
HK1	Hong Kong	FEA+	3	0.4	40	NC	40	NC
B3	Singapore	FEA	40	NC	10	0.4	40	NC
B6	Belgium	FEA	6	0.1	6	0.1	6	0.8
R47	Manchester	FEA	7	0.4	40	NC	40	NC
B4	Holland	FEA	20	0.4	40	NC	40	NC

* LG, Liverpool/Ghana, Carrying a 3.2 Md plasmid.
\+ FEA, Far East/American, carrying a 4.4 Md plasmid.
\+
\+ Not cured.

Plasmid analysis showed that in every case where curing was achieved it could be demonstrated by the absence of plasmid DNA, and that the coding for B-lactamase production had also been lost.
Low conctent rations of the three agents tested certainly had a curative effect on gonococcal B-lactamase production because the strains became sensitive to benzyl penicillin and gave a negative reaction with nitrocefin. Plasmid analysis of the strains suggests that ethidium bromide and acridine dyes eliminate the 3.2 and 4.4 Md plasmids which are known to carry the genetic code for B-lactamase production in N. gonorrhoeae although the other plasmids (2.6 and 24 Md) of which the 2.6 one is present in all gonococci and the 24 Md one in some were not affected.
We were unable to show any partial curing in which some organisms in a culture were plasmid bearing and others were not. When the change was produced it occurred in all organisms.
Examination of totally to demonstrate plasmids. Although the simply detected the expression of the plasmidcoding for the production of B-lactamase when non B-lactamase strains were subjected to plasmid analysis they were found to be without the relevant plasmids.
The other plasmids observed in gonococci, i.e. 2.4. and 24 M dal., which are not associated with B-lactamase production, remain present in strains which have reverted to penicillin sensitivity. Their function is at present unknown.

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