ACTIVITY OF ERYTHROMYCIN AGAINST STAPHYLOCOCCUS AUREUS

BY

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Erythromycin is a new antibiotic derived from *Streptomyces erythreus*. Its chemical and physical characteristics were described by McGuire *et al.* (1952), and its biological properties were investigated by the same workers and by other groups (Haight and Finland, 1952a, 1952b; Heilman *et al.*, 1952). Erythromycin was found to be active *in vitro* in very low concentrations against a wide range of Gram-positive organisms, but was inactive against Gram-negative organisms with the exception of *Neisseria*, *Haemophilus influenzae*, *Haemophilus pertussis*, and strains of *Brucella*.

Erythromycin, which can be given orally, has low toxicity for man and laboratory animals; it gives satisfactory concentrations in the blood and urine but not in the cerebrospinal fluid of normal persons (Heilman *et al.*, 1952). The ability of erythromycin to protect mice against experimental infections was found to parallel the sensitivity *in vitro* of the infecting organism and to be related to the blood concentration of the drug (McGuire *et al.*, 1952). Clinical trials of erythromycin have been reported by Heilman *et al.* (1952) and by Haight and Finland (1952c). Favourable results have been obtained in various streptococcal, pneumococcal, and staphylococcal infections. Erythromycin has been shown to be particularly effective against staphylococci, whether the strains were penicillin-sensitive or penicillin-resistant (McGuire *et al.*, 1952).

There is an increasing emergence of strains of *Staphylococcus aureus* which are resistant to penicillin, streptomycin, and to one or more of the "broad-spectrum" antibiotics. The high incidence, especially in hospitals, of infections caused by such strains makes it highly desirable to have a new antibiotic at our disposal which is effective against staphylococci. Since it seems likely that this will be the chief potential role of erythromycin, the present report is devoted to laboratory experience of the drug's activity against pathogenic staphylococci.

Materials and Methods

A supply of pure crystalline erythromycin (potency 915 µg./mg.) was provided through the courtesy of Abbott Laboratories. A stock solution of the white powder was made in distilled water in a concentration of 2 mg./ml. To aid solution a little ethyl alcohol was used. This stock solution could be stored in the frozen state at -20°C for as long as eight weeks.

Many of the strains of *Staph. aureus* used were recently isolated from patients with staphylococcal infections or from normal nasal or oral flora. Stock laboratory strains were also included.

Sensitivity tests were performed with serial dilutions of erythromycin either by a tube method with digest broth as the medium or by a plate method (modified from May and Morley, 1952) with Levinthal agar. Five-hour broth cultures of organisms to be tested were obtained by picking individual colonies from

the growth on blood-agar plates. In the tube test the final concentration of antibiotic ranged from 20 to 0.01 µg./ml. The inoculum was estimated to yield a final concentration of 5×10^3 organisms per ml. The sensitivity of the organism was taken to be the minimum final concentration of antibiotic that completely inhibited growth as judged by visible turbidity after 18-24 hours' incubation.

The inoculum in the plate test was one 2-mm. loopful of the broth culture (approximately 10^5 organisms). The sensitivity of the strain was expressed as the concentration of antibiotic which completely inhibited growth of the organism. The sensitivity of staphylococci to other antibiotics was estimated by a plate method (May and Morley, 1952).

In the production of resistant variants of originally sensitive strains of staphylococci two methods were employed. The first was that of Haight and Finland (1952e) in which the organism was subcultured serially at 48-hourly intervals on a series of 10% blood-agar plates containing increasing concentrations of erythromycin. The inoculum was one loopful of a broth suspension of the growth from the plate with the highest concentration of antibiotic allowing good growth. The second method was a modification of that of Barber (1953). The organism was subcultured serially on erythromycin ditch plates. These were of 10% blood agar with a ditch cut at one side of the plate and filled with the same medium containing at first 20 and later 100 µg. of erythromycin per ml. Subcultures were made from the edge of growth nearest to the ditch.

In both methods each parent strain was subcultured on antibiotic-free medium at the same intervals as the derivative strain.

Result of Experiments

Factors Influencing *in vitro* Sensitivity

The sensitivity of *Staph. aureus* G 1 was not affected by variations in the size of the bacterial inoculum, ranging from 5×10^4 to 50 organisms, in either the tube or the plate method. The effect of continued incubation was slight. In the tube test, incubated for 24 hours, *Staph. aureus* G 1 was inhibited by 0.25 µg. of erythromycin per ml. After 48-72 hours' incubation slight growth was visible in this tube. No new growth was visible in tubes containing higher concentrations of antibiotic.

It has been shown by Knox *et al.* (1952) that in sensitivity tests of mycobacteria with isoniazid the end-point is more unstable at some temperatures than at others, and that results obtained after arbitrary periods of incubation may vary widely. In tube sensitivity tests of *Staph. aureus* with erythromycin, variation of the temperature of incubation over a range 26-40°C. did not affect the results previously obtained.

The sensitivity of four strains of *Staph. aureus* was not affected by performing the tube test with broth to which 25% serum had been added. Plate tests with blood agar or nutrient agar in place of Levinthal agar gave identical results.

In comparative tests of 50 strains of *Staph. aureus* by the tube and plate method the results were identical with 40 strains. The sole discrepancy in the remaining 10 cases was that a scanty growth was observed on the plate corresponding in erythromycin concentration with the minimum inhibitory concentration in the tube test. On continued incubation this growth became complete, but no growth developed in plates containing higher concentrations of antibiotic.

The plate method has been adopted for routine sensitivity testing of large numbers of strains of *Staph. aureus*. Prepared

plates maintain unimpaired erythromycin potency for over two weeks after preparation if the plates are kept in the refrigerator until required.

Growth of Staphylococci in Varying Concentrations of Erythromycin

A five-hour broth culture of *Staph. aureus* G 1 was added in a final concentration estimated at 5×10^7 organisms per ml. to serial broth dilutions of erythromycin and to a tube of broth containing no antibiotic. After varying periods of incubation at 37° C., 0.1-ml. samples were taken from each tube and bacterial counts were made by the method of Miles, Misra, and Irwin (1938). At daily intervals the whole of 1-ml. samples was plated out from the tubes containing 4, 1, and 0.5 µg. of erythromycin per ml. The results are shown graphically in Fig. 1. With increasing concentrations of erythromycin the number of viable organisms re-

FIG. 1.—Effect of various concentrations of erythromycin (µg./ml.) on the growth of *Staph. aureus* G 1.

mained stationary for decreasing periods of time and then declined progressively. There was no growth in 4 or 1 µg./ml. after 24 hours. With 0.5 µg./ml. complete sterilization was not achieved even after 72 hours. At lower concentrations of erythromycin the number of organisms remained almost stationary for long periods or fell slightly and then began to rise. In the case of 0.1 µg./ml. the bacterial count was almost the same as in the control culture after 24 hours.

Sensitivity of a Large Number of Strains of *Staph. aureus*

A total of 213 strains were tested. All strains were coagulase-positive and fermented mannite. The distribution of sensitivity of staphylococci to erythromycin is shown in Table I. Thirty-three strains of *Staph. aureus* of known phage type were selected. These fell into each of the three main phage groups. There was no difference in erythromycin sensitivity between any of these strains. All were sensitive to 0.25–0.5 µg./ml. The sensitivity of 47 strains of

TABLE I.—Sensitivity of 213 Strains of *Staph. aureus* to Erythromycin (Plate Method)

	Minimum Inhibitory Concentration of Erythromycin (µg./ml.)					
	> 1	0.5	0.25	0.1	0.05	0.01
No. of strains sensitive to each concentration of antibiotic	0	157	49	6	1	0
Percentage of strains sensitive to each concentration	—	100	26.5	3.5	0.5	—

Staph. aureus to penicillin, streptomycin, chloramphenicol, and oxytetracycline ("terramycin") was tested. The strains selected included penicillin-resistant and penicillin-sensitive organisms and strains with varying combinations of resistance to other antibiotics. All were sensitive to 0.25–0.5 µg. of erythromycin per ml. whatever their behaviour to any of the other antibiotics. The results are shown in Fig. 2.

Resistance to Erythromycin

It is believed that resistance to antibiotics may develop by mutation of organisms in a "sensitive" strain to resistant forms. On contact with the antibiotic these resistant forms will be selected out. By serial subculture in the presence of antibiotic it is thus possible to develop strains whose antibiotic resistance is much greater than that of the parent. Two patterns of resistance have been described: (a) the penicillin pattern, in which resistance is gradual and "step-wise," depending on a series of small mutations; and (b) the streptomycin pattern, in which "one step" resistance of a high order can occur owing to selection of highly resistant variants in the original culture.

From an overnight culture of *Staph. aureus* G 1 a sample was taken for bacterial count and 1-ml. aliquots were spread evenly over the surface of 10% blood-agar plates containing erythromycin. Various concentrations of antibiotic were tested in separate experiments, 15 plates being used each time. The average inoculum was estimated at 2×10^8 organisms. No growth was discovered on plates containing 100, 50, 20, 10, or 5 µg. of antibiotic per ml. There was confluent growth on control plates with no antibiotic. In an experiment with 1 µg./ml., six plates gave no growth, and on eight plates a small number of colonies (2–10) appeared. On plates containing 0.5 µg. of erythromycin per ml. a variable number of colonies appeared on all plates.

These colonies could represent either mutants present in the culture or organisms which had undergone adaptation in some way during their contact with erythromycin. To decide this point the fluctuation test designed by Luria and Delbrück (1943) and adapted for work with antibiotics by Demerec (1948) was performed. From a single culture of staphylococcus G 1, small inocula (approximately 300 bacteria) were subcultured into 16 bottles of broth. After overnight incubation 1 ml. of each of 15 broth cultures was plated on to blood-agar plates containing 0.5 µg. of erythromycin per ml. From the 16th broth culture 15 samples were similarly plated (approximately 2×10^8 organisms per plate). After incubation the number of colonies on each plate was counted. The variation between plates was 15 times greater in the series of independent cultures than in the series of replicate platings of a single culture. These findings suggest that erythromycin-resistant bacteria in a culture of a sensitive organism arise spontaneously by mutation. If these bacteria originated by a process of adaptation when the culture and the antibiotic came into contact, a similar number of colonies would occur on all plates whatever the origin of the sample plated.

FIG. 2.—Comparative sensitivity of 47 strains of *Staph. aureus* to five antibiotics, tested by a plate method (May and Morley, 1952).

Development of Erythromycin-resistant Strains

Fourteen strains of staphylococci sensitive to 0.25–0.5 $\mu\text{g.}$ of erythromycin per ml. were serially subcultured by the method of Haight and Finland (1952e), as described above. All the strains increased in erythromycin resistance rapidly. Two strains grew on plates containing 80 $\mu\text{g.}/\text{ml.}$ after only

FIG. 3.—Changes in antibiotic resistance occurring during repeated subcultures of four strains of staphylococci on blood agar containing erythromycin.

five subcultures. The behaviour of four strains during 12 transfers is shown in Fig. 3. The parent strains subcultured on antibiotic-free agar retained their original sensitivity.

The resistant variants of all 14 strains of staphylococci have shown no significant change in resistance to erythromycin after 10 subcultures on antibiotic-free medium.

Metabolic Effects of Acquired Erythromycin Resistance

The 14 parent strains of staphylococci and their resistant variants were observed for colonial appearance, mannite fermentation, and coagulase production. The phage-pattern of six strains and their resistant variants was examined. None of these characteristics was changed following development of erythromycin resistance. The only

FIG. 4.—Growth curves of *Staph. aureus* G 82 and its derivative strain G 82v resistant to 80 $\mu\text{g.}$ of erythromycin per ml.

difference noted was that growth of resistant variants was less active than growth of the parent strain. The growth in broth of *Staph. aureus* G 82 and its variant resistant to 80 $\mu\text{g.}/\text{ml.}$ was estimated turbidimetrically. The growth rate of the resistant variant was slower than that of the parent strain (Fig. 4). Each resistant variant was examined to exclude the possibility that acquired resistance might be associated with the production of an erythromycinase. The test employed for this purpose was a modification of Got's plate test for penicillinase (Haight and Finland, 1952d). There was no evidence of erythromycinase production.

The 14 staphylococci and their resistant variants were tested for their sensitivity to penicillin, streptomycin, chloramphenicol, and oxytetracycline. In no case was the development of resistance to erythromycin associated with the development of cross-resistance to any of the other antibiotics. During the course of this investigation a sample of a new antibiotic, carbomycin ("magnamycin"), was obtained through the courtesy of Messrs. Charles Pfizer, Ltd. The antibacterial activity of this agent has been described by Tanner *et al.* (1952) and by English *et al.* (1952). The range of activity of carbomycin closely resembles that of erythromycin. Cross-resistance between the two antibiotics has been reported by Finland *et al.* (1952). Four of the staphylococci used in the present investigation, and erythromycin-resistant variants selected at various levels of erythromycin-sensitivity, were tested against the two antibiotics by the plate method already described. It will be seen from Table II that increase in resistance to erythromycin was accompanied by an increase in resistance to carbomycin. Each strain tested required two to four times as much carbomycin, weight for weight, as erythromycin to produce complete inhibition.

TABLE II.—Cross-resistance Between Erythromycin and Carbomycin

Strain of Staphylococcus	Sensitivity of Strain ($\mu\text{g.}/\text{ml.}$)	
	Erythromycin	Carbomycin
G 3	0.25	1.0
G 3v	10.0	40.0
G 6	0.5	5.0
G 6v	40.0	160.0
G 5	0.25	1.0
G 5v	80.0	160.0
G 7	0.5	0.5
G 7v	10.0	20.0

Three strains of staphylococci were subcultured serially on blood-agar plates containing erythromycin in a lateral ditch. There was a similar progressive increase in resistance, but no colonial variants were seen of the type described by Barber (1953) in her study of penicillin- and streptomycin-resistant staphylococci, nor were any erythromycin-dependent variants isolated.

Discussion

Laboratory tests with erythromycin have shown that it may be readily adapted to standard procedures used routinely for other antibiotics. The tube dilution and the agar-plate dilution methods of testing gave comparable results. Growth curves of *Staph. aureus* in varying concentrations of erythromycin show that concentrations of antibiotic higher than the minimum inhibitory concentration cause a reduction in the number of viable organisms, and may sterilize the culture, whereas with lower concentrations the organisms escape from the inhibitory effect of erythromycin after a period of bacteriostasis.

In the present study the sensitivity of 47 strains of *Staph. aureus* to erythromycin was shown to be independent of the pattern of sensitivity to four other antibiotics. Even strains which were resistant to penicillin, streptomycin, chloramphenicol, and oxytetracycline were completely inhibited by 0.5 $\mu\text{g.}$ of erythromycin per ml. Sensitivity to erythromycin was also shown to be independent of phage type of the strain.

Resistance of staphylococci to erythromycin developed readily *in vitro* but appeared to follow the "stepwise" pattern of acquired resistance to penicillin rather than that of streptomycin. From the experiments described above it would appear that a culture of a sensitive strain of staphylococcus may contain spontaneous mutants which are more resistant to erythromycin. Mutants resistant to high levels of erythromycin were not found in these experiments. However, by serial subculture on media containing erythromycin progressive increase of resistance occurred in 14 strains of staphylococci tested. These strains differed from each other in the speed of development of resistance, but in no case was the first "step" to a resistance more than four times that of the original strain. Apart from some reduction in rate of growth, the biological characteristics and phage pattern of the resistant variants were unchanged. Haight and Finland (1952e) observed that development of resistance was associated with loss of coagulase activity. This finding has not been confirmed in the present study or by later experience of the same group of workers (Purcell *et al.*, 1953). Resistant variants were not found to have developed cross-resistance to any other antibiotic except carbomycin. Similar experiences have been reported by Haight and Finland (1952e) and Finland *et al.* (1952). The fact that originally sensitive strains of staphylococci rapidly develop resistance *in vitro* and also *in vivo* (McGuire *et al.*, 1952) is a potential drawback to the use of erythromycin. However, recent work (Purcell *et al.*, 1953) shows that *in vitro* combinations of erythromycin with penicillin or with streptomycin will delay or depress the emergence of resistance to both erythromycin and penicillin or streptomycin.

Summary

Methods for sensitivity testing of staphylococci with erythromycin are described. Factors affecting these tests are discussed.

The sensitivity to erythromycin of a large series of strains of *Staph. aureus* is shown to be of the order of 0.5 µg./ml., and to be independent of their phage type or their behaviour to four other antibiotics.

The effect of erythromycin on the growth rate of staphylococci has been shown to vary according to the concentration of antibiotic. Concentrations above the minimum inhibitory concentration produce a fall in the number of viable organisms.

The development in staphylococci of resistance to erythromycin appears to follow a stepwise pattern resembling that seen with penicillin.

Resistant staphylococci were found to differ from the erythromycin-sensitive parent organism only by slight differences in growth rate and by the acquisition of cross-resistance to carbomycin.

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REFERENCES

- Barber, Mary (1953). *J. gen. Microbiol.*, **8**, 111.
 Demerec, M. (1948). *J. Bact.*, **56**, 63.
 English, A. R., Field, M. F., Szendy, Sophia R., Tagliani, Nancy J., and Fitts, R. A. (1952). *Antibiot. and Chemother.*, **2**, 678.
 Finland, M., Wilcox, Clare, Wright, S. S., and Purcell, E. M. (1952). *Proc. Soc. exp. Biol., N.Y.*, **81**, 725.
 Haight, T. H., and Finland, M. (1952a). *Ibid.*, **81**, 175.
 — (1952b). *Ibid.*, **81**, 188.
 — (1952c). *New Engl. J. Med.*, **247**, 227.
 — (1952d). *Amer. J. clin. Path.*, **22**, 806.
 — (1952e). *Proc. Soc. exp. Biol., N.Y.*, **81**, 183.
 Heilman, F. R., Herrell, W. E., Wellman, W. E., and Geraci, J. E. (1952). *Proc. Mayo Clin.*, **27**, 285.
 Knox, R., King, M. B., and Woodroffe, R. C. (1952). *Lancet*, **2**, 854.
 Luria, S. E., and Delbrück, M. (1943). *Genetics*, **28**, 491.
 McGuire, J. M., Bunch, R. L., Anderson, R. C., Boaz, H. E., Flynn, E. H., Powell, H. M., and Smith, J. W. (1952). *Antibiot. and Chemother.*, **2**, 281.
 May, J. R., and Morley, C. W. (1952). *Lancet*, **1**, 636.
 Miles, A. A., Misra, S. S., and Irwin, J. O. (1938). *J. Hyg., Camb.*, **38**, 732.
 Purcell, E. M., Wright, S. S., and Finland, M. (1953). *Proc. Soc. exp. Biol., N.Y.*, **82**, 124.
 Tanner, F. W., jun., English, A. R., Lees, T. M., and Routien, J. B. (1952). *Antibiot. and Chemother.*, **2**, 441.

SOME LESSONS FROM 4,000 UTERO-TUBAL INSUFFLATIONS*

BY

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Personal experience of just over 4,000 utero-tubal insufflations has taught several practical lessons. The foremost lesson, in my opinion, concerns the gas employed and its safety.

Rubin's Test

Utero-tubal insufflation was first described by Rubin in 1920, so that more than 30 years have elapsed to permit the evaluation of a large number of these tests by investigators all over the world and the accumulation of recorded complications and untoward sequelae. It must not be overlooked, of course, that details of mishaps and even fatalities might pass unpublished. An illustration of this is to be found in the report of the meeting of the Section of Obstetrics of the Royal Academy of Medicine in Ireland on November 7, 1947, in which a speaker made the bald statement that he knew of eight deaths in Ireland following the Rubin test. Nevertheless, a study of the replies to Rubin's (1947) detailed questionnaire, returned by 356 gynaecologists and involving 80,376 insufflations, must supply the data to answer the more important questions, including the safety of the procedure. Among all these insufflations, embolism occurred in 15 patients, of whom 3 died. These deaths are additional to the 10 known post-insufflation deaths from embolism reported in the literature; Frommolt (1925), 1 case; Moench (1927), 2 cases; Mansfeld and Dudits (1934), 1 case; Weitzman and Cohen (1937), 1 case; Dible *et al.* (1938), 1 case; Feiner (1942), 1 case; Finn (1945), 1 case; and Faulkner (1946), 2 cases. In all these fatal cases air or oxygen was used; in no instance is there a record of death from embolism following insufflation with carbon dioxide. Non-fatal but alarming clinical effects, in varying degrees, have been observed by many investigators, but these are rarely seen with the use of carbon dioxide.

At the Royal Samaritan Hospital for Women, Glasgow, insufflation has been employed for nearly 25 years, and at no time has any gas other than carbon dioxide been used. The test has been carried out by various members of the staff on an aggregate of over 6,500 occasions. My personal share of these experiences amounts to just over 4,000 insufflations. None of us has seen any evidence of embolism and there certainly has been no fatality. It is reasonable, therefore, to conclude from accumulated clinical experience that carbon dioxide is the gas of choice. Moreover, it must be pointed out that Rubin, in more than 10,000 insufflations personally carried out, with the exception of approximately the first 100 cases in which he used oxygen (a procedure which was quickly abandoned by him because of the disadvantage that it takes 24–100 hours to be absorbed from the peri-

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