Mutation of gonococci and meningococci *in vitro* to rifampin (rifampicin) resistance

H. SCHNEIDER, A. S. DOBEK, AND M. S. ARTENSTEIN

Department of Bacterial Diseases, Division of Communicable Diseases and Immunology,
Walter Reed Army Institute of Research, Washington, D.C. 20012, U.S.A.

Rifampin (rifampicin) is a semisynthetic antibiotic, derived from the rifamycins which are produced by *Streptomyces mediterranei*. A number of reports have described its use in the treatment of acute gonococcal infection with cure rates varying from 89 to 93 per cent. (Arya, Rao, and Nnochiri, 1971; Ongom, 1971; Panduro, 1971; and Willcox, Morrison, and Cobbold, 1970). In several reports, some of the treatment failures have responded to a second treatment with rifampin. *In vitro* studies of gonococcal strains have shown minimal inhibitory concentrations of rifampin to be less than 0.4 µg./ml., well within the range considered to be attainable in blood and urine after standard dose regimens (Dans, McGehee, Wilcox, and Finland, 1970).

Since problems related to rifampin resistance have been met with in other bacterial infections (Atlas and Turck, 1968; Beam, Newberg, Devine, Pierce, and Davies, 1971), the following study of rifampin resistance *in vitro* in gonococci was undertaken.

Methods

BACTERIAL STRAINS

Of the thirteen gonococcal strains used, ten were recent isolates from patients attending a local clinic; all were characterized bacteriologically as *Neisseria gonorrhoeae*. Three other strains (F-62, 7134, 13) were obtained from Dr. D. Kellogg, Center for Disease Control, Atlanta, Ga. Type 1 colonies of all gonococcal strains were stored in the lyophilized state within three to five passages after arrival in the laboratory. Eleven meningococcal strains were recent isolates from nasopharyngeal carriers; three strains were isolated from the cerebrospinal fluid of patients with meningitis. The latter strains were characterized by methods previously described (Evans, Arntstein, and Hunter, 1968). Agar plate cultures of all strains were grown at 37°C, in a humidified candle extinction jar. Broth cultures of meningococci were grown aerobically in a 37°C. waterbath shaker.

MEDIA

GC agar base (Difco) supplemented with Isovitalex (BBL) (GCI agar) was used for routine cultivation of all gonococcal strains. For isolation of strains from clinical

cases of gonorrhoea, GCI agar was further supplemented with the inhibitory antibiotic formulation, VCN (BBL). Mueller-Hinton (MH) agar and broth (Difco) were used to cultivate the meningococcal strains, and Eugon broth (BBL) was used as a suspension and dilution medium for both the gonococci and meningococci.

Rifampin used for rifampin-containing media was provided as a powder by the Pitman-Moore Division, Dow Chemical Co., Indianapolis, Indiana. Rifampin-containing GCI and MH selection agars were made, using a freshly prepared rifampin solution diluted so as to attain the desired final agar concentrations.

SELECTION AND ANALYSIS OF RIFAMPIN-RESISTANT MUTANTS

For the gonococci, each strain was heavily seeded onto four GCI agar plates, incubated for 5 to 6 hrs and then harvested using a total of 12 ml. Eugon broth. The harvests were plated in 0.1 ml. amounts onto GCI 0.4 µg./ml. rifampin selection agar both directly and after 10-fold concentration. After 48 hrs' incubation the rifampin-resistant colonies were counted and then analysed for resistance to higher levels of rifampin resistance by replication onto GCI agar containing 6.25 and 100 µg./ml. rifampin.

Meningococci were grown in MH broth at 37°C. in a waterbath shaker to an optical density of 0.20, using a Coleman Jr. spectrophotometer (equivalent to $5-6 \times 10^8$ cells/ml.). The meningococcal broth cultures were plated directly and after 10-fold concentration onto MH-0.4 µg./ml. rifampin selection agar and mutants analysed for higher levels of rifampin resistance by replication onto MH agar containing higher concentrations of rifampin. Estimates of the viable numbers of each strain applied to selection plates were made by plating serial dilutions of the plate harvests or broth cultures onto appropriate media without rifampin.

Results

Mutation rates for resistance to rifampin were determined for thirteen strains of gonococci. As shown in Table I, mutants resistant to 0.4 µg./ml. rifampin appeared at rates varying from 1×10^{-8} to 8.5×10^{-9} . Analysis of mutants of six of the thirteen strains for resistance to higher rifampin concentrations showed that a relatively large proportion were resistant to 6.25 µg./ml., and many to as much as

100 µg./ml., 400 times more rifampin than was present in the original selection medium (Table II). These data indicate that the selective pressure exerted by a low concentration of rifampin resulted in the single-step selection of mutants resistant to high rifampin concentrations.

TABLE I Rates of mutation to 0.4 µg./ml. rifampin resistance among thirteen gonococcal strains

Strains	Mutation rate
HR	1.0×10^{-8}
DA	1.6×10^{-8}
WL	1.9×10^{-8}
JO	3.0×10^{-8}
7134	1.0×10^{-9}
JA	1.0×10^{-9}
HU	1.5×10^{-9}
TO	1.8×10^{-9}
CR	2.7×10^{-9}
SH	5.2×10^{-9}
F62	5.5×10^{-9}
DO	5.7×10^{-9}
13	8.5×10^{-9}

TABLE II Analysis of 0.4 µg./ml.-resistant gonococcal mutants for resistance to higher concentrations of rifampin

Strain	No. of mutants tested	Rifampin concentration (µg./ml.)			
		6.25		100	
		No.	Per cent. ^a	No.	Per cent. ^a
13	57	17	30	14	25
F62	22	22	100	14	67
DA	110	28	25	7	6
CR	30	16	53	10	33
TO	32	2	6	2	6
7134	37	25	68	6	16

^aPercentage of mutants tested

The results of similar studies on fourteen strains of meningococci are shown in Tables III and IV. The rates of mutation observed at 0.4 µg./ml. rifampin varied from 1×10^{-7} to 1×10^{-8} . Compared to the gonococci, these mutation rates were greater by at least a factor of 10. Analysis of these mutants gave results similar to the results from the gonococcal mutants; a large proportion were resistant to 6.25 µg./ml. rifampin and many were resistant to 100 µg./ml.

Discussion

Mutation of gonococci *in vitro* to resistance to low concentrations of the antibiotic rifampin occurred at rates as high as 1×10^{-8} . Under similar conditions

TABLE III Rates of mutation to 0.4 µg./ml. rifampin resistance among fourteen strains of meningococci

Strain	Source	Serogroup	Mutation rate
NP-6	NP ^a	29E	1.0×10^{-7c}
NP-40	NP	29E	1.0×10^{-7c}
5319	CSF ^b	C	1.2×10^{-7}
NP-28	NP	C	1.4×10^{-7}
NP-42	NP	29E	1.5×10^{-7}
4857	CSF	C	1.5×10^{-7}
NP-94	NP	C	3.7×10^{-7}
NP-64	NP	29E	6.2×10^{-7}
4860	CSF	C	8.9×10^{-7}
NP-39	NP	C	1.0×10^{-8}
NP-97	NP	C	2.0×10^{-8}
NP-16	NP	C	2.3×10^{-8}
NP-12	NP	29E	3.0×10^{-8c}
NP-1	NP	C	3.0×10^{-8}

^aNasopharynx

^bCerebrospinal fluid

^cStrain resistant to 0.4 µg./ml. rifampin; rate calculated on numbers of mutants observed at 1.6 µg./ml.

TABLE IV Analysis of 0.4 µg./ml. resistant meningococcal mutants for resistance to higher concentrations of rifampin

Strain	No. of mutants tested	Rifampin concentration (µg./ml.)			
		6.25		100	
		No.	Per cent. ^a	No.	Per cent. ^a
4857	100	24	24	18	18
4860	51	47	90	42	82
NP-1	75	55	73	18	24
NP-15	206	50	24	0	0
NP-39	126	89	71	10	8

^aPercentage of mutants tested

rifampin-resistant meningococcal mutants were found to occur at rates at least ten times greater. Significantly, selection of mutants of both species at low (0.4 µg./ml.) concentrations of rifampin resulted in the selection of many mutants that were resistant to as much as 100 µg./ml. rifampin.

Clinical studies with rifampin have shown the frequent emergence of rifampin-resistant organisms. In the treatment of urinary tract infections, Atlas and Turck (1968) found that rifampin-resistant *Escherichia coli*, *Proteus mirabilis*, and *Pseudomonas* emerged and persisted in the urines of patients during treatment. Recovery of rifampin-resistant tubercle bacilli from a small number of the patients was reported by Vall-Spinoza, Lester, Moulding, Davidson, and McClatchy (1970).

Perhaps more pertinent to the present study of gonococci is the experience with rifampin in the chemoprophylaxis of meningococcal nasopharyngeal carriers. After the initial report of Deal and Sanders

(1969) of the efficacy of rifampin in eradication of the carrier state, a number of larger field studies were undertaken. In each study where post-treatment meningococcal strains were tested for rifampin sensitivity, resistant strains were found to have emerged (Beam and others, 1971; Devine, Johnson, Rhode, Pierce, Hagerman, and Peckinpaugh, 1971; Eickhoff, 1971; Guttler, Counts, Avent and Beaty, 1971; Weidmer, Dunkel, Pettyjohn, Smith, and Leibovitz, 1971). Indeed, in one study, transmission of rifampin-resistant meningococci was detected (Beam and others, 1971). However, systemic disease due to such strains did not occur in these limited trials.

On the basis of these experiences and with the knowledge that resistant gonococcal mutants are easily detected *in vitro*, it seems likely that clinically significant resistance to rifampin can be expected if the drug is employed widely for treatment of gonococcal infections. It appears desirable that any future clinical studies of rifampin therapy of gonorrhoea be designed so that gonococci isolated from treatment failures can be investigated for rifampin resistance.

Summary

Rifampin-resistant mutants were detected *in vitro* in thirteen strains of gonococci by selection on agar containing low concentrations of the antibiotic. Mutation rates varying from 1.0×10^{-8} to 8.5×10^{-9} were observed. Many of the mutants were found to be resistant to very high concentrations of rifampin, indicating that the selective pressure exerted by low concentrations of rifampin resulted in the selection of single-step high-concentration resistant mutants. Similar studies of fourteen meningococcal strains showed that resistant mutants occurred at rates up to ten times greater. These results suggest that gonococcal resistance to rifampin is likely to become a clinically significant problem if rifampin therapy comes into wide usage.

We thank Dr. G. Landmann who provided the opportunity for us to obtain strains from her patients and Dr. D. Kellogg who provided several other gonococcal strains. We also thank Mr. E. Dixon for his competent technical assistance.

References

- ATLAS, E., and TURCK, M. (1968) *Amer. J. med. Sci.*, **256**, 247
- ARYA, O. P., RAO, S. K., and NNOCHIRI E. (1971) *Brit. J. vener. Dis.*, **47**, 184
- BEAM, W. E., NEWBERG, N. R., DEVINE, L. F., PIERCE, W. E., and DAVIES, J. A. (1971) *J. infect. Dis.*, **124**, 39
- DANS, P. E., MCGEHEE, R. F., WILCOX, C., and FINLAND, M. (1970) *Amer. J. med. Sci.*, **259**, 120
- DEAL, W. B., and SANDERS, E. (1969) *New Engl. J. Med.*, **281**, 641
- DEVINE, L. F., JOHNSON, D. P., RHODE, S. L., PIERCE, W. E., HAGERMAN, C. R., and PECKINPAUGH, R. O. (1971) *Amer. J. med. Sci.*, **261**, 79
- EICKHOFF, T. C. (1971) *J. infect. Dis.*, **123**, 414
- EVANS, J. R., ARTENSTEIN, M. S., and HUNTER, D. H. (1968) *Amer. J. Epidemiol.*, **87**, 643
- GUTTLE, R. B., COUNTS, G. W., AVENT, C. K., and BEATY, H. N. (1971) *J. infect. Dis.*, **124**, 199
- MALMBORG, A. S., MOLIN, L., and NYSTRÖM, B. (1971) *Chemotherapy*, **16**, 319
- ONGOM, V. L. (1971) *Brit. J. vener. Dis.*, **47**, 188
- PANDURO, J. (1971) *Ibid.*, **47**, 440
- VALL-SPINOSA, A., LESTER, W., MOULDING, T., DAVIDSON, P. T., and MCCLATCHY, J. K. (1970) *New Engl. J. Med.*, **283**, 616
- WEIDMER, C. E., DUNKEL, T. B., PETTYJOHN, F. S., SMITH, C. D., and LEIBOVITZ, A. (1971) *J. infect. Dis.*, **124**, 172
- WILCOX, R. R., MORRISON, G. D., and COBBOLD, R. J. C. (1970) *Brit. J. vener. Dis.*, **46**, 145

Addendum

In a recent report which came to our attention after submission of this manuscript, Malmberg, Molin, and Nyström (1971) reported 25 (12.5 per cent.) failures after treatment of 200 cases of gonorrhoea with a single oral dose of 900 mg. rifampin. Eleven of the 25 gonococcal strains isolated from these treatment failures at the time of their first follow-up visit were found to be rifampin-resistant. In only one of the eleven cases was the strain isolated before treatment found to be rifampin-resistant. In one instance they observed the transmission of a post-treatment rifampin strain to another contact.

Mutation *in vitro* des gonocoques et des méningocoques vers la résistance à la rifampin (rifampicin)

SOMMAIRE

Des mutants-résistants à la rifampin furent trouvés *in vitro* dans 13 souches de gonocoques, par sélection sur gélose contenant de faibles concentrations de l'antibiotique. On observa des taux de mutation allant de $1,0 \times 10^{-8}$ à $8,5 \times 10^{-9}$. Beaucoup de mutants furent trouvés résistants à de très hautes concentrations de

rifampin, ceci indiquant que l'effet sélectif exercé par de faibles concentrations de rifampin avait entraîné, après un seul contact, une haute proportion de mutants résistants. Des études similaires sur 14 souches de méningocoques montrèrent que les mutants-résistants

atteignaient des taux jusqu'à 10 fois plus grands. Ces résultats suggèrent que la résistance du gonocoque à la rifampin est susceptible de devenir un réel problème clinique si le traitement par la rifampin devient largement employé.