RESISTANCE OF PROPIONIBACTERIA TO ANTIBIOTICS USED IN THE TREATMENT OF ACNE

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SUMMARY. Strains of propionibacteria resistant to clindamycin or clindamycin and erythromycin were isolated from four patients with acne, three of whom were receiving clindamycin. Four strains of *P. acnes* and one of *P. granulosum* with moderate levels of tetracycline resistance were isolated from 25 patients with acne being treated with tetracycline. A similar increase in tetracycline resistance was achieved by training sensitive strains *in vitro*. *P. acnes* was sensitive to sulphonamide and trimethoprim but some strains of *P. granulosum* were resistant to sulphonamide. Similar reports of clindamycin and erythromycin resistance from the USA suggest resistance may be increasing in isolates from patients with acne.

INTRODUCTION

Antibiotics are used with considerable success in the treatment of patients with acne, although not all respond. The propionibacteria that are part of the normal bacterial flora of the skin, *Propionibacterium acnes* in particular, have been implicated in the pathogenesis of acne, especially in the inflammatory stages of the disease (Marples, Downing and Kligman, 1971; Cunliffe *et al.*, 1981; Holland, Ingham and Cunliffe, 1981).

Treatment with antibiotics may reduce the number of propionibacteria and this correlates with reduced levels of free fatty acids and reduction in the severity of disease. Treatment with tetracycline or minocycline by mouth in normal therapeutic doses reduced the numbers of *P. acnes* in skin washings and was accompanied by clinical improvement (Marples and Kligman, 1971; Leyden, McGinley and Kligman, 1982). However, clinical improvement, with a fall in free fatty acid levels (Cunliffe *et al.*, 1973) but without reduction in bacterial numbers, also occurred in patients with mild or moderate acne on low-dose tetracycline therapy, which suggests that inhibition of lipase activity that is responsible for the production of free fatty acids (Marples *et al.*, 1971) rather than reduction of bacterial numbers was an important factor in successful therapy (Cove, Cunliffe and Holland, 1980). A marked fall in numbers of *P. acnes* in comedones occurred with clindamycin but not erythromycin therapy although there was clinical improvement with both agents (Resh and Stoughton, 1976; Stoughton, 1979).

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Studies of antibiotic sensitivity of large numbers of propionibacteria have indicated that this group of organisms is generally sensitive to a wide range of antibacterial agents, including those used for acne therapy (Martin, Gardner and Washington, 1972; Chow, Patten and Guze, 1975; Tally *et al.*, 1975; Wang *et al.*, 1977; Hoffer, Niederau and Pulverer, 1980). This contrasts with the sensitivity of coagulase-negative staphylococci on the skin that are often resistant to one or more antibiotics (Corse and Williams, 1968). Some isolates of *P. acnes* have slightly raised levels of resistance to tetracycline compared with the majority (Chow *et al.*, 1975) and Gould and Cunliffe (1978) reported the isolation of tetracycline-resistant strains during clinical treatment although the total number isolated over a 10-year period was small. Recently, reports from the USA have indicated that clindamycin- and erythromycin-resistant strains can be isolated from patients undergoing therapy. Crawford *et al.* (1979) isolated clindamycin- and erythromycin-resistant strains of *P. acnes* from comedones of about 20% of patients who were using topical lotions containing 1% (w/v) of either drug. Resistant strains (MIC > 25 µg/ml) have also been isolated from previously untreated acne patients (Guin, Huber and Gierlerak, 1979) including one strain of *P. granulosum* resistant to tetracycline, erythromycin and clindamycin and a strain of *P. acnes* resistant to tetracycline.

We have looked for resistant strains of propionibacteria in patients undergoing antibiotic therapy for acne and in some individuals without acne. Patients had been on antibiotic therapy for 4 months or more and, as far as possible, were those who had not responded well to therapy. It was assumed that resistant strains might be found in this group of patients.

MATERIALS AND METHODS

Bacterial strains. A total of 253 strains of propionibacteria were isolated from 69 subjects (table I). The 28 patients receiving antibiotic therapy included 20 patients attending the Dermatology Clinic at Guy's Hospital and eight patients from St John's Hospital. Swabs from the patients at St John's Hospital were kindly provided by Dr W. C. Noble. Strains isolated from children were provided by Ms K. Nördstrom, Institute of Dermatology, London. *P. acnes* NCTC737 was used as a reference strain.

Media. Blood agar (BA) plates (Tissue Culture Services) were used routinely for cultures. Peptone-yeast-glucose medium with cysteine 0.1% (w/v) as a reducing agent and adjusted to pH 6.9 (PYGC), was used as broth or solidified with agar (Oxoid no. 1 or New Zealand agar, 1% w/v). Carbohydrate media for fermentation tests were PYGC containing 1% (w/v) of the

TABLE I
Identity and source of strains of propionibacteria tested for antibiotic sensitivity

Group of subjects	Number of subjects	Number of strains isolated and tested			
		<i>P. acnes</i>	<i>P. granulosum</i>	<i>P. avidum</i>	Total
Acne patients on antibiotic therapy	28	165	7	4	176
Acne patients untreated	2	7	1	0	8
Non-acne patients	10	31	5	0	36
Normal children	29	...	...	...	33
Total	69	203	13	4	253

carbohydrate in 10 ml-volumes in standard test tubes to a depth of about 6.5 cm. PYGC and media for fermentation tests were based on the Anaerobe Laboratory Manual (Holdeman and Moore, 1972). Plates were incubated in an anaerobic jar (Gaz Kit, Oxoid). Liquid cultures containing cysteine were incubated aerobically unless otherwise stated. DSTA (Tissue Culture Services) was used for disk sensitivity tests and DST (Oxoid) agar or PYGC for MIC tests.

Sampling methods. Skin washings of the forehead or back were taken with a glass cylinder (34 mm diameter) held against the skin (Williamson and Kligman, 1965); 2 ml of Triton X-100 0.1% in 0.1M phosphate buffer (pH 7.9) were introduced and the skin surface rubbed for 1 min with a teflon rod with a flattened end. The fluid was removed, the procedure repeated and the two samples pooled. Samples were plated on non-selective medium and incubated anaerobically for 4–5 days at 37°C.

Identification. After three single-colony sub-cultures for purification, propionibacteria were recognised as characteristic gram-positive rods that failed to grow aerobically on blood agar. Strains were identified as *P. acnes*, *P. granulosum* or *P. avidum* on the basis of the following tests: acid from glucose, sucrose, maltose or sorbitol, aesculin hydrolysis, indole production, nitrate reduction and gelatin liquefaction (Cummins and Johnson, 1974). *P. acnes* isolates were agglutinated by *Corynebacterium acnes* antiserum No. 554 (Difco).

Storage. Bacteria were maintained on nutrient medium under anaerobic conditions. Strains were stored on glass beads at –70°C (Feltham *et al.*, 1978) as follows: 3–5-day cultures were emulsified in PYGC broth containing glycerol 10% (w/v) to give a dense suspension of *c.* 10⁹ cfu/ml. A small volume was used to moisten the surface of several glass beads in a screw-capped glass vial. Excess culture suspension was removed and the vials stored at –70°C. Each bead carried about 10⁶ bacteria and viability was well maintained for the 18-month test period. Viable counts showed a fall of about 10-fold in this time.

Determination of minimum inhibitory concentrations. Doubling dilutions of tetracycline, erythromycin (supplied by Lilly Research Laboratories) or clindamycin were made in PYGC agar and of sulphamethoxazole or trimethoprim in DST agar. Antibiotic-containing media were inoculated with a multiple inoculator loaded with a 48-h liquid culture diluted 1 in 1000 in saline to give semi-confluent growth on the antibiotic-free control plates. Lysed horse blood (5–7%) was added to DST agar. Plates were incubated for 4 days at 37°C. The MIC was the concentration that gave a greater than 50-fold reduction in growth compared with the control plate, or complete inhibition of growth; the latter was the most common end-point. Erythromycin and clindamycin were dissolved and diluted in ethyl alcohol; other antibiotics were dissolved in distilled water. The range of concentrations was 0.048–100 µg/ml. *P. acnes* NCTC737 was included as a standard sensitive strain in each series of tests. Occasionally MIC values varied by two-fold in tests on different occasions; with sulphamethoxazole and trimethoprim the variation was as much as four-fold.

Training experiments. Volumes (0.5 ml) of 2-day PYGC cultures were inoculated into a series of tubes of arithmetic or doubling dilutions of antibiotic and incubated for 5–7 days at 37°C. Volumes of 0.5 ml from tubes containing the highest concentration of antibiotic that allowed greater than 50% growth when compared visually with the control, were inoculated into a further series of antibiotic-containing media. When there appeared to be an increase in resistance of the culture, MIC determinations were made directly from 1000-fold dilutions of cultures. Cultures were checked for purity and tested for growth in anaerobic but not aerobic conditions on blood agar, colonial appearance and antibiotic-sensitivity pattern.

RESULTS

Up to 10 colonies of propionibacteria were isolated from washings taken from patients and tested for antibiotic sensitivity. It is possible that all the isolates from an individual were of the same strain because the tests used for identification did not distinguish between strains of a species. Antibiotic sensitivity tests showed some differences in resistance between individual isolates from the same patient; therefore, several colonies from each patient were screened. MIC determinations were

performed on all isolates. With a few exceptions, most strains were sensitive to the five antibiotics tested (table 2). The 33 strains of propionibacteria isolated from children were all sensitive to the five antibiotics.

Tetracycline and minocycline sensitivity

Some strains of *P. acnes* and *P. granulosum* showed a small but reproducible increase in level of resistance (MIC 3.1 µg/ml) to tetracycline (table II). These strains were isolated from patients who had been treated with tetracycline for 4 months or longer with the exception of one patient who had received tetracycline therapy intermittently.

Moderately resistant strains of *P. acnes* were isolated from four such patients and of *P. granulosum* from one. However, strains with increased resistance were not isolated from the remaining 20 patients receiving similar courses of tetracycline. Tetracycline-resistant strains were not found amongst 77 strains of propionibacteria from 12 adults and 29 children who were not receiving therapy.

Attempts were made to increase the level of tetracycline resistance of moderately resistant or sensitive strains by successive culture in liquid medium containing tetracycline. With strains showing moderate levels of tetracycline resistance, very little or no increase in resistance was achieved after nine sub-cultures, but the MIC of normally-sensitive strains (MIC 0.39–0.78 µg/ml) could be increased to 6.25 µg/ml by similar passage. It was also observed that strains were able to grow in liquid medium with concentrations of tetracycline about four-fold higher than would be expected from their MIC in solid media. When MIC tests in liquid culture were incubated under anaerobic conditions the MIC values were very similar to those obtained under aerobic conditions (table III).

MIC's of minocycline were about four-fold lower than tetracycline MIC values

TABLE II

Minimum inhibitory concentration of antibiotics for strains of propionibacteria isolated from subjects with and without acne

Antibiotic	MIC (µg/ml)	Number of test strains with the given MIC			MIC for reference strain <i>P. acnes</i> NCTC737
		<i>P. acnes</i> (n=203)	<i>P. granulosum</i> (n=13)	<i>P. avidum</i> (n=4)	
Tetracycline	0.39–0.78	179	8	4	0.39–0.78
Clindamycin	3.12	24	5		
	≤0.048–0.097	184	11	4	0.048–0.097
	0.39	4			
	6.25	4			
Erythromycin	>100.00	11	2		
	≤0.048–0.097	192	11	4	0.048–0.097
	>100.0	11	2		
Sulphamethoxazole	0.78–3.12	201			0.78–3.12
	6.25–12.5		6	4	
	>100.0		7		
Trimethoprim	0.39–1.56	201			0.39–1.56
	0.78–3.12		13	4	

TABLE III

Minimum inhibitory concentrations of tetracycline for three test strains in aerobic and anaerobic conditions of incubation

Strain	MIC of tetracycline ($\mu\text{g/ml}$) for the given strain		
	in broth with		in agar with anaerobic incubation
	aerobic incubation	anaerobic incubation	
<i>P. acnes</i> NCTC737 Tet* Sm‡	25.0	6.25	6.25
<i>P. acnes</i> Tet†	25.0	3.12	3.12
<i>P. acnes</i> NCTC737 Sm‡	3.12	1.56	0.78–1.56

* After passage through tetracycline-containing broth.

† Tetracycline-resistant clinical isolate.

‡ High-level streptomycin-resistant mutant.

with 17 strains of *P. acnes* and two strains of *P. granulosum* with low or moderate sensitivity to tetracycline. Strains with a tetracycline MIC of 0.39–0.78 $\mu\text{g/ml}$ had minocycline MIC's of 0.097–0.195 $\mu\text{g/ml}$ and strains with a tetracycline MIC of 3.12 $\mu\text{g/ml}$ had minocycline MIC's of 0.78 $\mu\text{g/ml}$.

Clindamycin and erythromycin sensitivity

Clindamycin-resistant strains were isolated from four of the 28 patients studied (table IV). Three had been receiving clindamycin therapy, one topically and two orally, for 4 months or more. The fourth patient was not known to have been treated with clindamycin or erythromycin. The four strains isolated from the patient who was using a topical preparation of clindamycin 1% (w/v) showed moderate resistance to clindamycin (MIC 6.25 $\mu\text{g/ml}$) but were sensitive to erythromycin. Ten strains of *P. acnes* isolated from one patient on oral clindamycin therapy were resistant to clindamycin and erythromycin (MIC > 100 $\mu\text{g/ml}$) and were moderately resistant to tetracycline (MIC 3.12 $\mu\text{g/ml}$). From the third patient on clindamycin therapy, two strains resistant to clindamycin and erythromycin were isolated. These were identified as atypical strains of *P. granulosum* that did not ferment maltose or sucrose. Only one of eight strains of *P. acnes* isolated from the fourth patient, who was receiving oral tetracycline therapy, was resistant to clindamycin and erythromycin (MIC > 100 $\mu\text{g/ml}$).

Sulphamethoxazole and trimethoprim sensitivity

All the isolates of *P. acnes* tested were sensitive to sulphamethoxazole and trimethoprim independently (table II). However, isolates of *P. granulosum* and *P. avidum* from subjects with or without acne had increased resistance to sulphamethoxazole (MIC 6.25–12.5 $\mu\text{g/ml}$) and some strains of *P. granulosum* were fully resistant (MIC > 100 $\mu\text{g/ml}$) (table II). Synergy was demonstrated with both sulphamethoxazole-sensitive and -resistant strains when combinations of sulphamethoxazole and

TABLE IV
Minimum inhibitory concentrations of antibiotics for propionibacteria isolated from patients treated with antibiotic for 4 months or longer

Number of strains tested	Number of patients from whom strains were isolated	MIC ($\mu\text{g/ml}$) of				Treatment given
		Tetracycline	Clindamycin	Erythromycin	Sulphamethoxazole	Trimethoprim
<i>P. acnes</i>						
10	2	3-12	≤ 0.097	0.048	1.56	0.78
4	1	3-12	0.39	0.048	3-12	1.56
4	1	0.39	6.25	0.097	1.56	1.56
10	1	3-12	> 100.0	> 100.0	1.56	0.78
1	1	0.39-0.78	> 100.0	> 100.0	...	...
<i>P. granulosum</i>						
5	1	3-12	0.097	0.097	> 100.0	3-12
2*	1	0.78	> 100.0	> 100.0	> 100.0	Clindamycin

* Atypical strains.

trimethoprim in ratios of 2–32:1 were used in plate MIC tests. Synergy was also demonstrated in disk tests.

DISCUSSION

Tetracycline and several other antibiotics are effective in the treatment of acne. Oral clindamycin was introduced in the early 1970's, followed by topical preparations of clindamycin, erythromycin and tetracycline (Resh and Stoughton, 1976; Stoughton, 1979; Adams, Cooke and Cunliffe, 1981). Several studies have established the efficacy of these topical preparations, particularly clindamycin (Resh and Stoughton, 1976; Adams *et al.*, 1981) which is used extensively in the USA (Stoughton, 1979). However, in view of the association between topical and oral therapy with clindamycin and antibiotic-associated pseudomembranous colitis (Cohen, McNeill and Wells, 1973; Milstone, McDonald and Scholhamer, 1981), care in its use is indicated (Adams *et al.*, 1981). The relatively small number of patients being treated with clindamycin in this study reflects the conservative use of this drug.

Less significance is now attributed to the direct role of free fatty acids in the inflammatory reaction in acne (Puhvel and Sakamoto, 1977; Webster, McGinley and Leyden, 1981). This view is supported by the evidence of Weeks *et al.* (1977) who showed that topical application of potent inhibitors of bacterial lipase did not alleviate the severity of acne lesions although they rapidly reduced free fatty acid levels. However, some of the products of comedonal bacteria act as chemotactic agents (Puhvel and Sakamoto, 1980). The lipase of *P. acnes* is chemotactic for human polymorphonuclear leucocytes (Lee *et al.*, 1982) and hyaluronidase and phosphatase are chemotactic for monocytes (Gould *et al.*, 1979). Some substances are directly chemotactic and with others complement is involved. A possible mechanism for antibiotic action was proposed for tetracycline which has been shown to inhibit lipase activity in *P. acnes* (Hassing, 1971; Weaber, Freedman and Eudy, 1971; Marples *et al.*, 1971; Cove *et al.*, 1980). Production of lipase was delayed at concentrations of tetracycline that had little effect on growth (Webster *et al.*, 1981; Unkles and Gemmell, 1982) and a similar effect was produced by erythromycin (Webster *et al.*, 1981). Tetracycline inhibited lipase production to a greater extent in *P. granulosum*, whereas clindamycin inhibited lipase production by *P. acnes* and *P. granulosum* at concentrations sub-inhibitory for growth (Unkles and Gemmell, 1982).

Some antibiotics have been shown to act directly as anti-inflammatory agents. Clindamycin, erythromycin and tetracycline inhibit chemotaxis of human leukocytes at concentrations below therapeutic blood levels (Esterly, Furey and Flanagan, 1978). There is very little information on the antibiotic concentrations attained in comedonal material although therapeutic concentrations of clindamycin are achieved in some people using topical application of clindamycin (Guin *et al.*, 1979). Smith and Waterworth (1961) could not detect inhibitory activity attributable to tetracycline in comedonal material from patients on tetracycline therapy. Therapeutic levels are reached on the skin surface within 4–8 days with normal doses but they are not always detectable in patients on low-dose regimes (Rashleigh, Rife and Goltz, 1967).

Response to antimicrobial chemotherapy is sometimes slow and fails to alleviate the symptoms in some patients (Leyden, 1976; Gould and Cunliffe, 1978). A number of unrelated factors that might contribute to failure of therapy have been considered,

including antibiotic resistance in propionibacteria (Leyden, 1976; Cunliffe *et al.*, 1981) but because resistance was not commonly observed it seemed unlikely to be an important cause of treatment failure (Leyden, 1976). However, Crawford *et al.* (1979) found a substantial incidence of resistance in propionibacteria isolated from patients on topical erythromycin or clindamycin after treatment for 8 weeks or longer; strains were resistant to both drugs. Phenotypically similar strains were derived from sensitive strains *in vitro* but the genetic basis of their resistance was not necessarily similar (Crawford *et al.*, 1979). The present results confirm that such strains can be isolated from patients on oral clindamycin therapy. The isolation of a resistant strain of *P. acnes* in the absence of clindamycin or erythromycin therapy is similar to the isolation of a resistant strain of *P. granulosum* by Guin *et al.* (1979) before the start of antibiotic therapy. Other workers have failed to isolate resistant strains from patients receiving therapy. In some studies, the absence of resistance may have been a result of the relatively short period of observation (Bernstein and Shalita, 1980) but in studies over longer periods this is unlikely to account for the absence of resistant strains (Leyden, 1976; Cunliffe *et al.*, 1981).

In spite of the extensive use of tetracycline for acne therapy there is a low incidence of resistance. The MIC values reported here confirm those obtained in other studies, where few strains with an MIC > 6.25 $\mu\text{g/ml}$ have been isolated and most were inhibited at lower concentrations. The greater sensitivity of strains to minocycline compared with tetracycline (Chow *et al.*, 1975; Hoeffler, Ko and Pulverer, 1976) was also confirmed. In contrast with the generally low levels of tetracycline resistance, the strains of *P. acnes* isolated by Guin *et al.* (1979) with MIC values > 25 $\mu\text{g/ml}$ are of interest. Moreover, they reported a strain resistant to ampicillin (MIC > 25 $\mu\text{g/ml}$).

The results of sulphonamide sensitivity tests did not confirm the finding of Hoeffler *et al.* (1976) that all strains of *P. acnes* were resistant to sulphamethoxazole (MIC > 100 $\mu\text{g/ml}$). The isolates of *P. acnes* in this study were uniformly sensitive (MIC 0.78–3.12 $\mu\text{g/ml}$) but several strains of *P. granulosum* were resistant (MIC > 100 $\mu\text{g/ml}$).

Antibiotic therapy has the disadvantage of selecting a resistant bacterial flora and it has been suggested that this is more likely to occur with the topical use of drugs (Noble, 1981). The source of resistant strains isolated from these patients is not known, but there are a number of possibilities. These include reinfection or recolonisation of the skin by resistant strains from another source after reduction of the sensitive flora or selection of resistant cells from the original population. Antibiotic resistance may be determined by plasmid or chromosomal genes. Clindamycin-resistant mutants can be isolated *in vitro* (Crawford *et al.*, 1979; Brown, unpublished observations) and chromosomal mutations could, therefore, account for resistant strains, particularly in patients undergoing long-term antibiotic therapy. The effect of bacterial resistance on the outcome of antibiotic therapy is not known but must be considered in studies of the response to therapy because it seems likely that the incidence of resistance will increase with the use of these antimicrobial agents.

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