

SHORT COMMUNICATION

Demonstration of Intracellular Growth of Gonococci in Human Phagocytes using Spectinomycin to Kill Extracellular Organisms

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The use of spectinomycin to kill extracellular bacteria in phagocytosis tests with gonococci and human polymorphonuclear phagocytes allowed the demonstration of a greater degree of intracellular survival and growth than in previous tests using penicillin.

INTRODUCTION

On average, 49% of the gonococci in urethral exudates from male patients with acute gonorrhoea are associated with polymorphonuclear (PMN) phagocytes (Veale *et al.*, 1979). Electron microscopic observations suggest that the majority of gonococci associated with PMN phagocytes are intracellular and that, judged morphologically, some of them are capable of surviving and dividing (Ward *et al.*, 1972; Farzadegan & Roth, 1975; Novotny *et al.*, 1975; Ovchinnikov *et al.*, 1976). If a substantial number of intracellular organisms could survive and multiply intracellularly, they would play a major role in the pathogenesis of the disease. Hence, assessing the extent of such survival and growth is important. Whether or not intracellular survival and growth occur can only be established by counts of intracellular gonococci at different times after phagocytosis or from evidence of active metabolism, for gonococci that appear intact and dividing in electron micrographs may have been recently ingested and about to be killed.

Studies on intracellular survival and multiplication of bacteria in phagocytes usually require a bactericidal medium which will kill all extracellular organisms without substantially affecting those that are intracellular. Until now, the most effective bactericidal medium for studying gonococcal survival in human phagocytes has been a mixture of penicillin (at low concentration; 0.2 to 0.4 $\mu\text{g ml}^{-1}$) and fresh human serum; survival of gonococci for up to 15 h in human peripheral blood PMN phagocytes was demonstrated using this mixture (Veale *et al.*, 1976, 1979; Witt *et al.*, 1976). At higher penicillin concentrations there was substantial killing of intracellular organisms by penetration of the antibiotic into the phagocytes (Veale *et al.*, 1976), as has been noted in phagocytosis tests with other bacteria and antibiotics (Patterson & Youmans, 1970; Frost *et al.*, 1972; Hart, 1973; Cole & Brostoff, 1975). Even with 0.4 $\mu\text{g ml}^{-1}$ penicillin some antibiotic apparently penetrated the phagocytes and the numbers of viable intracellular gonococci found after 4 to 6 h incubation were usually low (Veale *et al.*, 1976, 1979). However, these intracellular gonococci appeared to be growing, since they were killed by the addition of high concentrations of penicillin after 4 h incubation with lower concentrations (Veale *et al.*, 1979); penicillin kills only growing organisms (Ghuysen, 1977), a property used recently in the demonstration of intracellular multiplication of *Salmonella typhimurium* (Lowrie *et al.*, 1979).

Table 1. *Survival and growth of gonococci (strain BSDH) within human phagocytes incubated with spectinomycin*

Gonococci and phagocytes were mixed in a ratio of 20:1 (expts 1 and 4) or 40:1 (expts 2, 3 and 5). Viable counts (the average of those in duplicate tubes sampled at the stated time) were made on the deposit of infected phagocytes (about 80% PMN phagocytes) after the ingestion period (when extracellular organisms were present) and after incubation with antibiotics and 50% fresh human serum; they were not successive samplings of the same population. Figures in parentheses are the corresponding viable counts in the supernatant media. Where <2 organisms were detected in the supernatant media of experimental tubes, the deposits of the control tubes (without phagocytes) which contained viable gonococci before incubation with spectinomycin also contained <2 organisms after incubation with the antibiotic.

Expt and blood donor	10 ⁻³ × Viable count of gonococci in deposit before incubation with spectinomycin	Time of incubation with spectinomycin (h)	Viable count of gonococci in deposit after incubation with fresh human serum and spectinomycin		
			Spectinomycin concn (μg ml ⁻¹)		
			5	10	100
1 (BC)	8	2	140 (60)	10 (<2)	95 (<2)
		6	680 (<2)	240 (<2)	150 (<2)
		6*	<2 (<2)	<2 (<2)	<2 (<2)
2 (BC)	31	2	810 (70)	1300 (<2)	960 (<2)
		6	1600 (<2)	3700 (<2)	1300 (<2)
		6*	360 (<2)	55 (<2)	35 (<2)
3 (SC)	72	2	5800 (260)	2900 (<2)	420 (<2)
		6	2500 (890)	2500 (<2)	1800 (<2)
		6*	1700 (<2)	230 (<2)	<2 (<2)
4 (SC)	81	2	330 (75)	320 (<2)	75 (<2)
		6	1700 (150)	1600 (<2)	5 (<2)
		6*	85 (<2)	260 (<2)	—
5 (SM)	55	2	130 (45)	145 (<2)	250 (<2)
		6	1100 (15)	4400 (<2)	10 (<2)
		6*	5 (<2)	490 (<2)	5 (<2)

* Penicillin (to a final concentration of 200 μg ml⁻¹) was added to these tubes 4 h after the addition of spectinomycin. In some tubes, the deposits were not incubated in medium containing spectinomycin but with penicillin (0.4 μg ml⁻¹) as used previously (Veale *et al.*, 1979); the viable counts found in the phagocyte deposits after 6 h incubation (without extra addition of penicillin at 4 h) in experiments 1, 2, 3, 4 and 5 were 5 (<2), 420 (<2), 2200 (<2), <2 (<2) and 85 (<2), respectively.

The difficulty of quantitative assessment of gonococcal survival and multiplication in phagocytes cultured in a medium containing penicillin is the least satisfactory aspect of the previous work. Here we describe much greater survival and multiplication of gonococci in human phagocytes when spectinomycin was used instead of penicillin in the external medium.

METHODS

Bacteria. *Neisseria gonorrhoeae* strain BSDH was derived, stored and cultured as described previously (Veale *et al.*, 1975; Penn *et al.*, 1977).

Test for intracellular survival using human PMN phagocytes. The test described by Veale *et al.* (1979) using techniques described previously (Veale *et al.*, 1976; Witt *et al.*, 1976) was used. In essence, the test consists of two parts: a preliminary ingestion period of 1 h when phagocytes ingest gonococci and, together with some extracellular gonococci, settle on the flat portions of Leighton tubes, and then a period of intracellular survival and multiplication in the presence of a bactericidal medium that kills extracellular gonococci (which is shown by appropriate controls) (Veale *et al.*, 1976, 1979; Witt *et al.*, 1976). The following modifications were made: (i) after the initial 1 h ingestion period, spectinomycin hydrochloride (Upjohn Co., Kalamazoo, Mich., U.S.A.; 5 to 100 μg ml⁻¹) was added to some Leighton tubes instead of benzylpenicillin; (ii) after

4 h incubation with spectinomycin, penicillin (20 μl , in Parker medium 199) was added to a final concentration of 200 $\mu\text{g ml}^{-1}$ in some tubes and incubation was continued for 2 h; (iii) penicillinase (Riker Laboratories, Loughborough, Leics.; 1000 units in 0.1 ml medium 199) was added to all tubes incubated with penicillin prior to sampling.

RESULTS AND DISCUSSION

The results of five experiments with differing concentrations of spectinomycin, representative of a total of 13, are shown in Table 1. With 5 μg spectinomycin ml^{-1} , organisms were often detected in the supernatant medium and in control tubes (without phagocytes) indicating incomplete killing of extracellular organisms. The lowest concentration of spectinomycin that consistently killed all extracellular organisms was 10 $\mu\text{g ml}^{-1}$ and thus this was the optimum concentration for the tests. The numbers of viable intracellular organisms were usually considerably greater at this concentration of spectinomycin than at the corresponding optimum penicillin concentration, i.e. 0.4 $\mu\text{g ml}^{-1}$ (footnote to Table 1). Furthermore, the viable counts usually showed clear evidence of gonococcal multiplication between 2 and 6 h incubation. At high concentrations of spectinomycin (100 $\mu\text{g ml}^{-1}$), the numbers of surviving intracellular organisms were usually reduced indicating that some intracellular penetration of antibiotic had occurred. Addition of penicillin after 4 h incubation in the presence of spectinomycin considerably reduced the number of intracellular organisms detected at 6 h (Table 1) indicating that these organisms were growing.

These results support our previous evidence (Veale *et al.*, 1976, 1979; Witt *et al.*, 1976) that gonococci are capable of surviving and multiplying inside human phagocytes to a substantial extent and therefore intracellular survival and multiplication is probably important in the pathogenesis of gonorrhoea. Spectinomycin is a better agent than penicillin in tests for intracellular survival and multiplication of gonococci as it combines effective bactericidal activity extracellularly with a reduced effect on intracellular organisms. This reduced effect may be due to less penetration into the phagocytes or possibly, as for streptomycin (De Duve *et al.*, 1974), to a reduced effectiveness in the phagocyte vacuoles due to a low pH. Penicillin is, however, a useful agent for determining whether the intracellular organisms are growing.

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